"Studies in the Molecular Organisation of the Nuclear Envelope".

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In accordance with the regulations laid down by the University, I hereby declare that some parts of the work described in this thesis are in the press.

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The work described in this thesis, except where indicated to the contrary, is entirely my own and the thesis was composed by myself.

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Abstract

Immobilised lactoperoxidase has been developed as a probe of the molecular organisation of the nuclear envelope. In particular, it has been used to identify proteins of the nuclear pore complex (which is believed to be the principle site for nucleocytoplasmic transport of ribonucleoprotein) and to investigate the extent to which the nuclear membranes are differentiated from rough endoplasmic reticulum.

The outer annulus of the nuclear pore complex is shown to comprise at least 14 polypeptides, only two of which (N1 and N2) are major components of the nuclear envelope as a whole. A third major component of the nuclear envelope (N3) is located in the fibrous meshwork that underlies and interconnects the pore complexes, and which represents the peripheral aspect of the nuclear matrix.

The polypeptides of the nuclear envelope and rough endoplasmic reticulum are examined with respect to their distribution and organisation. It is firmly established, contrary to widely-held beliefs, that the nuclear membranes are a highly specialised membrane system quite distinct from the rough endoplasmic reticulum.

Abbreviations.

ATP	Adenosine 5' - Triphosphate.
Con A	Concanavlin A
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
GO	Glucose oxidase
LPO	Lactoperoxidase
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
PMSF	Phenylmethyl-sulfonylfluoride
RNA	Ribonucleic acid
mRNA	Messenger RNA
rRNA	Ribosomal RNA
tRNA	Transfer RNA
hnRNA	Heterogenous nuclear RNA
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
Tris	/Tris (hydroxymethyl)aminomethane/
u	micro or microns

C O N T E N T S.

1. General Introduction.

1.1 Structure of the Nuclear Envelope

The nuclear envelope is a characteristic feature of, and represents the most complex membrane system within, the eukaryote cell. It is a porous, concentric double membrane system, separating the nucleus from the cytoplasm; and the great interest shown in this structure stems from the belief that, as a barrier between nucleus and cytoplasm, it plays a crucial role in nucleocytoplasmic exchanges and hence in gene expression. The structure and biochemistry of the nuclear envelope have been the subject of exhaustive review in recent years (see Feldherr 1972; Siebert 1972; Zbarsky 1972; Kay and Johnson 1973; Kessel 1973; Berezney 1974; Franke and Scheer 1974; Kasper 1974; Fry 1976b; Harris and Agutter 1976; Wunderlich *et al*. 1976; Franke 1977; Harris 1978) and will only be summarised here (see Fig. 1).

The outer nuclear membrane is continuous with the inner nuclear membrane at the level of the pore complex, and is occasionally viewed as being continuous with the rough endoplasmic reticulum (Watson 1955; de Groodt *et al*. 1958; Whaley *et al*. 1960; Gibbs 1962; Hadek and Swift 1962; Fawcett 1966; Franke and Scheer 1974). It bears ribosomes (Watson 1955; Palade 1955) and provides a surface to which microtubules, microfilaments and other structural elements of the cytoplasm may attach. (see Franke and Scheer

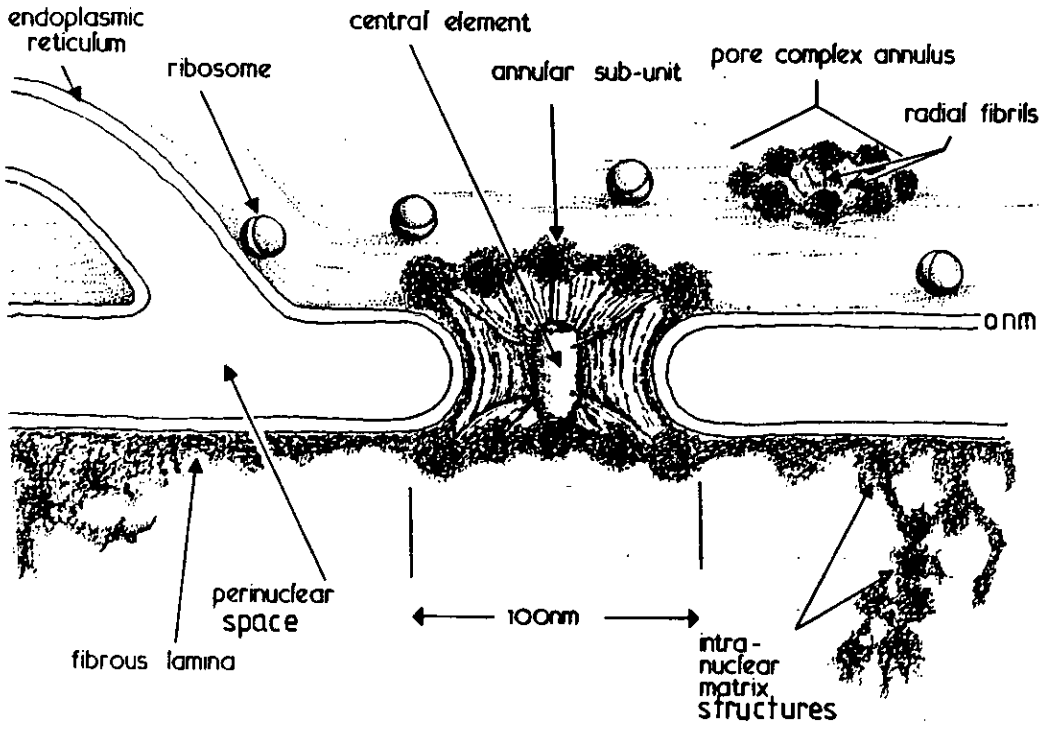

endoplasmic reticulum
ribosome
central element
annular sub-unit
pore complex annulus
radial fibrils
onm
perinuclear space
100nm
fibrous lamina
intra-nuclear matrix structures

Fig.1

1974).

The inner nuclear membrane abutts a fibrous lamina (Fawcett 1966; Aaronson and Blobel 1975) and it is presumably to this latter structure that elements of the nucleoplasm attach. The fibrous lamina represents the peripheral aspect of the nuclear matrix (which ramifies throughout the nucleus) and provides a skeletal base for the nuclear pore complexes. It is believed that the fibrous lamina can modulate, inter alia, both lipid distribution and fluidity in nuclear membranes (Wunderlich et al. 1978). At several places the inner and outer nuclear membranes unite, leaving small circular areas where no membrane interposes between nucleoplasm and cytoplasm; these are the nuclear pores and the structures that bound them are known as nuclear pore complexes.

1.2 The Nuclear Matrix.

The nuclear matrix consists of three main components; a residual nuclear envelope (comprising the fibrous lamina and associated pore complexes), a residual nucleolus and an extensive granular and fibrous matrix which extends throughout the interior of the nucleus from the nucleolus to the surrounding nuclear envelope (Berezney and Coffey 1976; Berezney and Coffey 1977). Newly replicated DNA is closely associated with the nuclear matrix (Berezney and Coffey 1975; Berezney and Buchholtz 1978) and heterogeneous nuclear RNA is associated with a non-chromatin nuclear

ribonucleoprotein network connected to the fibrous lamina (Faiferman and Pogo 1975; Herman et al. 1978).

The principle pathway by which nascent RNA is transported from the nucleus to the cytoplasm is believed to be the nuclear pore complex (Franke and Scheer 1974a; Wunderlich et al. 1976) but the mechanism of transport between the site of RNA synthesis and the pore complex is unknown. Since the nuclear matrix directly connects pore complexes with residual components of interchromatinic structures, and with the belief that newly synthesised extranucleolar RNA may be closely associated with the nuclear matrix (Berezney and Coffey 1976), it has been suggested that the nuclear protein matrix may provide a skeletal passageway for RNA transport (Berezney and Coffey 1976). Since RNA undergoes considerable post-transcriptional modification prior to its possible appearance in the cytoplasm (Heinrich et al. 1978), the nuclear matrix might also be seen as a possible site for RNA processing as well as transport to the pore complex.

1.3 The Pore Complex.

The nuclear pore complex is not a discrete structure (which is why a method for its isolation has proved elusive) for it is contiguous with the fibrous lamina and with the nuclear membranes at the point of fusion of the inner and outer nuclear membranes. It should not therefore be considered as a subcellular

organelle in its own right, although such an idea has been mooted (Abelson and Smith 1970; Faberge 1974; Harris and Agutter 1976). The ultrastructure of the pore complex has been studied in detail and although there is still considerable controversy over its fine structure, certain features of the pore complex are beyond reasonable dispute.

1) The pore orifice is bounded by an annulus on both its cytoplasmic and nucleoplasmic surfaces; and the annuli are composed of granular subunits, which usually show an eightfold radial symmetry.

2) Eight irregularly shaped projections extend, either from the pore wall or the annuli, into the lumen and also demonstrate an eightfold symmetry.

3) A dense central element, be it tubular, granular, or fibrillar, is present in many pore complexes. It may represent ribonucleoprotein material actually <u>in transit</u> through the pore orifice or, more probably, it may be a structural feature of the pore complex. The central element is not seen in every pore complex and may be present in one pore complex although absent in several adjacent ones.

The number of pore complexes per unit area of nuclear surface and the total number per nucleus are highly variable. Both the number and distribution of nuclear pore complexes varies from one cell type to another and can vary within the same cell at different

stages in its development and after stimulation by phytohaemagglutinin and Con A (Merriam 1962; Maul *et al*. 1971; Wunderlich *et al*. 1974). The mature amphibian erythrocyte (in which transcriptional activity is exceedingly low) is characterised by only about 3 pores/um^2 and a total of only 150-300 pores/nucleus, whereas in a lampbrush-stage amphibian oocyte the density is about 60 pores/um^2 and the total number of pores is more than 50 x 10^6/nucleus. However, there is no simple correlation between the nuclear pore frequency and nucleocytoplasmic exchange rates since *mature* oocytes, which display a low metabolic activity, have nuclear pore frequencies nearly ten times greater than stimulated lymphocytes or HeLa cells which have a high metabolic activity (Wunderlich *et al*. 1976).

The exact chamical composition of nuclear pore complexes is unknown but digestion studies using proteolytic enzymes, RNase and DNase have indicated that the pore complex is composed of protein (Merriam 1961; Clerot 1968; Beaulaton 1968; Koshiba *et al*. 1970) and RNA (Mentre 1969; Agutter *et al*. 1977) but not DNA (Cole 1969; Mentre 1969; Koshiba *et al*. 1970).

1.4. Permeability Properties

Many molecules and macro-molecules exchange rapidly and continuously between nucleus and cytoplasm through the nuclear envelope, and these exchanges fre-

quently proceed against concentration gradients. The nuclear envelope appears to exert little restriction on the movement of most low molecular weight organic substances (Horowitz and Fernichel 1970; Kohen et al. 1971; Horowitz 1972; Horowitz and Moore 1974; Frank and Horowitz 1975). However many ions, small molecules and macromolecules are unevenly distributed between the nucleus and cytoplasm and the question arises whether the nuclear envelope plays an active role in establishing and maintaining these asymmetries.

There is absolutely no hard evidence that the nuclear envelope may act as a permeability barrier to, or may actively transport, ions. The evidence that a concentration gradient for Na^+ and K^+ exists between the nucleus and cytoplasm (Abelson and Duryee 1949; Langendorf et al. 1966; Century et al. 1970; Horowitz and Fenichel 1970; Gullasch and Kaufmann 1974) and that in some cells there is a potential difference between the nucleoplasm and exterior (Loewenstein 1964; Loewenstein et al. 1966; Badr 1974; Gullasch and Kaufmann 1974), may be explained in terms of the nucleoplasm providing a pool of fixed anionic charges which adsorb cations; thus the nucleus both binds and concentrates cations, and the fixed charge produces a potential difference with the cytoplasm (see Ling 1970).

Most of our information about the permeability

of the nuclear envelope to macromolecules is derived from micro-injection studies in which macromolecules are introduced into the cytoplasm and their appearance in the nucleus is monitored (Bonner 1975a + b; Feldherr 1962; 1966; 1968; 1971; 1972; 1975; 1978; Gurdon 1970; Gurdon et al. 1976; Paine and Feldherr 1972; Paine 1975; Paine et al. 1975; De Robertis et al. 1978). Thus, although the nuclear envelope appears to offer little resistance to the diffusion of ions and small molecules it exhibits some selectivity with larger molecules. Histones and bovine serum albumin (mol. wt 67,000) rapidly penetrate the amphibian oocyte nuclear envelope (Gurdon 1970) but proteins with a molecular weight greater than about 69,000 are severely restricted (Bonner 1975a). Nuclei will take-up labelled nuclear proteins injected into the cytoplasm but not labelled cytoplasmic proteins so injected (Bonner 1975b); moreover, nuclear proteins of mol. wt 120,000 are concentrated with the same efficiency as smaller ones (De Robertis et al 1978). It has therefore been suggested that nuclear proteins may contain in their molecular structure a signal that enables them to accumulate selectively in the nuclear compartment (De Robertis et al. 1978). This is not to imply that there is an active mechanism, mediated by the nuclear envelope, to such sequestration. If the affinity of the nucleoplasm for these proteins is high, then a facilitated diffusion would adequately

account for the process. Accumulation of proteins below 69,000 mol. wt would thus reflect the binding properties of the nucleoplasm and be independent of the nuclear envelope; whereas, in contrast, proteins greater than 69,000 mol. wt would require facilitation by the nuclear envelope, and their accumulation would reflect both the properties of the nuclear envelope and of the nucleoplasm.

The mechanism by which mRNA and rRNA are transported from the nucleus to the cytoplasm is unknown although it does appear to be an active process. Stevens and Amos (1972) have shown that cooling of HeLa cells reduces the transport of rRNA but not tRNA (which as a small molecule presumably leaves the nucleus by diffusion) and Bier (1965) has shown that oxygen deprivation inhibits overall RNA transport but not synthesis. Several groups have attempted to demonstrate a requirement for ATP in *in vitro* efflux studies (Schneider 1959; Ishikawa *et al.* 1969; Ishikawa *et al.* 1970; Raskas 1971; Raskas and Okubo 1971; Brunner and Raskas 1972; Schumm and Webb 1972; Agutter *et al.* 1976b) but only Schumm and Webb (1972) and Agutter *et al.* (1976) have shown clearly that the mechanism requires actual hydrolysis of ATP. Yasuzumi and Tsubo (1966) have located ATPase activity histochemically within the pore complex, and so it is an attractive idea that a nuclear pore complex ATPase

is involved in translocating newly synthesised RNA from the nucleus into the cytoplasm; but as yet, there is no really hard evidence.

1.5 Proteins.

Although proteins comprise the bulk of the nuclear envelope, they have not been extensively fractionated or characterised. In part this is a reflection of their intractibility and in part a measure of the difficulty of isolating sufficient quantities of the envelope for fractionation.

On average more than 20 different polypeptides, with molecular weights ranging from 16,000 to greater than 200,000, can be distinguished by SDS polyacrylamide gel electrophoresis (Franke et al. 1970; Matsuura and Ueda 1972; Monneron et al. 1972; Bornens and Kasper 1973; Blanchet 1974; Aaronson and Blobel 1975; Shelton et al., 1976; Jackson 1976; Wilson and Chytil 1976; Virtannen 1977). Approximately 55% of the total nuclear envelope proteins are divided into the two molecular weight ranges 47,000 to 60,000 and 64,000 to 74,000 (Bornens and Kasper 1973). Three bands predominate above all others at molecular weights of 80,000, 74,000 and 64,000 in chicken erythrocyte nuclear envelopes (Jackson 1976) and at 74,000, 70,000 and 53,000 in rat liver nuclear envelopes (Bornens and Kasper 1973). Too much reliance should not be placed on these molecular weight estimates,

which were obtained using differing electrophoretic techniques.

Discontinuous buffer gel systems provide much greater resolution than those used by early workers and many more polypeptide species may be identified. In these systems, the three major bands, hereafter referred to as N1, N2, and N3 all migrate at between 62,000 and 69,000 mol. wt (Aaronson and Blobel 1975; Dwyer and Blobel 1976; Virtanen 1977).

The nuclear envelope polypeptide composition has been compared with that of endoplasmic reticulum (Franke et al. 1970; Matsuura and Ueda 1972; Monneron et al. 1972; Bornens and Kasper 1973; Wilson and Chytil 1976; Harris 1978) and plasma membrane fractions (Jackson 1976; Shelton et al. 1976; Wilson and Chytil 1976) and although some homologies exist between the nuclear envelope and endoplasmic reticulum polypeptides, (but see Chapter 5) those of the plasma membrane and nuclear envelope seem quite different (contrary to findings of Blanchet 1974).

1.5.1. Amino Acid Analysis.

Bornens and Kasper (1973) have performed an amino acid analysis on nuclear envelope proteins solubilised in SDS and fractionated by gel filtration. They found that the amino acid composition of individual fractions of proteins did not differ greatly from that of the whole envelope. A general trend for both acidic

and basic residues was noted, in which the amounts of glutamic acid and aspartic acid decreased, whereas the sum of lysine, histidine and arginine increased, in going from high to low molecular weight polypeptides. Most envelope polypeptides had an acidic character and no polypeptides with an histone - like acidic/basic ratio were present. The ratios of non-polar residues ranged from 46.3 to 52.6 moles % and at first sight these values seem extraordinarily high (the major intrinsic protein of the erythrocyte membrane, for example, has 33 moles % non-polar residues. Ho and Guidotti 1975; Jenkins and Tanner 1977); but this can be accounted for by their inclusion of glycine and proline in the non-polar (hydrophobic) class, when they are more usually categorised as polar. If the non-polar ratio is recalculated to include valine, methionine, leucine, isoleucine, phenylalanine and tyrosine then the ratios of non-polar residues range from 25.3 to 36.67 moles %; ratios which are more generally typical.

1.5.2. Glycoproteins

Glycolipids are not found in the nuclear envelope (Keenan et al. 1970; 1972; Kleinig 1970) but the presence of glycoproteins in nuclei and isolated nuclear envelope has frequently been reported and in many cell types. (c.f. Franke et al. 1976).

Nuclear envelope glycopeptides are predominantly neutral in character (Kawasaki and Yamashima 1972). Although sialic acid has frequently been described as a component of nuclei and nuclear envelopes (Bosmann 1973; Keshgegian and Glick 1973; Marcus et al. 1965; Zbarsky 1972) very low levels have also been reported (Kashnig and Kasper 1969; Kawasaki and Yamashima 1972; Philipp et al. 1976; Franke et al. 1976) and these could be accounted for by as little as 1% contamination by plasma membranes (Franke et al. 1976).

Periodic acid-Schiff (PAS) staining of SDS polyacrylamide gels of isolated rat liver nuclear envelope has revealed a prominent glycoprotein at 160,000 and several minor bands between 50,000 and 74,000 mol. wt (Bornens and Kasper 1973; Kasper 1974). Con A binding polypeptides have been identified at 180,000, 34,000 and minor bands at 50,000 - 65,000 mol. wt by the more sensitive peroxidase method (Virtanen 1977a). The 180,000 mol. wt Con A binding polypeptide may well be identical to the 160,000 mol. wt polypeptide revealed by PAS staining.

Lectins such as Con A and wheat germ agglutinin have been shown to induce the aggregation of purified nuclei (Nicolson et al. 1972; Stoddart and Price 1977) suggesting a surface disposition of the glycopeptides. However, a proportion of the outer

nuclear membrane is always lost during isolation procedures with the possible exposure of glycoproteins of the cisternal surfaces of the nuclear envelope. Monneron and Segretain (1974), using a Con A peroxidase method, localised Con A binding to the cisternal surfaces of calf thymocyte nuclear envelope and to the ribosomes on the outer nuclear membrane. The nucleoplasmic surface of the inner nuclear membrane and the lumen of the pore complex were never labelled. Nuclei treated with detergents (Triton X-100 and deoxycholate) failed to show Con A binding, suggesting an exclusively membrane disposition for Con A binding glycopeptides. Labelling procedures must always be regarded with caution. In this case nuclei bound nearly 50% as much Con A in the presence of 0.2M α methyl mannoside as in its absence. Further, non-specific binding by peroxidase may be a problem and controls against the presence of an endogenous peroxidase are essential. In principle, the use of Con A-ferritin conjugates to detect Con A binding sites is to be preferred and by this means, Virtanen (Virtanen and Wartiovara 1976; Virtanen 1977) has demonstrated the presence of Con A binding sites solely on the cisternal surfaces of the inner and outer nuclear membranes.

1.5.3 Colchicine Binding Proteins.

There exists an enormous literature on the associations of the nuclear envelope with microtubules

both at the cytoplasmic and nucleoplasmic surfaces (for refs see Franke and Scheer 1974). This association has been particularly well demonstrated by the use of fluorescently labelled antibodies to tubulin in cultured cells (Weber et al. 1975; Osborn and Weber 1976) in which there is an extensive cytoplasmic skeleton of tubulin concentrated around the nucleus. Moreover, it would appear that the centriole, which is firmly attached to the outer nuclear membrane (Bornens 1977), may act as a microtubule organising centre for cytoplasmic microtubules (Osborn and Weber 1976).

Isolated nuclear envelopes bind colchicine (Stadler and Franke 1972; Stadler and Franke 1974), a property which is considered to be specific for the microtubule protein tubulin (Borisy and Taylor 1967; Weisenberg et al. 1968; Weisenberg 1972). However, both nuclear envelopes and microsomal membranes bind luminocolchicine as effectively as colchicine, whereas tubulin does not bind luminocolchicine (Wilson 1970). Stadler and Franke (1974) have interpreted this data as indicating a lack of specificity and have suggested that colchicine, which is hydrophobic, merely interacts with the hydrophobic domains of the two membranes. Such an interpretation is not necessarily correct however, for there is no reason to believe that colchicine and luminocolchicine were bound to the same sites. The

nuclear envelopes consistently bound more colchicine than microsomal membranes and extraction of the lipid did not decrease the efficacy of binding. It must however be considered unlikely that microtubular components, even if strongly associated with the nuclear envelopes *in vivo*, would co-purify with nuclear envelopes; for the conditions of preparation (4°C) are liable to dissociate microtubules. The most compelling evidence against colchicine binding to nuclear envelopes representing binding to tubulin, lies in the kinetics of binding - which are both quite different and more complex than is the case for brain supernatant (Stadler and Franke 1972; 1974). The association between the centriole and the nuclear envelope might lead one to suspect that at least a portion of observed colchicine binding represents binding to tubulin but Stadler and Franke (1974) conclude that only a minute proportion of the envelope proteins in rat liver nuclear envelopes could be constituted by tubulin. The finding that the nuclear matrix also binds colchicine (Berezney and Coffey 1976) re-opens the issue, but one must question whether such findings are important in view of the already well established association between microtubules and the nuclear envelope (see Franke and Scheer 1974). Perhaps the most interesting observation has been that colchicine inhibits RNA transport in isolated liver nuclei

(Schumm and Webb 1974). Furthermore, colchicine impairs the temperature induced changes in membrane particle distribution observable by freeze-etch electron microscopy (Wunderlich et al. 1973).

1.5.4. Selective Extraction Procedures.

Nuclear envelopes are, by nature, extremely insoluble. Even high concentrations of urea and SDS fail to solubilise a considerable amount of nuclear envelope protein (Franke 1974b). This has prompted some workers to use extremely vigorous extraction procedures in the hope of gaining some selectivity. For example, Jackson (1976) has used 0.1M NaOH to selectively remove polypeptides N1 and N2 but not N3 from chicken erythrocyte nuclear envelopes.

Kasper (1974) has reported a survey study performed on the solubility of the nuclear envelope in varying concentrations of urea, guanidine-HCl, acetic acid, pyridine, EDTA (pH 7.2), NaBr, acetamide and formamide and their N,N-dimethyl derivatives. In no case was a selective extraction of proteins achieved and high molecular weight aggregates tended to be formed.

Non-ionic detergents are increasingly being used to extract components from nuclear envelopes (Monneron 1974; Aaronson and Blobel 1975; Dwyer and Blobel 1976; Berezney and Coffey 1976; Shelton 1976;

Krohne et al. 1978). The attraction of using non-ionic detergents such as Triton X-100 lies in their being less apt to denature proteins than other solubilising agents. Extraction of purified nuclear envelopes removes proteins in the 50,000 mol. wt region along with 95% of the phospholipid, leaving an insoluble residue known as the 'pore-complex lamina fraction' (Aaronson and Blobel 1974; 1975; Dwyer and Blobel 1976; see also Berezney and Coffey 1976) composed largely of bands N1, N2 and N3.

Shelton (1976) has reported that Triton X-100 will selectively solubilise a greater proportion of nuclear envelope proteins if $MgCl_2$ is included in the extraction medium at concentrations of approximately 500mM; but, as he pelleted his material for only 20 minutes and at 27,000g, his conclusions cannot really be justified.

1.5.5. Pore Complex Proteins.

Despite at least two attempts (Aaronson and Blobel 1975; Harris 1977), the nuclear pore complex has not been isolated from mammalian cells. However, extraction of rat liver nuclear envelopes with Triton X-100 leaves a residue in which pore complexes are enriched and can be clearly identified. This residue has been termed the pore complex-lamina fraction (Aaronson and Blobel 1975; Dwyer and Blobel 1976)

and is largely composed of the major nuclear envelope polypeptides N1, N2 and N3; but it has not been possible to distinguish between components of the pore complex and those of the peripheral lamina (see Chapter 4). Similar results were obtained by Riley and Keller (1976) in an examination of nuclear ghosts from HeLa cells. The nuclear ghost components (which include residual pore annuli) show dramatic cell cycle dependant organisational differences but the polypeptide composition exhibits little variation (Riley and Keller 1978).

Very recently, Krohne et al. (1978) examined the residual polypeptides from amphibian oocyte nuclear envelopes in which nuclear pore density is very high and in which the peripheral lamina is minimal or absent. The fraction, greatly enriched in nuclear pore complexes, was largely composed of N2 and a polypeptide at 150,000 mol. wt; and these presumably represent pore complex proteins.

1.6 Discussion.

The nuclear envelope is the most complex membrane system within the eukaryote cell and physico-chemical analysis of its constituent proteins has extended little beyond the relatively trivial establishment of their electrophoretic profile. Although there is a great deal of information about enzyme activities associated with nuclear envelope preparations

(see Franke 1974a + b), some of which undoubtedly catalogues cross contamination from other membrane systems, such information loses much of its value in the absence of detail as to both where in the envelope the activities are located and what are their relationships to other enzyme activities.

Clearly, it would be a great advantage to our understanding of the function of the nuclear envelope if simple subfractionation methods allowed the separation of the inner and outer nuclear membranes and of the pore complex, but because these structures are continuous with one another, rather than discrete, it is difficult to see how this might be brought about. Moreover, techniques which do seek to fractionate the nuclear envelope into inner and outer membrane fractions and thence to establish enzyme profiles (Zbarsky 1972), are doomed in the absence of a suitable marker enzyme for either of the membranes with which to validate the separation.

Citric acid is believed to preferentially remove the outer nuclear membrane from isolated nuclei (Smith et al. 1969; Taylor et al. 1975; Virtanen et al. 1977); but the technique would almost certainly destroy the majority of enzyme activities and may even fix proteins into the membranes (it is also worth noting that Bornens 1968 considered that citric acid treatment of nuclei

removed both membranes).

Several methods for purifying nuclear envelopes involve floatation of partially purified envelopes in sucrose gradients. In certain cases, this leads to the production of light and heavy membrane fractions consisting primarily of single membrane vesicles (Zbarsky et al. 1969; Kashnig and Kasper 1969; Berezney et al. 1972) and it could be that, in effect, separation of the inner and outer membranes has already been achieved. However, lacking a suitable marker for either membrane, one is not able to say whether this is so. Ribosomes would of course be a good marker of the outer nuclear membrane, but they are frequently stripped off during membrane isolation.

One is therefore left with an apparently circular dilemma:- one cannot study the enzymes of the inner or outer nuclear membranes in isolation without first separating the two membranes, and one cannot validate any separation of the two membranes in the absence of a marker enzyme. An attractive way out of the problem would seem to be to develop a means of labelling the cytoplasmic surface of the nuclear envelope: with its development would come the means of validating any envelope subfractionation scheme, and of probing the molecular organisation of the nuclear envelope. Such a labelling technique has therefore been developed.

In the next chapter (Chapter 2) techniques for isolating nuclei and nuclear envelopes are reviewed and a method for preparing nuclear envelopes of very high purity and integrity is described. In Chapter 3, the development of a simple means of labelling the cytoplasmic surface of the nuclear envelope is described. Its use has led to the identification of the major proteins of the nuclear pore complex (Chapter 4) and to the clear establishment, contrary to current dogma, of the outer nuclear membranes differentiation from rough endoplasmic reticulum (Chapter 5).

2 The Isolation of Nuclear Envelopes from Whole Tissue.

2.1 Introduction

2.1.1 Terminology

Many physical, chemical and enzymic methods for the isolation of nuclear envelopes have been described but frequently they are misleadingly titled. The term 'nuclear envelope' usually refers to a double membrane system separating the nucleoplasm from the cytoplasm. The membranes are studded with very highly ordered structures known as pore complexes and frequently bear ribosomes on the cytoplasmic surface of the outer nuclear membrane. However two factors must be born in mind when describing the nuclear envelope 1) The pore complexes and their associated lamina may also be isolated as an integral part of the nuclear matrix (Berezney and Coffey 1977) and it would be foolish to argue that the pore complexes belong more corrently to either the nuclear membrane system or the nuclear matrix system for the nuclear membranes and the nuclear matrix are patently parts of the same physiological structure. 2) The outer nuclear membrane is apparently contiguous with the endoplasmic reticulum (but see Chapter 5) and unless there is a barrier to diffusion proteins and lipids will diffuse between the nuclear membranes and the endoplasmic reticulum.

In the light of the above, it must be considered that the nuclear envelope does not exist as a discrete physiological structure but only as a preparative artefact. However the term 'nuclear envelope' can be useful if

retained to describe those preparations of double nuclear membranes which retain ordered pore complexes. Some such preparations exist, particularly those prepared by manual dissection from cells with giant nuclei (the original technique is that of Callan et al. 1949, Callan and Tomlin 1950 but especially good preparations are those of Scheer 1972 and Scheer 1973). The great majority of 'nuclear envelope' preparations exhibit vesiculation, separation of the inner and outer membranes, and the nuclear pore complexes are not always well preserved (see the preparations of Zbarsky et al. 1969; Monneron et al. 1972). In these instances the term 'nuclear membranes' is more applicable even if in selected micrographs some double membranes bearing pore complexes are evident.

2.1.2 General consideration of isolation methods.

There now exists a wide variety of methods for preparing nuclear envelopes all of which, except that of Price et al. (1972), require the prior isolation of nuclei. Although non-aqueous methods for preparing nuclei have provided useful information with regard to the compartmentation of water soluble components of nuclei (see Siebert et al. 1973), all methods of preparing nuclei as a preliminary to the isolation of nuclear envelopes rely on aqueous techniques (e.g. Chaveau 1956; Widnell and Tata 1964; Blobel and

Potter 1966). Since there is no reliable set of enzymic markers for determining the purity of a given nuclear envelope preparation (see section 2.26) and because it can frequently be very difficult to distinguish between nuclear and other membranes in thin section, it is better to prepare nuclear membranes from a well defined preparation of highly purified nuclei than to isolate them directly from a homogenate.

The broad aims in this thesis are to try and identify proteins of the nuclear pore complex and to establish whether there are proteins truely common to both the nuclear membranes and the rough endoplasmic reticulum. The latter aspect may be reduced to the question "is there a barrier to diffusion of proteins between the nuclear membranes and the rough endoplasmic reticulum?". These aims set constrains on the type of nuclear envelope preparation that can be used. In particular, it is important to obtain envelopes containing pore complexes in a very high degree of preservation and therefore, by the same token, with as little separation of the inner and outer membranes as possible. It is worth emphasising that these are purely ultrastructural requirements. In a largely chemical study such as this, it is not necessary that enzyme activities be retained although this may well prove to be concomittent with a high degree of ultrastructural integrity.

A most important feature of any preparative method is its reliability and reproducibility. The method of Harris and Milne (1974) appears in published electron micrographs (Harris and Agutter 1976) to provide the most ultrastructurally intact nuclear 'envelopes' of all mass isolation techniques and could well be considered on this basis alone as probably the most useful method for preparing nuclear envelopes in a study such as this. However in my hands the method proved to be grossly unreliable. Furthermore, the ultrastructure of the isolated envelopes did not appear to be as good as one might have expected. As a result of this lack of reproducibility I was eventually forced to use the Kay procedure (Kay et al. 1972) to prepare nuclear envelopes. Although this did not provide material with as high a degree of integrity as is apparently possible with the Harris and Milne procedure, it did prove to be more reliable.

2.2 Factors important in the isolation of nuclei

2.2.1 Aims

The aims of the preparative method must be to provide nuclei in the highest yield, purity and integrity in the very minimum of time. It is important to obtain a high yield both from the point of view of obtaining a representative fraction of a tissue's nuclei and also in order to provide material in quantities

sufficient for biochemical analysis. Unfortunately, the aims outlined above, though not necessarily mutually exclusive, tend, in practice, to conflict.

Although it is not necessary to maintain the integrity of nuclei as such, if the final aim is the purification of nuclear envelopes, it is at least necessary to ensure that the pore complexes of the nuclear envelope show good structural preservation and that no selective loss of the outer nuclear membrane occurs. Quantitative measurements of the proportion of outer nuclear membrane that is retained after isolation of nuclei can and should be made by morphometric methods.

Yield, purity and integrity will all be greatly influenced by the type of tissue, method of cellular disruption, isolation medium and centrifugation procedures employed.

2.2.2. Choice of Tissue

Since all organs are heterogeneous in cell type the use of tissue culture cells (Hildebrand and Okinaka 1976) or avian erythrocytes (Zentgraf et al. 1971; Blanchet 1974; Shelton et al. 1976) is sometimes favoured. However, the cost of using tissue culture cells to prepare quantities of nuclear envelope sufficient for biochemical analysis is often prohibitive and it is more difficult to isolate clean nuclei from cultured cells. Avian erythrocytes are of course cheap

and usually readily obtainable but nuclear pore density is low.

Liver tissue (derived either from rat, pig or ox) is the most commonly used tissue for preparing nuclear envelopes. The livers from only a dozen adult rats can provide enough nuclear envelope for quite extensive biochemical analysis, and the nuclei may be prepared in pure form without recourse to the use of detergents. The tissue is soft and easily dispersed in a homogeniser. In addition, rats, which are comparatively cheap, can be fed controlled diets and be bred from known strains. The use of animal organs does suffer from the disadvantage of cellular heterogeneity. In rat liver, only about 70% of cells are hepatocytes (Herzfeld et al. 1973) and heterogeneity is further complicated by variations in nuclear ploidy, but other tissues tend to be even more heterogeneous.

2.2.3. Methods of Cellular Disruption

Ideally one requires a method of homogenisation with a low range of shearing stresses which, though sufficient to liberate nuclei, do not actually damage them. Other organelles such as mitochondria and lysosomes should also remain intact to decrease the risk of contamination and proteolysis. Moreover, contamination by cellular organelles such as mitochondria can be monitored by microscopy much more

easily if the organelles are intact than if they are fragmented and adsorbed onto the nuclear surface. Dounce and Potter-Elvehjem homogenisers are frequently used, but machines such as the Waring blender or Ultra-turax, with their greater range of shearing stresses, are more useful in bulk isolation procedures or when a more fibrous tissue is used (Berezney et al. 1970; Widnell et al. 1967). Nitrogen cavitation has been used to release nuclei from avian erythrocytes (Shelton et al. 1976) but the technique would not be applicable to liver tissue.

2.2.4. Isolation medium

The composition of the isolation medium is critical for one must stabilise nuclei and other cellular organelles without at the same time promoting aggregation or activating proteolytic and transaminase activities. Unfortunately those media that are most likely to stabilise nuclei by the inclusion of relatively high millimolar concentrations of calcium and magnesium are also prone to cause aggregation in sucrose media and promote proteolysis. Sucrose must be included in all media for although isolated nuclei are freely permeable to sucrose (Kodama and Tedeschi 1968) it is necessary to stabilise osmotically other organelles such as mitochondria and lysosomes. The concentration of sucrose and the ratio of tissue to medium may be

optimised to balance the requirements of yield, purity and integrity. Typically, 0.32 M sucrose is used at a medium to tissue ratio during homogenisation of 3:1. Higher concentrations of sucrose during homogenisation, as used in the Chaveau procedure (Chaveau 1956), tend to produce local heating effects, damaged nuclei and low yields.

No one pH may yet be described as the optimal pH for the isolation of nuclei. Most methods that have subsequently yielded nuclear envelope preparations use a pH of between 6.1 and 7.6 (Agutter 1972; Zbarsky 1969). Use of a higher pH would result in chromatin decondensation and lead eventually to nuclear lysis; very much lower pH would both extract intranuclear components and disrupt the outer nuclear membrane. Within the range around pH 7 the most important factor in deciding which pH is most suitable is the degree of contamination found (Fry 1976).

Inclusion of millimolar concentrations of magnesium, calcium or monovalent ions is essential to stabilise the nucleoplasm (Hogebroom et al. 1948; Potter 1955) and may contribute to the stability of pore complex material. Magnesium or monovalent ions are generally preferred to calcium which can cause organelle clumping in dense sucrose media and may also activate proteolytic and transaminase activities. PMSF, EGTA and tetrathionite are useful precautions

against proteolysis but EDTA cannot be used to inhibit metal ion dependant proteolysis since this would cause nuclear lysis. Octanol has been added to decrease foaming during homogenisation (Franke et al. 1970) but its use has been critisised as possibly causing fixation of contaminating protein onto nuclei (Wunderlich et al. 1976).

Detergents such as Triton X-100 and the Tweens have frequently been used in the isolation of nuclei from tissue culture cells (see review by Smuckler et al. 1976) in order to decrease cytoplasmic contamination, but the use of such agents is, of course, almost entirely precluded in a study directed towards either the proteins or the lipids of the nuclear envelopes.

2.2.5. Centrifugation Procedures

Nearly all centrifugation schemes for preparing nuclei are variations of the methods of Widnell and Tata (1964) and Blobel and Potter (1966); both of which are derivatives of the original dense sucrose procedure of Chaveau (1956). The Blobel and Potter (1966) method employs a single step discontinuous density gradient purification procedure of high yield and purity. However it severely restricts the amount of tissue that can be processed. At least three times as much tissue can be processed if nuclei are first concentrated from the homogenate using a

short low speed centrifugation. Against the great advantage of using a low speed spin to concentrate nuclei must be weighed the possibility that this will increase the likelihood of adsorbing cytoplasmic contaminants onto the nuclear surface; total yield may be increased at the expense of purity. Such a consideration is supported by the evidence (Kasper and Kubinski 1971) that purified nuclear membrane can form a hybrid complex with microsomal membrane. In addition, the more times nuclei are pelleted and resuspended the greater is the likelihood of damage to the outer nuclear membrane.

2.2.6. Tests of Purity.

Many enzyme tests, with their inherent problems of enzyme relocation, activation and destruction, have been used to assess the purity of nuclear fractions. Many such tests are of dubious or no value. Isolated rat and bovine liver nuclei and nuclear membranes demonstrably contain enzymes such as glucose-6-phosphatase (Berezney et al. 1972; Kay et al. 1972; Kartenbeck et al. 1973; but see also Franke et al. 1970; Agutter 1972b), rotenone insensitive NADH cytochrome C reductase (Kashnig and Kasper 1969; Zbarsky et al. 1969; Berezney et al. 1972) and also the cytochrome b5 (Franke et al. 1970; Kasper 1971; Berezney et al. 1972; Matsuura and Ueda 1972), all

of which are characteristic components of the endoplasmic reticulum but whose established presence in isolated nuclei and nuclear envelopes at high activities point more to the nuclear envelope being a specialised form of endoplasmic reticulum, than to contamination of nuclei by endoplasmic reticulum.

Similar problems relate to the use of cytochrome oxidase and cardiolipin as markers of mitochondrial contamination (see Berezney et al. 1972; Berezney and Crane 1972; Zbarsky 1972; Franke 1974; Jarasch and Franke 1974; Kasper 1974; Franke et al. 1976; Wunderlich et al. 1976; Jarasch and Franke 1977). Controversy rages around whether the small levels of cytochrome oxidase and cardiolipin found in nuclear preparations can or can not be accounted for by estimates of contamination based on other mitochondrial markers. In a careful study, Jarasch and Franke (1974) correlated the amounts of cytochrome oxidase found in nuclear envelope preparations with the amounts of cardiolipin and mitochrondrial type cytochromes (a, a_3, b, c) present, as well as with the activities of the NADH dehydrogenase complex (mitochondrial) and the mitochondrial contamination determined by morphometric analysis. They were not able, however, to correlate the cytochrome oxidase activities with succinate dehydrogenase (another

mitochondrial marker). Berezney's group considered that both cytochrome oxidase and cardiolipin were truely endogenous to the nuclear membranes and have even sought to demonstrate this by a reinterpretation (Wunderlich et al. 1976) of Jarasch and Franke's data. Jarasch and Franke (1977) now claim to produce highly purified nuclear envelope completely devoid of both cytochrome oxidase and cardiolipin: but since their method of preparing nuclear envelope was rather rigorous one may presumably argue that they have merely extracted these components from the preparation. Because cardiolipin and cytochrome oxidase have for long been regarded as mitochondrial markers, the burden for proof must fall heavily on those who would claim that they are also present in nuclei; and as yet, the evidence is not overwhelmingly convincing.

Succinate dehydrogenase does appear to be a valid marker for mitochondrial contamination, as do rotenone sensitive NADH dehydrogenase and monoamine oxidase (although the latter is contrary to the claims of Gorkin 1971). However succinate dehydrogenase is easily inactivated by the experimental stress experienced during isolation of nuclear membranes (Jarasch and Franke 1974) and so is best used as a marker during stages of isolating nuclei rather than nuclear envelopes; monoamine oxidase activity is easily masked even at intermediate levels and so may be missed.

As regards markers of plasma membrane contamination, 5' nucleotidase has been demonstrated cytochemically in whole nuclei (Sikstrom et al. 1976) and so may be of little use as a marker of plasma membrane contamination.

The overall picture for well known marker enzymes being useful measures of contamination in isolated nuclei is one of such uncertainty that Franke et al. (1976) have begun to use morphometric determinations of membranous contamination in ultrathin sections of isolated nuclei. The drawback with this technique is that the procedures of fixation and embedding involved in the preparation of a tissue for electron microscopy may in themselves violate the integrity of the nuclear membranes and thus give a falsely pessimistic impression of both the degree of integrity and contamination. Despite this, the technique probably gives the most reliable estimate of membranous contamination.

2.3. Materials and Methods in the Isolation of Nuclei

On the basis of considerations outlined above it was decided to use rat liver as a source of nuclei (although pig liver was used early on) and to use the method of Kay et al. (1972), with some modification, to prepare nuclei. The modifications included the use of PMSF as a proteolytic inhibitor, buffering of all sucrose media, the use of a swing-out rotor rather

than a fixed angle rotor to provide a cleaner separation of nuclei and lastly, the homogenate in dense sucrose was layered over a pad of clean sucrose prior to ultracentrifugation in order to minimise contamination by small dense particles and soluble cytoplasm.

Integrity of the nuclear envelope was estimated by morphometric determinations of the outer nuclear membrane in ultrathin sections of isolated nuclei. Purity was estimated on the basis of morphometric determinations and by succinate dehydrogenase assay.

2.3.1. Experimental.

Nuclei were isolated by a dense sucrose procedure similar to that of Kay and Johnston(1972). Three female Wistar rats (250 grams), fed ad libitum, were killed by cervical dislocation and their livers were removed as rapidly as possible and placed in ice cold homogenisation buffer (10% w/v sucrose 3mM $MgCl_2$ 0.2mM PMSF 0.2mM $NaHCO_3$ pH 7.4). All subsequent operations were performed at 0-4°C. Connective tissue was cut away and the livers were minced finely with scissors. The fluid was decanted and 20 grams of chopped liver weighed into a beaker containing 60 ml of ice cold homogenisation buffer. The liver suspension was homogenised in 2 x 40 ml aliquots in a motor driven glass/teflon Potter-Elvehjem homogeniser. Each aliquot was initially dispersed with 2 passages (15 secs) of a

wide clearance pestle (500 rpm), before being completely dispersed using a medium-fit pestle (approx. 25 passages). After this procedure, only one cell per 20 nuclei could be seen in the homogenate under phase contrast. The homogenate was then filtered through two layers of fine nylon mesh (Boots Co. Ltd. Nylon straining bag - fine mesh) and centrifuged at 700 g max in the 6 x 100 swing-out rotor of an MSE Mistral 4L centrifuge for ten minutes. The supernatant was discarded and the pellet was resuspended in 50 mls of homogenisation buffer by shaking. The suspension was centrifuged as before, the supernatant was again discarded and the pellet of crude nuclei was resuspended into 90 mls of dense sucrose (2.4M sucrose 1mM $MgCl_2$ 0.2mM PMSF 0.5mM $NaHCO_3$ pH 7.4) by vigorous shaking. The refractive index of the homogenate was measured using a refractometer and the sucrose concentration adjusted to 59%(w/w) sucrose. This suspension was layered over 12 mls of dense sucrose in 38 ml capacity centrifuge tubes and the interface of the two layers was stirred with a spatula. The tubes were centrifuged at 60,000 g max (18,000 r.p.m.) in the 6 x 38 ml swing-out rotor of an M.S.E. Prepspin 50 centrifuge for 80 minutes, which pelleted the nuclei.

After centrifugation the heavy brown plaque at the top of the tubes was loosened (by rimming with a spatula) and removed along with the supernatant by quickly inverting the tubes and allowing them to drain

thoroughly. The walls of the tubes were wiped clean with a tissue and then rinsed with distilled water. The pellet of clean nuclei was gently resuspended into 5 mls of sucrose buffer (10% w/v sucrose 1mM $MgCl_2$ 0.2mM PMSF 0.2mM $NaHCO_3$ pH 7.4) using a syringe and 6 inch 17 gauge needle. Finally, the suspension of nuclei was diluted to 50 mls with sucrose buffer and centrifuged at 700 g max in the 6 x 50 ml swing-out rotor of an M.S.E. Mistral 4L centrifuge for 5 minutes. This final pellet was designated purified nuclei.

2.3.2. Assay Methods

Except where stated to the contrary, all reagents used were of analytical reagent grade quality (A.R.).

1) Succinate dehydrogenase

Succinate dehydrogenase was assayed by a modification of the method of Singer (1975), by the reduction of phenazine methosulphate (PMS) by succinate and its dehydrogenase. The assay mixture was as follows, all solutions being pre-incubated at 38°C.

0.75 mls	0.2M sodium phosphate buffer pH 7.8
0.45 mls	10mM potassium cyanide (neutralised-HCl)
0.2 mls	0.6M succinic acid, adjusted to pH 7.8 with NaOH
1.0 mls	H_2O
0.1 mls	sample

The above mixture was incubated in 3 ml spectrophotometer cuvettes for 10 minutes in order to permit activation of the enzyme. Water rather than succinate was added to the reference cell. Finally, the following were rapidly added to the cuvettes and the optical density at 600 nm followed continuously at 38°C in a Beckman DB spectrophotometer coupled to a Fisons Vitatron recorder set to 0-0.2 OD full scale deflection (linear).

0.1 ml 15mM dichloroindophenol (Sigma)

0.2 ml 9mM phenazine methosulphate (Sigma)

All enzyme preparations were freeze-thawed and briefly sonicated in order to assure free penetration of the dye. Activity is calculated from the absorbance decrease, using the millimolar extinction coefficient for DCIP of 19.1 at 600 nm.

2) Protein

Protein was assayed by the method of Lowry et al. (1951) except that incubation in copper alkali solution was performed at 60°C for 45 minutes thereby decreasing variability of membrane protein assay. Several procedures described in the thesis resulted in spuriously high Lowry protein readings. In particular Triton X-100, Tris and cystamine all interfered with the reaction. Therefore, except where the Triton X-100/protein ratio was extremely high, and in consequence the real protein level impossible to determine, samples were dialysed exhaustively against distilled water prior to assay.

3) DNA

DNA was assayed by the Giles' and Myers' modification (Giles and Myers 1965) of the method of Burton (1956). Analar diphenylamine (BDH) was recrystallised three times from ethanol before use. Acetaldehyde (May and Baker) was redistilled once.

To 0.25 mls sample was added 0.05 mls 30% w/v A.R. perchloric acid (Fisons) and the mixture was incubated at 70°C for 20 minutes with vortexing every 2 minutes. Organic matter persisting after this time was removed by centrifugation at 2,000 g for 20 minutes. 0.25 mls of the PCA digest was then added to 0.4 mls of diphenylamine reagent (4% w/v diphenylamine, 0.4% w/v acetaldehyde in glacial acetic acid) and the mixture was incubated for 16-24 hours at 32°C. The optical density at 595 nm was read against a reagent blank. Any absorbance at 700 nm was subtracted from this value. Because sucrose interferes with the reaction, all samples were dialysed prior to assay. Deoxyadenosine monophosphate (Sigma) was used as standard and DNA values calculated according to the assumption that G + C content of DNA = 50%, the ratio -

$$\text{ug deoxypurinenucleotide /ug DNA} = {}^{1}/_{2.0}$$

4) RNA

RNA was assayed by a modification (P.S. Agutter, unpublished) of the orcinol method of Schneider (1957). Reagent grade orcinol (BDH) was recrystallised from toluene before use.

To 0.25 mls of sample was added 0.05 mls 30%

($^{w}/w$) perchloric acid (Fisons) and the mixture was incubated at 70°C as in the DNA assay. Insoluble material was removed by centrifugation. The supernatant was mixed with 2 mls $FeCl_3$ reagent (0.5 mls 10% $^{w}/v$ $FeCl_3$ in 100 mls 12N HCl), 0.15 mls orcinol solution (1.5 grams orcinol in 25 mls ethanol - can be kept stored at -20°C for several weeks) and was incubated at 100°C for 20 minutes. The tubes were cooled in crushed ice and the optical density at 660 nm was read within 20 minutes (colour is unstable).

D-ribose was used as standard and the assumption was made that ug D-ribose/ug RNA = $^{1}/2.37$ (P.S. Agutter 1972a).

DNA gives a slight but measurable colour with orcinol, thus making a spurious contribution to the apparent amount of RNA present. Compensation was made for this by measuring the amount of DNA present by the diphenylamine reaction and subtracting the value this would contribute to the orcinol reaction. This was done from a plot of the interference of calf thymus DNA (Sigma) in the orcinol reaction.

5) Phospholipid

Lipid was extracted from membrane samples according to Bligh and Dyer (1959) and the extract was evaporated to dryness. The dry sample was incubated in 0.15 mls 70% ($^{w}/w$) perchloric acid for 15 minutes at 145°C in a hot oven. If organic matter persisted

after this time, 10 microlitres of 30% ($^{w}/v$) A.R. H_2O_2 (BDH) was added and the incubation continued for a further 90 minutes.

Phosphate was assayed according to Chen et al. (1956). Samples containing 1-5 ug of phosphorous were adjusted to 2 mls with water followed by the addition of 2 mls colour reagent (2% $^{w}/v$ ascorbic acid, 0.5% $^{w}/v$ ammonium molybdate in 0.6M sulphuric acid) and incubated at 37°C for 2 hours. The solutions were cooled to room temperature and the optical density at 820 nm was read against a reagent blank. KH_2PO_4 was used as standard.

Before assay, all glassware was soaked in chromic acid for 7 days followed by exhaustive washing in double glass distilled water, soaking in concentrated Decon for 24 hrs and further extensive washing in double distilled water.

Assuming an average molecular weight for phospholipid of 800 and a mol. wt of 95 for phosphate,

ug phosphate/ug phospholipid = 95/800

2.3.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Proteins were separated by polyacrylamide gel electrophoresis in the discontinuous buffer system of Laemmli (1970) using an electrophoresis module of the type described by Studier (1973). Electrophoresis was performed in 2 mm thick slab gels comprising a

stacking gel (3.75% w/v acrylamide, 0.1% w/v N,N' methylenebisacrylamide) and a resolving gel (16% w/v acrylamide, 0.094 % w/v N,N'-methylenebisacrylamide).

The gels were cast between glass plates separated by 2.00 mm perspex spacers. The plates and spacers were clamped together with metal spring clips and the base was sealed with plasticine; dimensions, 12 x 12 x 0.2 cm. Resolving gel solution (30 ml), polymerised by the addition of ammonium persulphate to 0.025% (w/v) and N,N,N',N'-tetramethylenediamine (TEMED) to 0.025% (v/v), was quickly poured between the glass plates and the surface was carefully overlayed with distilled water. After an hour, the overlay was removed with a syringe and the surface of the polymerised gel was washed with distilled water. Stacking gel, polymerised by the addition of ammonium persulphate to 0.625% (w/v) and TEMED to 0.083% (v/v), was rapidly layered onto the resolving gel and a perspex comb, providing 12 sample wells 6.5 mm wide, was quickly inserted into the surface of the gel solution such that the distance between the surface of the resolving gel and the base of the comb was 1.5 cm. The gel polymerised within 8 minutes and the comb could then be removed. The wells formed by the comb were immediately rinsed with distilled water to remove any unpolymerised acrylamide and the gel was then ready for electrophoresis.

Stacking gel buffer : 0.1% w/v SDS, 125mM Tris. HCl pH 6.8

Resolving gel buffer : 0.1% w/v SDS, 375mM Tris. HCl pH 8.7

Tank buffer: 0.1% w/v SDS, 250mM Tris, 192.5mM Glycine pH 8.3

After removal of the plasticine, the slab was sealed into the electrophoresis module with high vacuum grease (Edwards High Vacuum) and retained in position with metal spring clips. Samples, never greater than 25ul, were loaded into the wells formed by the perspex comb using a Hamilton syringe. The samples were electrophoresed at 20mA until the bromophenol blue tracker dye reached the resolving gel when the current was raised to 40mA; running time was approx. 3.5 hours.

Sample preparation

Samples were precipitated in 2 volumes ethanol at -20°C for 16 hours prior to electrophoresis in order to decrease the presence of detergent and salts. Pelleted samples were then washed with an equal volume of distilled water (to remove excess ethanol) and resuspended into 5 volumes of a solution containing 3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 1mM EDTA and 62.5mM Tris. HCl pH 6.8. The samples were then incubated for 10 minutes at 70°C and for 5 minutes at 100°C. Particulate material

remaining after this time was removed by centrifugation at 3,000 g max for 5 min. Samples could be stored at -20°C for many months without obvious deterioration. Protein standards used were

E. coli β-galactosidase	MW 130,000
Bovine serum albumin	MW 68,000
Chick brain tubulin	MW 55,000
Rabbit muscle actin	MW 46,000
Lactate dehydrogenase	MW 35,000
β-lactoglobulin	MW 17,500
Beef heart cytochrome C	MW 12,500

Fixation and Staining

After electrophoresis, gels were removed from between the glass plates and fixed and stained according to Fairbanks *et al.* (1971) while gently agitating in a shaker bath. The sequence was as follows -

A. 25% (v/v) isopropanol, 10% (v/v) glacial acetic acid, 0.025% (w/v) Coomassie blue OVERNIGHT using 400 ml (or more) stain per gel.

B. 10% (v/v) isopropanol, 10% (v/v) acetic acid and 0.0025% Coomassie for 6-9 hrs.

C. 10% (v/v) acetic acid for several short changes - over a period of 2-3 days.

Drying of gels for Autoradiography and Fluorography

Gels were dried down onto thick filter paper under vacuum at 90°C using a 'Bio-Rad Gel Slab Dryer'

(Model 224). The whole procedure took less than 3 hours and was accomplished without cracking of the gels. Dry gels were either exposed directly to X ray film (Kodak X-Omat H film) or the film was first flash exposed, backed with an intensifying screen (Ilford Fast Tungstate) and closely opposed to the gel in a cassette at -70°C for 3-10 days. The fluorographic method appeared to be about 10 times faster than straight forward autoradiography but gave slightly inferior resolution.

Electrophoresis Reagents

Superior resolution was obtained if acrylamide (BDH) was recrystallised from chloroform and if N N'-Methylenebisacrylamide (BDH) was recrystallised from acetone. Use of specially purified acrylamide from BDH or from Bio-Rad Laboratories resulted in significantly inferior resolution (recrystallisation of the specially purified acrylamide marketed by BDH failed to improve the quality of resolution).

Other reagents:

A.R. Glycine (Fisons)

Trizma base (Sigma)

Trizma HCl (Sigma)

SDS. specially purified (BDH) - or Fisons if recrystallised from ethanol.

2.3.4 Microscopy

Phase contrast microscopy

Homogenates were routinely examined for cellular disruption under a Vickers phase contrast microscope using a X 40 objective. Purified nuclei were monitored for purity and integrity, and nuclear envelope was examined for chromatin contamination, under a Zeiss Ultraphot microscope and X 40 objective.

Electron microscopy

Electron micrographs were taken with a Philips EM 300 operating at 80KV. Kodak 'Estar' sheets were used in the camera.

a) Embedding and thin sectioning.

Material was pelleted in Eppendorf tubes and fixed for one hour by overlaying with 3.4% ($^{w}/v$) glutaraldehyde, 2mM $MgCl_2$, 200mM sodium cacodylate, pH 7.2 at $4^{o}C$. After fixation the pellet was washed three times at ten minute intervals by overlaying with glutaraldehyde-free cacodylate buffer and then post-fixed at room temperature in 1% ($^{w}/v$) osmic acid (Fisons), 100mM cacodylate, 2mM $MgCl_2$, pH 7.0 for one hour. The pellet was then washed twice at ten minute intervals with cacodylate buffer and finally left overnight in cacodylate buffer.

The next morning, the pellet was removed from the Eppendorf tube and dehydrated through the following alcohol series and propylene oxide.

30% EtOH	10 mins
30% EtOH, 1% ($^{w}/v$) uranyl acetate	90 mins in the dark
50% EtOH	30 mins
75% EtOH	10 mins
100% EtOH	10 mins (X2)
100% Propylene oxide	10 mins (X3)

The dehydrated pellet was placed in 5 mls of 25% ($^{v}/v$) embedding mixture/75% propylene oxide and placed on a rotary agitator overnight.

Embedding mixture

50 mls hardener (dodecenylsiccinic anhydride)

50 mls resin (Araldite CY212)

1½ mls accelerator (benzdimethylamine)

The next day the pellet was placed in fresh 100% embedding mixture and left on a rotary agitator for a further 8 hours. Finally the pellet was placed in fresh embedding mixture in a BEEM capsule and incubated at 60°C for 48 hours after which the resin was fully polymerised.

Thin sectioning was performed on an LKB ultra-microtome using either glass or diamond knives. When sectioning the pore-lamina fraction of the nuclear envelope it always proved necessary to use diamond knives. The sections were stained with uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963) before examination.

b) Negative staining

Negative staining of unfixed material with 2% ($^{w}/v$) ammonium molybdate, pH 7.2, was performed using the single droplet technique of Harris and Agutter (1970). A single drop of sample (sucrose free) and a single drop of stain were placed on a small sheet of parafilm. Membrane suspension was picked up from the parafilm on a carbon coated specimen grid. Most of the suspension was then drawn off with a filter paper and a drop of stain was picked up in the same manner. After 20 secs, excess stain was drawn off with a filter paper and the thin film of membrane and stain was allowed to dry.

Morphometry

Electron microscopic examination of subcellular fractions is, as here, normally performed on material collected by centrifugation. Quantitative morphological information is thus difficult to obtain because the distribution within a pellet is far from homogeneous and the number of particle profiles seen in a section does not supply a direct estimate of the number of particles in a preparation. Baudhuin (1967) has introduced a filtration method for pelleting very thin pellicles of particles on millipore filters. The main advantages of this technique are that it produces heterogeneity solely in the direction perpendicular to the

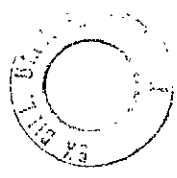
surface of the pellicle and that sections covering the whole depth of the pellicle can be photographed in a single field; sampling can then be effectively random. Lacking the apparatus needed for this technique, the following measures were taken in order to obtain a good estimate of different membrane profiles.

1) Samples were pelleted from a thick sludge of purified nuclei in order to minimise centrifugal separations.
2) Samples were randomly orientated within the plastic blocks.
3) Sections were cut from different angles within a block and from more than one block.
4) Electron micrographs were taken at low magnifications in order to obtain a large field, and analysed at higher magnifications.

Morphometric determinations of nuclear integrity and membranous contamination were carried out on electron micrographs taken at between 3,000 and 5,000 diameters magnification. Negatives were displayed on a Carl Zeiss (Jena) Dokumator DL-2 Microfilm reader and examined at magnifications between 6.5 and 17.5 diameters. Length measurements were made at 6.5 diameters using a 'Map Measure' and converted to microns original membrane.

Nuclear membranes were classified as membrane profiles which were associated with nuclear chromatin in at least one site, and which contained pore complexes

(Franke et al. 1976). The circumference of very small vesicles was approximated to three times the largest diameter.



2.4 Characterisation of Isolated Nuclei

2.4.1 Yield of Nuclei

Nuclear preparation	Recovery of DNA%
1	76
2	83
3	81

Table 1. Estimates based on the recovery of DNA from the initial homogenate

2.4.2 Composition

Protein %	DNA %	RNA %	Phospholipid %
75	20	3	2

Table 2. Values based on the approximation that Protein + DNA + RNA + Phospholipid = 100%. In nuclei and in membrane material prepared from nuclei, phospholipid accounts for 62-70% of the total lipid (Gurr et al. 1963; Keenan et al. 1970; Kleinig 1970).

2.4.3 Succinate Dehydrogenase

Fraction	SDH activity	% activity of mitochondria
mitochondria		
preparation 1	0.43	
2	0.54	
3	0.50	
Mean	0.49	100
nuclei		
preparation 1	0.0019	
2	0.0024	
3	0.0032	
mean	0.0025	0.5

Table 3. Activity of succinate dehydrogenase is expressed as umoles DCIP reduced per minute per milligram of protein. Mitochondria were prepared as 200,000 g.min pellets from post-nuclear supernatants and washed three times.

2.4.4 Morphometric analysis.

a) analysis of membrane contamination

Nuclear preparation	Membrane profile lengths nuclear (u)	other (u)	nuclear mem/total %
1	682	24	97
2	678	41	94
3	849	72	92
4	1094	83	93

Table 4. Values are expressed in microns of membrane profile. Nuclear membranes are all membrane profiles which are associated with nuclear chromatin in at least one site and which contain pore complexes.

b) integrity of the outer nuclear membrane

nuclear preparation	membrane profile length		ONM/INM %
	inner	outer	
1	366	315	86
2	353	323	92
3	427	375	88

Table 5. Determination of the extent to which the inner nuclear membrane is covered by outer nuclear membrane in purified nuclei. Values are expressed in microns of membrane profile in thin section.

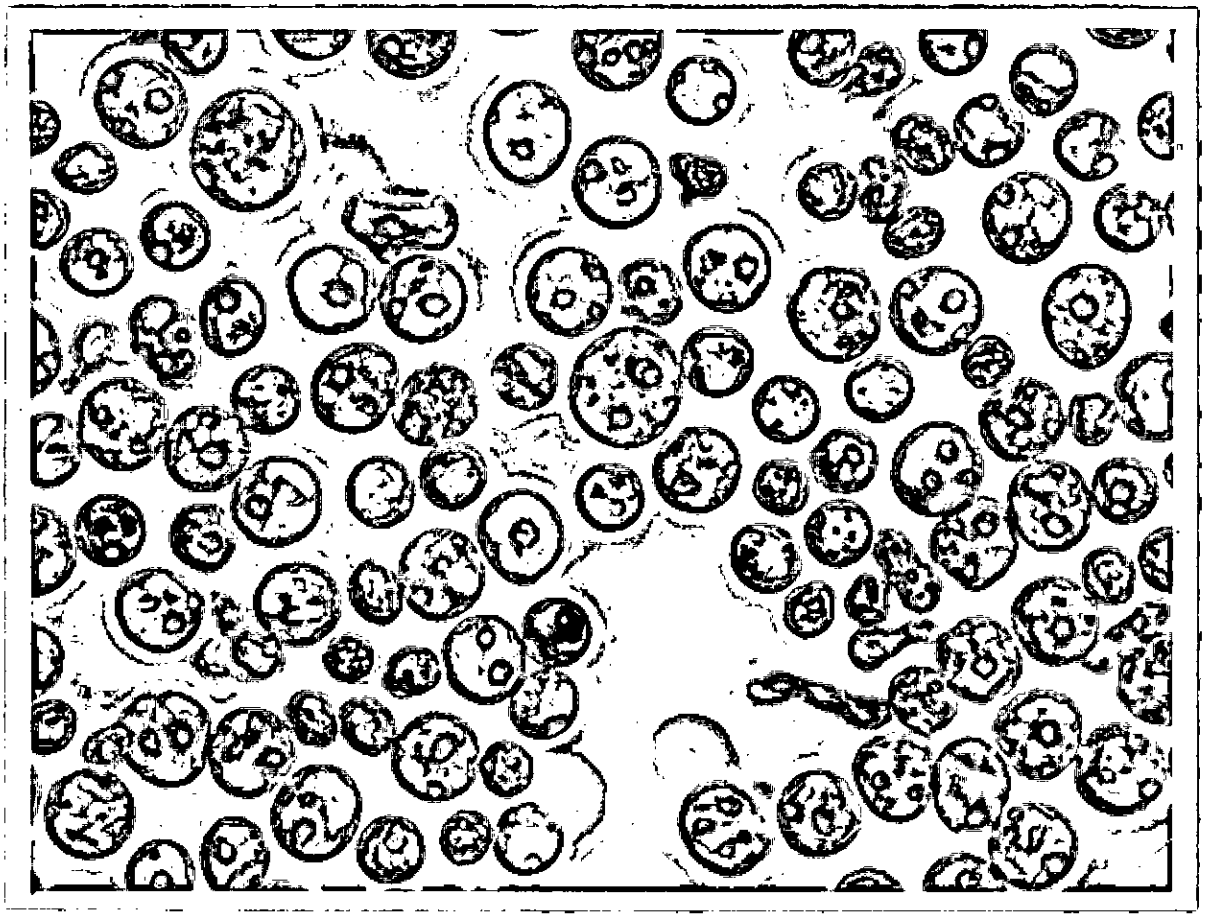


Plate 1 Phase contrast micrograph of purified nuclei (X 1300). In this survey micrograph approx. 100 nuclei can be seen. Although 4 grossly distorted nuclei are apparent, the majority of nuclei are rounded and nucleoli are quite distinct. No mitochondria or membrane vesicles are evident.

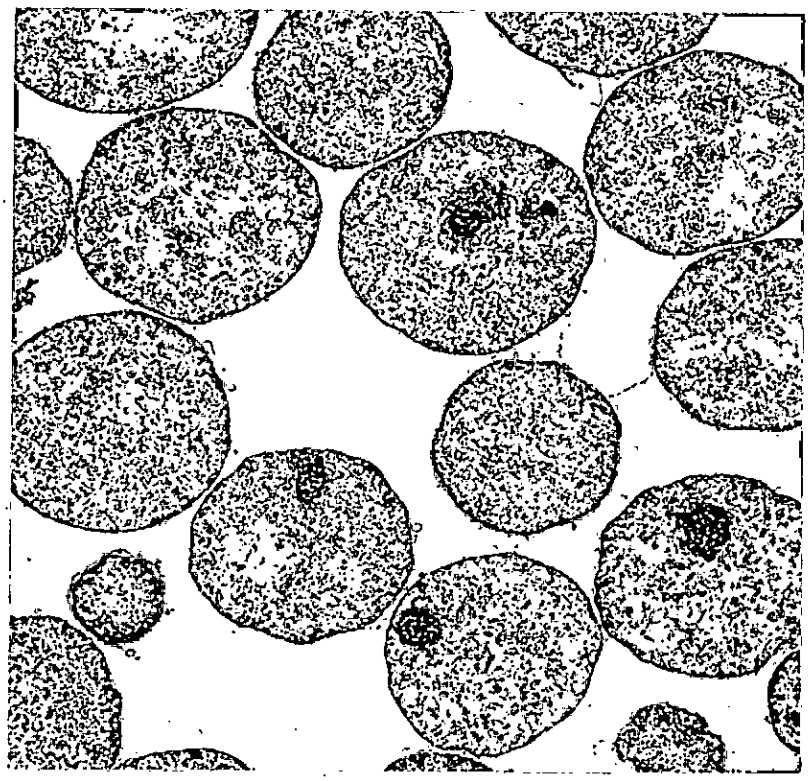


Plate 2 Survey electron micrograph of purified nuclei (X4,000). The nuclei are rounded and nucleoli are quite distinct. There appears to be very little leakage of intra-nuclear components. The outer nuclear membrane of some nuclei has been peeled away in places and this appears to be due to nuclei sticking together and then parting. The inner and outer nuclear membranes are in very close apposition which resembles the *in vivo* state.

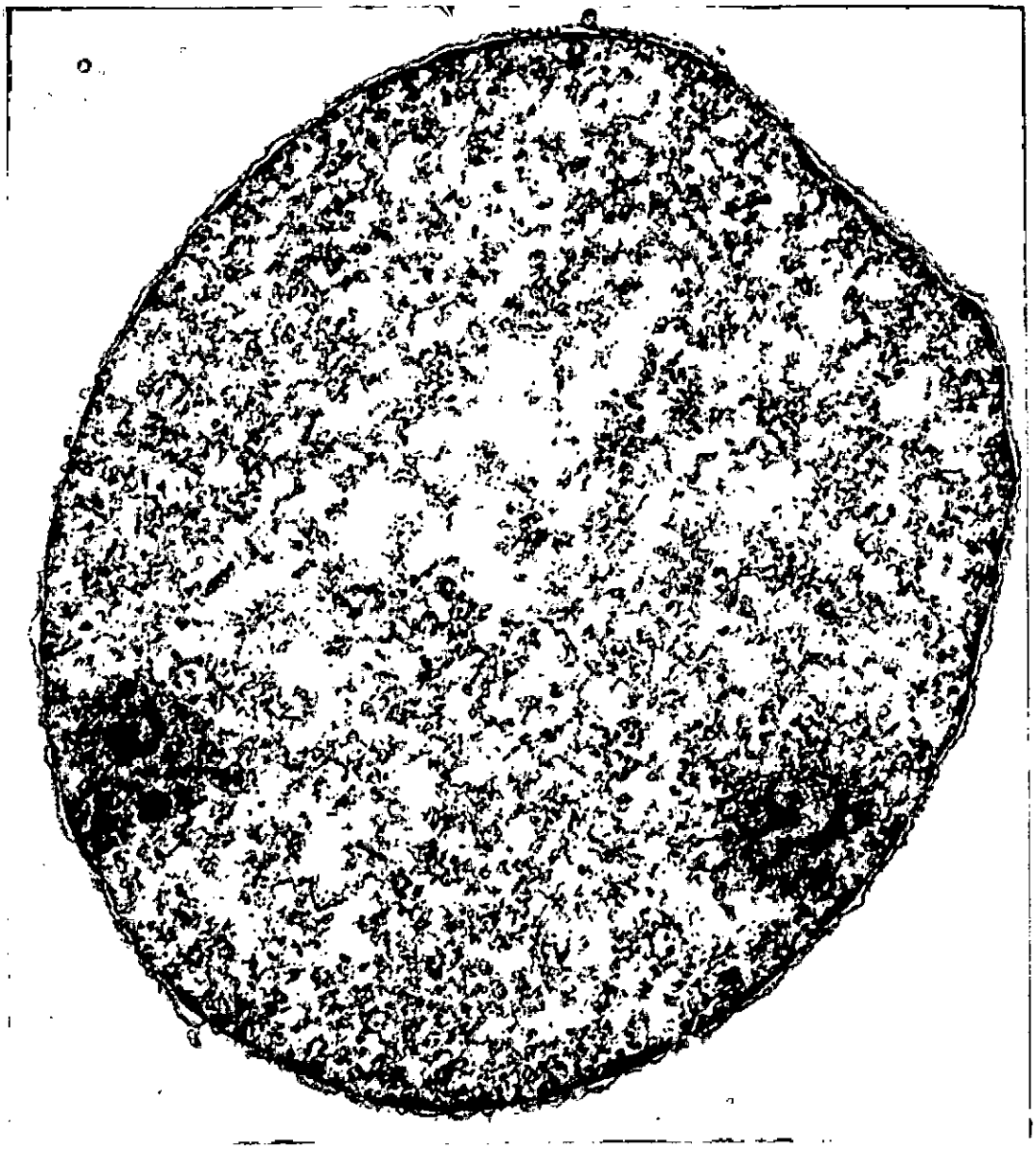


Plate 3 Electron micrograph of single nucleus (X21,000). The inner and outer nuclear membranes are clearly resolved. Pore complexes are seen to fuse the two membranes and ribosomes can be discerned on the cytoplasmic surface of the outer membrane. In places, the outer nuclear membrane has been completely removed but this represents the loss of only a small proportion of the outer membrane. Little cytoplasmic contamination can be seen adhering to the cytoplasmic surface of the outer nuclear membrane.

2.5 Discussion.

Nuclei are prepared at a yield greater than 75% (Table 1) by chemically mild means providing a sample that is both sufficient for biochemical analysis and presumably representative of the tissue as a whole. Electron micrographs of purified nuclei show little cytoplasmic contamination and demonstrate a high degree of integrity of the nuclear envelope. The low degree of cytoplasmic contamination of nuclei is confirmed by succinate dehydrogenase assay (Table 3) an RNA/DNA ratio of 0.15 (Table 2) and by morphometric analysis (Table 4). The integrity of the nuclear envelope is demonstrated both by the high proportion (90%) of inner membrane covered by outer membrane (Table 5) and the close apposition of the two membranes which resembles the *in vivo* state (dense sucrose procedures can sometimes result in considerable loss of the outer nuclear membrane and in gross swelling of the envelope cisterna and blebbing of the outer nuclear membrane - see Kartenbeck *et al*. 1973).

Purity with regard to membranous contamination from other organelles is equal to or greater than that achieved by Franke *et al*.(1976) who used a much more rigorous and lengthy centrifugation procedure, involving two dense sucrose steps, to provide

the most highly characterised nuclei for the preparation of nuclear envelope so far published.

Whilst the aims of providing nuclei of high yield, purity and integrity have been met, the preparation time of three hours from sacrifice of animals to obtain purified nuclei, must be regarded as rather long. However, shorter preparation times using conventional centrifugation techniques could only result in greater contamination, a lower yield or both.

It is concluded that the nuclei prepared by this procedure provide an acceptable starting material for the preparation of nuclear envelope.

2.6 The release of nuclear envelopes from purified nuclei

2.6.1 General considerations

The release of nuclear envelope from purified nuclei requires the prior destruction of the links between the nuclear matrix and its peripheral layer known as the fibrous lamina. The fibrous lamina and its associated pore complexes are presumably leant greater stability than the rest of the nuclear matrix by virtue of their close association with the nuclear membranes. This point has so far been almost completely ignored (exception: see Wunderlich *et al*. 1976). A preparation of nuclear envelopes is not necessarily pure merely if it is completely devoid of DNA - although this

may be a useful starting indicator. One may for example consider two theoretical possibilities. 1) An envelope preparation which though containing DNA, possesses little matrix material or 2) an envelope preparation which, though completely devoid of DNA is highly contaminated with matrix. The second case is of course the more likely for the matrix may be very closely structured with or provide a skeletal framework for DNA in the nucleus; but the examples serve the point that a low DNA content in a nuclear envelope preparation is not necessarily a good indication of purity. Variable quantities of matrix associated with nuclear envelopes may be expected to show in the phospholipid/protein ratios of different preparations. Certainly values do vary from 1.06 (Zbarsky et al. 1969) to 0.21 (Franke et al. 1973) in preparations of rat liver nuclear envelopes which contain less than 3% DNA (see Table 6). Of course, these differences may in part be accounted for by different methods of chemical determination and by differences in the degree to which the nuclear envelopes are cross contaminated by other cellular membranes. Nonetheless, such huge disparities in the phospholipid/protein ratio do suggest rather fundamental differences in the character of the two preparations. Envelope preparations which, low in DNA, show separation of the inner and outer membranes

and considerable loss of pore complex material may be expected to show a comparatively high phospholipid/protein ratio. Preparations which, low in DNA, show close apposition of the inner and outer membranes and high integrity of the pore complexes (and therefore fulfil the definition of 'envelope' - see section 2.1.1) will be expected to show a rather lower phospholipid/protein ratio.

2.6.2 Methods for disrupting nuclei

a) Manual disruption

The simplest method of disrupting nuclei is that of manually bursting the nuclei with a fine pipette (Scheer 1972). However this can only be applied to giant nuclei and could never provide the substantial quantities of material needed for biochemical analysis. The greatest use of this technique is in rapidly providing, by mild means, superbly preserved nuclear envelopes for ultrastructural analysis.

b) Sonication

Sonication has been extensively used in bulk isolation procedures in combination with extraction in either low or high salt solutions (Agutter 1972b; Franke 1966, 1967, 1970; Jarasch et al. 1973; Kartenbeck et al. 1973; Kashnig and Kasper 1969; Moore and Wilson 1972; Philipp et al. 1976; Stavy et al. 1973; Yoo and Bayley 1967;

Table 6. Phospholipid/protein ratios of nuclear envelope preparations.

Tissue	Phospholipid/Protein Ratio	%DNA	Reference
Rat liver	0.36	8	Agutter (1972b)
Bovine Liver	0.32	1.1	Berezney et al. (1972)
Rat liver	0.69	0.4	Bornens and Courvalin (1978)
Rat liver	0.21	2.2	Franke et al. (1973)
Rat liver	0.35	3.4	Kartenbeck et al. (1973)
Rat liver	0.50 (light fraction)	0	Kashnig and Kasper (1969)
Rat liver	0.39 (heavy fraction)	0	Kashnig and Kasper (1969)
Rat liver	0.40	3.9	Kay et al. (1972)
Rat liver	0.20	3	Milne et al. (1978)
Rat liver	0.31(5)	0.6	Monneron et al. (1972)
Rat liver	1.06 (light fraction)	0.4	Zbarsky et al. (1969)
Rat liver	0.57 (heavy fraction)	1.6	Zbarsky et al. (1969)

Zbarsky et al. 1969; Zentgraf et al. 1971) but there is a tendency to produce small, frequently single, membrane fragments and vesicles. Sonication is difficult to control precisely and can lead to both excessive fragmentation and poor yields.

c) Low ionic strength methods

The commonly cited advantage of low ionic strength methods is that of 'mildness' but they may also lead to contamination by non-membranous elements; in particular, nucleoplasmic proteins, ribonucleoproteins and nucleolar components. Evaluations of such contaminants is of course difficult since clear-out markers often do not exist.

Although the nucleus is not an osmometer and cannot be lysed by low ionic strength media per se, use can be made of the fact that divalent cations are necessary for chromatin condensation (Anderson and Wilbur 1951; Mirsky and Osawa 1961). Nuclei can be ruptured by decondensing chromatin in low ionic strength media containing only very low (< 0.2mM) concentrations of divalent cations (Harris and Milne 1974; Kay et al. 1972). The membranes may then be liberated from chromatin by digestion with DNase. Both the Harris and Milne (1974) and the Kay et al. (1972) procedures provide large membrane fragments and some nuclear 'ghosts' which demonstrate well preserved pore complexes. However, low ionic strength procedures tend towards rather higher DNA contents than high ionic strength methods and the need for

elevated temperatures during the DNase digestion may lead to proteolysis and enzyme inactivation.

d) High ionic strength methods

The fact that nuclei will lyse in high ionic strength media has been used by several groups to prepare nuclear envelopes (Mentre 1966; Monneron et al. 1972; Nozawa et al. 1973). The Monneron method, in particular, has been adapted and used successfully by other groups (Fukushima et al. 1976; Spangler et al. 1975; Sikstrom et al. 1976). Lysis occurs via the disruption of salt bridges leading to rapid swelling of chromatin and breakage of the nuclear envelope. This can produce a very gelatinous state from which it is difficult to remove membranes. However, the inclusion of glycerol has been found to decrease this tendency (Monneron et al. 1972). Nuclei from different tissues vary in their tendency toward gel formation (Barrack and Coffey 1974); a feature which may be related to the presence of endogenous nucleases (Wunderlich et al. 1976; see also Harris and Agutter 1976; Hewish and Burgoyne 1973).

The Monneron method produces membranes with recognisable pore complexes (Monneron et al. 1972; Harris and Agutter 1976) but the preparation is highly vesicular (Fry 1976). Fukushima (1976) claims that the preparation meets Agutter's (1972b) morphological criteria in consisting of double membranes containing pore

complexes but this is not immediately obvious from the published micrographs. Certainly pores are present but annular material is largely removed and in negative stain the pores seem fairly empty. One important objection to the use of high ionic strength media is that they will extract extrinsic membrane components. Moreover, high concentrations of KCl or NaCl are reported to remove pore complex material (Mentre 1969; Agutter 1972b). It would seem that maximal preservation of nuclear envelope morphology and molecular components can only be gained at the expense of a certain degree of nucleoplasmic contamination.

e) Polyanion Methods

Natural and synthetic polyanions can produce rapid nuclear swelling (Kraemer and Coffey 1970; Coffey et al. 1974) and thereby rupture the nuclear envelope. Addition of sufficient polyanion (particularly heparin) can render the chromatin completely soluble and large 'ghosts' of nuclear membranes may be obtained either in a simple one step centrifugation (Bornens 1973, 1977a; Bornens and Courvalin 1978) or by density gradient centrifugation (Hildebrand and Okinaka 1976; Wilson and Chytil 1976). The use of phosphate buffer appears crucial to this procedure. Although the reason for this is not immediately clear, the fact that EDTA may be substituted for phosphate suggests that the importance lies in the chelation of divalent cations. The isolated

membranes, which contain less than 0.4% DNA, are completely devoid of pore complexes although pores are present (showing, rather unusually, a diaphragm). As such, the Bornens procedure probably provides one of the cleanest membrane preparations derived from nuclei (it is, incidentally, described as an 'envelope' preparation). The method has several advantages:

1) Nuclear disruption can be achieved rapidly at 0-4°C without enzymic digestion which requires elevated temperatures or extended periods of incubation at low temperatures.

2) Nuclear lysis can be produced at physiological pH and without subjecting nuclei to high non-physiological salt concentrations.

3) The method achieves the astonishingly high yield of nearly 100%.

4) Contamination by DNA is very low.

5) An association between the centriole and the nuclear membranes can be demonstrated (Bornens 1977b).

The details of enzyme activities associated with the membranes after heparin treatment have yet to be published but even if heparin proves damaging to enzyme activities the method should become useful in studies of the membrane or non-pore complex proteins. Unfortunately, insufficient experimental detail of the Bornens procedure has been published to readily enable the method to be repeated. We have been unable to prepare

nuclear membranes using the Bornens procedure without recourse to the use of either EDTA or DNase digestion.

2.6.3 Choice of Preparative Method

Only two preparative methods, both using low ionic strength media, meet the requirements set out in section 2.1.2; those of Harris and Milne (1974, see also Harris and Agutter 1976; Agutter *et al*. 1977; Milne *et al*. 1978) and Kay *et al*. (1972 see also Kay and Johnston1977). As mentioned earlier (section 2.1.2), the Harris and Milne procedure proved to be grossly unreliable and although the method is described as 'rapid' it is in fact rather lengthy - taking, on average, four hours to obtain membranes from washed nuclei.

One is left with the Kay procedure which has several strong advantages. At the ultrastructural level, the membranes show the presence of numerous pore complexes. At high power, the annular subunits of the pore complex and central granule of the pore are quite evident. The membrane fragments are large and many whole 'ghosts' can be seen under phase contrast microscopy. Moreover, the outer membrane bears distinct ribosomes. There is however some separation of inner and outer membranes. The Kay method is rapid, taking only 80 minutes to obtain membranes from washed nuclei, and has been used in modified form by other groups (Aaronsonand Blobel 1975; Aaronson 1978; Dwyer and Blobel 1976; Franke *et al*. 1976).

There are some disadvantages to the method; in particular, the use of elevated temperatures (23°C) during the DNase digestions which may result in proteolysis. Jackson (1976) has noted that there are differences in the high molecular weight protein components of nuclear envelopes prepared from chicken erythrocytes in the presence and absence of proteolytic inhibitors. Apart from this one report, there are no other indications of proteolytic activity, or measures to counteract their potential presence, described in nuclear envelope preparations. I have been unable to detect changes in the polypeptide composition of rat liver nuclear envelopes prepared by the Kay procedure in the presence or absence of PMSF. Furthermore, if nuclear envelopes were prepared from nuclei that had been stored at 4°C for 24 hours, there was still no noticeable change in the polypeptide composition. Proteolysis does not, therefore seem to be a serious problem in the preparation of nuclear envelopes from rat liver. The use of a high pH (8.5), which is essential to the success of the Kay procedure, could remove extrinsic proteins and may inactivate nucleoside triphosphatase activity (Porteous et al. 1978). Further, low salt conditions can be plagued by the artefactual adsorption of proteins (section 2.6.2.C). The problems of either loss of extrinsic proteins or adventitious adsorption of proteins onto membranes are ones that bedevil all sub-cellular

Table 7. Composition of Rat Liver Nuclear Envelopes prepared by radically different procedures

References	Procedure	Density (gm/cm^3)	% Composition*			
			Protein	Phoso-lipid	RNA	DNA
Kartenbeck et al. (1973)	Sonication 2m NaCl extraction	1.21-1.23	67.1	23.5	6.1	3.4
Kashnig and Kasper (1969)	Sonication 10% ($^w/_v$)	1.16-1.18	64.5	32.1	3.4	0.0
	K citrate extraction	1.18-1.20	67.4	26.1	6.6	0.0
Monneron et al. (1972)	0.5M $MgCl_2$ extraction	1.18	73	23	3	0.6
Harris and Milne (1974) Milne et al. (1978)	Low salt DNase digestion	1.21	75	15	7	3
Kay et al. (1972)	Low salt DNase digestion Elevated pH.	-	65.7	26.7	3.6	3.9
Bornens (1977a) Bornens and Courvalin (1978)	Heparin treatment	1.18	57.5	39.6	2.4	0.4

* 100% = Protein + DNA + RNA + Phospholipid.

studies. Except where one is dealing with firmly established soluble enzymes such as lactate dehydrogenase (Evans 1978), there is no way out of this problem. One can extract a membrane with high and low salt, raise and lower the pH and then argue that the proteins that remain are, by virtue of their strong association with the membrane, truly membrane proteins. But it is not possible to resolve the problem of loosely associated or 'extrinsic' proteins.

The advantages of the Kay procedure may be listed as follows:

1) The membranes show a high degree of ultrastructural integrity and largely conform to the definition of 'envelope' (section 2.1.1.).

2) The method is reliable and has been used by other groups.

3) Preparation time in only 80 minutes.

4) The technique is 'mild' as demonstrated by high glucose-6-phosphatase activity. This is a rather labile enzyme, completely absent in the preparations of Agutter (1972b) and Franke et al. (1970).

The method does require some modification to reduce the level of contamination by DNA (presumably there is histone, non-histone and matrix protein concomitant with this) which at 3% is unacceptably high. Moreover, since both the preparation of nuclei and the preparation of envelopes are performed in low ionic

strength media, some precautions are also necessary to reduce the potential for adventitious adsorption of cytoplasmic proteins. Modifications, similar to those used by others (Dwyer and Blobel 1976) have been adapted and include the following:

1) Inclusion of a proteolytic inhibitor in all digestion media.
2) Omission of β mercaptoethanol as an unnecessary component of the digestion media (Harris and Agutter 1976).
3) Alterations in the tissue to media ratios and a differing centrifugation scheme aimed at an improvement in the yield and integrity of the envelopes.
4) Extraction of the final envelope pellet with 2M NaCl to remove residual chromatin and adventitiously adsorbed cytoplasmic protein.

2.7. Materials and Methods in the Isolation of Nuclear Envelopes.

Step 1 (Incubation with DNase at pH 8.5): A pellet of nuclei derived from 20 grams of rat liver was resuspended by the addition of a few drops of glycerol and vortexing. To the suspension was added, with vigorous vortexing, 7.5 mls H_2O followed by 375 ul DNase 1 (100 ug/ml H_2O. Sigma type DNEP) and 30 mls of a solution of 10% Sucrose, 10mM Tris. HCl, 0.1mM $MgCl_2$ and 0.1mM PMSF pH 8.5. The

mixture was incubated at 22°C for 15 minutes with vortexing every 5 minutes. After 15 minutes the digestion was slowed by the addition of 40 mls ice cold distilled water and the suspension centrifuged at 18,000 r.p.m. (40,000 g max) for 15 minutes and at 4°C in the 6 x 100 ml rotor of an MSE high speed 18 centrifuge, yielding a supernate (D_1s) and pellet (D_1p).

Step 2 (Incubation with DNase at pH 7.5): The D_1p fraction was resuspended, using a syringe and fine gauge needle, into 7.5 mls of a solution of 10% sucrose, 10mM Tris. HCl, 0.1mM $MgCl_2$ and 0.1mM PMSF pH 7.5. To this suspension 375 ul DNase (100 ug/ml) were added. After incubation for 20 minutes at 22°C the digestion was slowed by the addition of 8 mls ice cold distilled water and the suspension centrifuged for 10 minutes at 4°C and at 16,000 r.p.m. (20,000 g. avg.) in the 10 x 10 titanium rotor of an MSE Prepspin 50 centrifuge, yielding a supernate (D_2s) and a pellet (D_2p) of crude nuclear envelopes.

Step 3 (High salt extraction of crude nuclear envelopes): The D_2p fraction was thoroughly resuspended, using a syringe and fine gauge needle, into 0.25 mls of an ice cold solution of 10% sucrose, 10mM Tris.HCl, 0.1mM $MgCl_2$ pH 7.5 followed by 3.75 mls of an ice cold solution of 10% sucrose, 2.0M NaCl, 5mM $MgCl_2$, 10mM Tris. HCl pH 7.5. Incubation of the mixture on ice for 10 minutes followed by centrifugation for 10 minutes at 4°C as in step 2,

yielded a supernate (D_2Ss) and a pellet (D_2Sp) of highly purified nuclear envelopes.

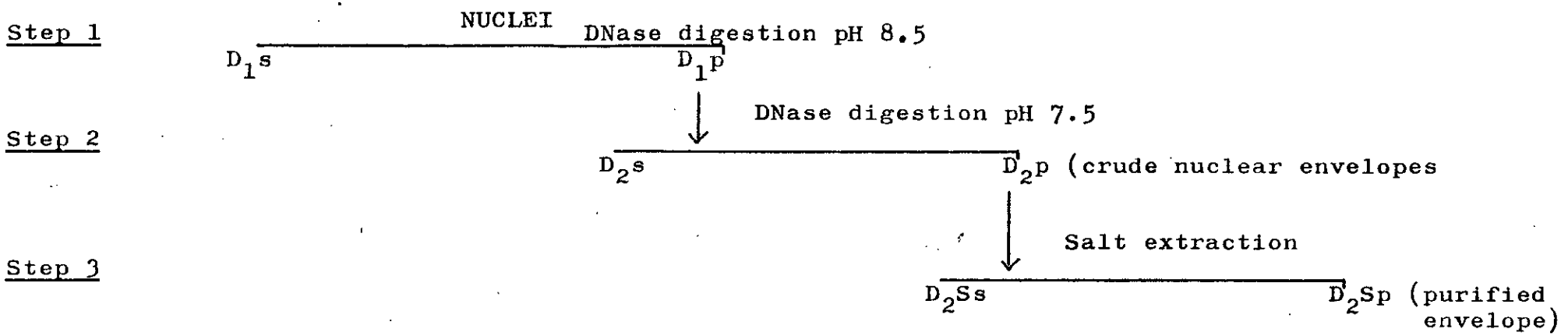


Fig. 1. Flow diagram for preparation of nuclear envelopes from isolated rat liver nuclei. Small s and p indicate supernate and pellet after centrifugation.

2.8. Characterisation of Isolated Nuclear Envelopes

2.8.1. Chemical Analysis of Nuclear Envelope Fractions (Table 8)

Fraction	Protein	DNA	RNA	Phospholipid	Phospholipid/Protein Ratio
crude envelopes (D_2p)					
preparation 1	81.9	4.2	3.9	10	0.12
2	80.4	5.3	4.8	9.5	0.12
3	76.4	5.0	4.5	14.1	0.18
mean of D_2p fractions	79.5	4.8	4.4	11.2	0.14
purified envelopes (D_2Sp)					
preparation 1	79.2	0.6	4.0	16.2	0.20
2	76.2	0.4	3.9	19.5	0.26
3	79.6	0.8	4.2	15.4	0.19
mean of D_2Sp fractions	78.3	0.6	4.0	17	0.22

* Calculations based on the approximation Protein + DNA + RNA + Phospholipid = 100%

2.8.2. Morphology of Isolated Envelopes

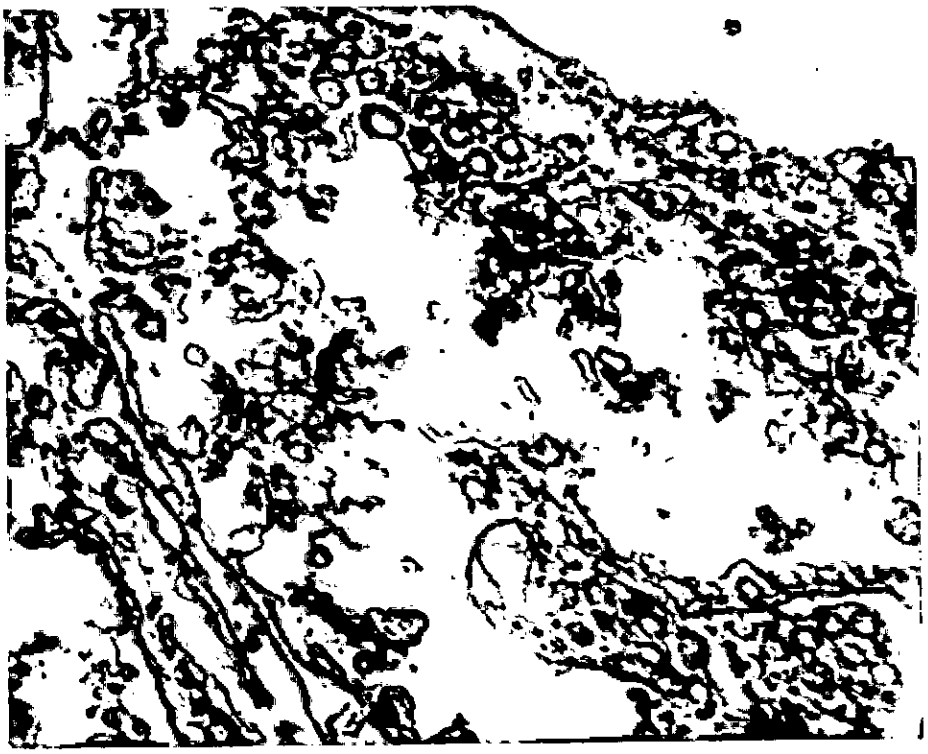


Plate 1. Survey micrograph D_2p fraction (crude envelopes) in thin section. Magnification x 40,000. In this large field more than 50 pore complexes are seen in tangential section. The pore annulus sits well proud of the membrane and subunits are clearly resolved in many pore complexes. The centre of the pores is often occupied by a distinct densely staining particle, the "central granule" and fibres may be seen radiating from this to the periphery. Membranes in transverse section (bottom right) show comparatively close apposition.

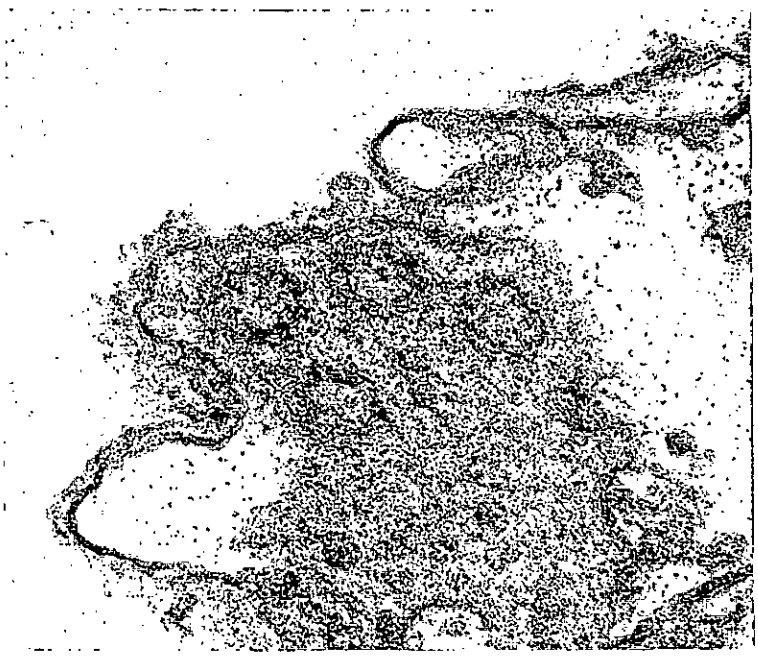


Plate 2 High power (x 126,000) micrograph showing details of the nuclear pore complexes seen in tangential section of the D_2p (crude envelope) fraction. The central granule is present in three of the pores shown and annular subunits are quite distinct. Detail within the pores is not well resolved although material does appear to evaginate from the subunits into the pores.

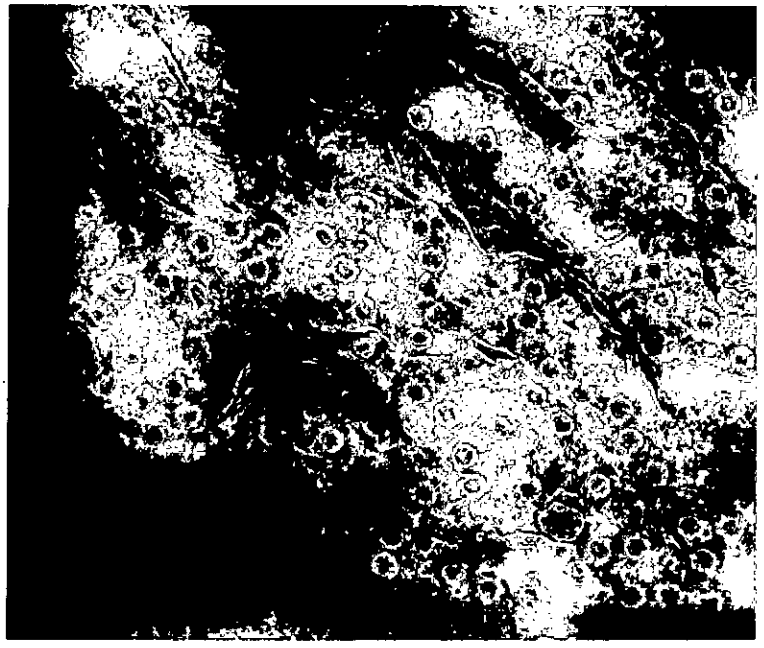


Plate 3 Details of nuclear pore organisation seen in negative stain (ammonium molybdate). Nuclear ghost from D_2p fraction showing more than 100 pore complexes. Central granules may be seen in many pores and the annulus is a prominent feature of the pore complexes. Detail within the annular ring is not as well resolved as in fixed and embedded material and no detail can be seen within the pore margin. (×35,000)

Plate 4 High power micrograph (x 67,000) of nuclear envelope fragments (D_2p fraction) in negative stain. The pore annulus is very sharply resolved although the annular subunits are not distinct. Little detail can be seen within the pores aside from the central granules.

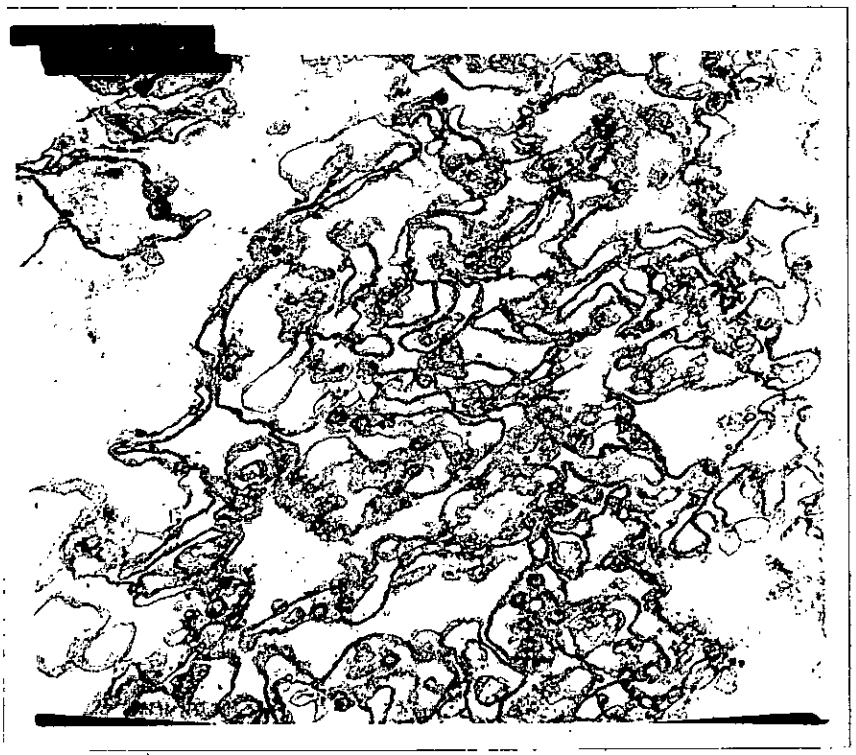


Plate 5 Survey micrograph of D_2Sp fraction (purified envelope) in thin section. Magnification x 27,000. Many pores are seen in tangential section showing a prominent annulus and densely staining central granule. When seen in transverse section the membranes show comparatively close apposition but there is little evidence of ribosomes.

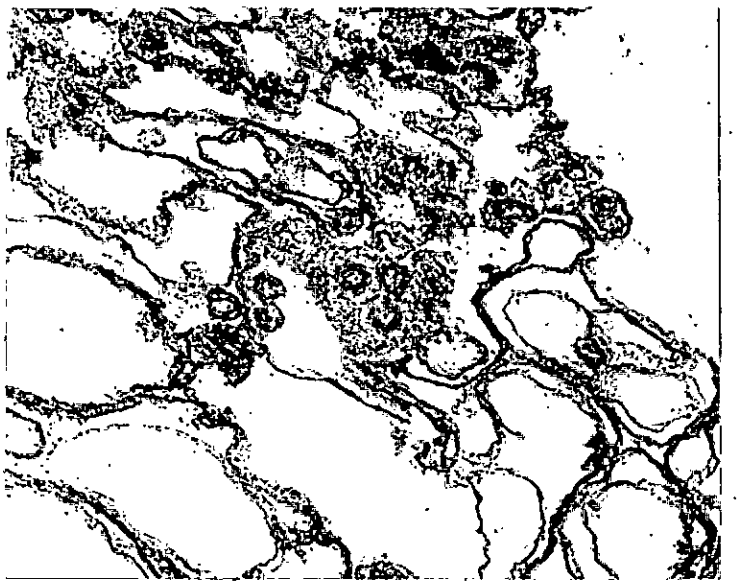


Plate 6 Higher power micrograph of D_2Sp fraction in thin section. Magnification x 54,000. Nuclear pore complexes are seen in tangential section. Although annular material is prominent, detail within the annulus is not apparent. Central granules are present within the pores and material does appear to evaginate from the annular margin into the pores.

Plate 7 Details of purified envelopes seen in transverse section (x 40,000). Some membranes are closely apposed but there is also a degree of separation of inner and outer membranes. Pore complexes seen in transverse section indicate the 'envelope' nature of the membrane preparation (section 2.1.1).

2.8.3 Polypeptide Composition of Isolated Nuclei and Nuclear Envelopes

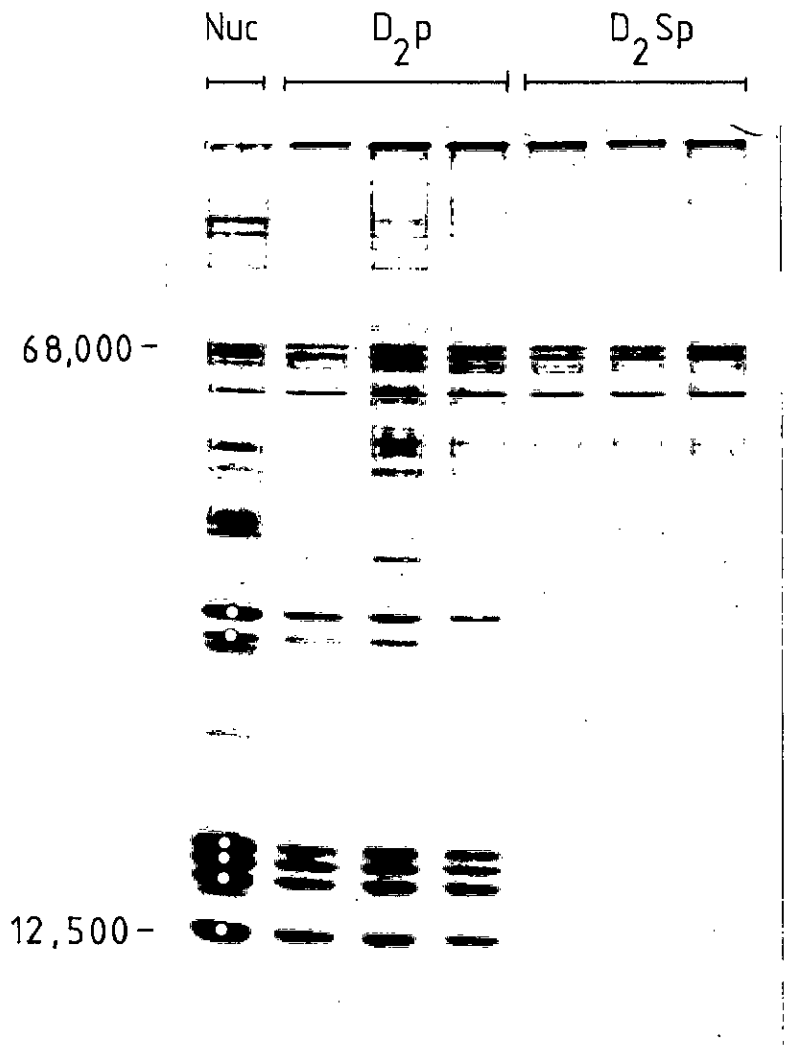


Figure 2 SDS polyacrylamide gel electrophoresis of polypeptides in nuclei and nuclear envelope fractions. Histone bands are indicated by dots. Numbers to the left of nucleus slot indicate molecular weight of standards. Note the significant histone contamination present in the D_2p fraction but absent in the D_2Sp fraction. Envelope fractions are particularly enriched in three polypeptides between 69,000 and 60,000 mol. wt and in giant polypeptides greater than 100,000 mol. wt.

2.9 Discussion

In seeking to provide an envelope preparation that is chemically pure (in so far as both DNA and histones are almost completely removed) yet which retains a high degree of ultrastructural integrity, the Kay procedure has been substantially modified.

The sequential use of low ionic strength, high pH and high ionic strength media in the preparation of any membrane system classifies the procedure as biochemically rigorous. If we consider peripheral proteins to be those proteins, external to the non-polar region of the lipid bilayer, that can be removed by washing with high and low salt media or by elevating the pH, then the method described has, by definition, the capacity to remove peripheral proteins. However, not all peripheral proteins are removed by this procedure. When further extracted with 0.1M NaOH, two major polypeptides at 80,000 and 74,000 (Jackson 1976) are removed from the membrane (along with 5% of the lipid). Such proteins may also be classed as 'peripheral' according to the definition of Steck and Yu (1973) but they clearly differ in the strength of their association with the membranes from those which may remove by high or low salt extraction.

The use of high ionic strength extraction in the preparation of nuclear envelopes may be justified on the following grounds.

1) A decrease in the DNA content of isolated envelopes from 4.8% to 0.6%.

2) Almost complete removal of histone contamination (see Fig. 2).

3) An increase in the phospholipid/protein ratio from 0.14 to 0.22 (Table 8).

4) Theoretical removal of adventitiously adsorbed (or weakly bound peripheral) proteins.

5) A significant increase in chemical purity without a concomitantly large decrease in ultrastructural integrity of the envelope.

The purified envelopes show an 8.5 fold enrichment of phospholipid over isolated nuclei and a 340 fold decrease in the DNA/phospholipid ratio. On the assumption that nuclear phospholipid is confined to the nuclear membranes and that DNA, despite its close association, is not a constitutive part of the nuclear envelope, then these figures indicate a substantial degree of chemical purity. Electron microscopy of the purified fraction does not reveal any contaminating nucleoli and the clean profiles shown by the membranes in transverse section suggest a considerable removal of the nuclear matrix.

It is particularly interesting that the envelopes, despite removal of ribosomes, still contain RNA even after extraction in conditions that remove the bulk of DNA. RNA is a consistent feature of all nuclear envelope preparations including those that are completely devoid of DNA (Kashnig and Kasper 1969; Matsuura and Ueda 1972)

or which have only trace amounts (Bornens 1968). The RNA content is non ribosomal and its resistance to pancreatic and T1 ribonuclease (Kashnig and Kasper 1969) suggests that the RNA is inaccessible to the enzyme or that it may exist as an RNA-protein complex immune to attack by ribonuclease (Kasper 1974). Although there is a great deal of experimental evidence that pore complex structures contain RNP (see Scheer 1972; Franke and Scheer 1974), there is as yet no data as to which RNP species is associated with the pore complex, and the possibility that pore complexes are not simply gateways for the eventual entry of RNP into the cytoplasm but also sites of final processing and assembly of ribosomes (Franke and Scheer 1970) remains to be established. Despite Kashnig and Kasper's claim that the RNA is resistant to ribonuclease, it has been found that the central granule is at least in part ribonucleoprotein for it can be completely removed with RNase (Mentre 1969) and the annulus is also sensitive to RNase (Agutter et al. 1977). In the Agutter preparation of RNase treated membranes, whilst RNase has clearly had effect upon the annulus, the central granules are still quite evident.

At the ultrastructural level, the preparation shows a comparatively high degree of integrity. Membrane fragments are large and many whole 'ghosts' are present. The inner and outer membranes are mostly closely apposed and the annulus and central granule are prominent

features of the pore complexes. Particular attention must be paid to the state of the annulus. Prior to high salt extraction annular sub-units are quite distinct in thin section. After high salt extraction the annulus appears as a rather homogeneous ring, annular subunits cannot be distinguished and it is doubtful whether optical rotational enhancement techniques would improve their resolution. One must consider the alteration of annular ultrastructure in one of two ways. Either one regards it merely as the partial collapse or rearrangement of the structure with no actual loss of protein, or as the physical extraction of annular components. If this is to be viewed in the light of actual extraction of annular components, and as has been discussed earlier in relation to membrane proteins - this is a distinct possibility in such a biochemically rigorous procedure, then this will have serious repercussions in a subsequent study of the pore complex proteins. On the other hand one has to weigh the fact that without the high salt extraction one would not have great confidence in the preparations purity. This poses the question of whether one can accept the loss of some peripheral proteins if, on balance, their loss largely resolves the problem of the adventitious adsorption of proteins. If one is to move forward then this question must be answered in the affirmative.

The preparative method may be regarded as providing very pure nuclear envelopes with a comparatively high degree of integrity. The annulus appears to have suffered some

extraction of its components or at least a decrease in its integrity and because of this and the fact that there is some separation of inner and outer membranes, the preparation does not completely meet the definition of 'envelope' set out in Section 2.1.1. Nevertheless, for a chemically very pure preparation, a prerequisite for the study to be undertaken, the preparation does exhibit a sufficiently high degree of integrity and 'envelope' character for meaningful data to be gained on the membrane and pore complex proteins.

The method described above for preparing nuclei and nuclear envelopes has therefore been used in the identification of proteins of the nuclear pore complex (see Chapter 4) and in an examination of the extent to which the nuclear membranes are differentiated from rough endoplasmic reticulum (Chapter 5).

3. The Development of a Probe for the Proteins of the Cytoplasmic Surface of the Outer Nuclear Membrane.

3.1. Introduction.

Several methods have been developed for labelling the cell surface (for recent reviews see Hubbard and Cohn 1976; Hynes 1976). These were mostly developed with a view to studying the erythrocyte membrane and their use cannot necessarily be extended to other surface or cytoplasmic membrane types. Labelling of subcellular organelles presents especial problems in regard to the permeability, purity and orientation of a given membrane.

Maddy (1964) developed the first non-permeating membrane probe (4-acetamido-4-iso-thiocyano-2,2 stilibene disulphonate) and this was followed some time later by other low molecular weight chemical labels (Berg 1969; Bretscher 1971), by site specific probes (Hokin et al. 1966 see also review of the use of lectins - Nicolson 1974) and by enzymic methods (Phillips and Morrison 1971; Steck and Dawson 1974; Kinzel and Mueller 1973; Brewer and Singer 1974). However, none of these agents may be applied immediately to the labelling of the nuclear surface. The very great structural complexity of the nuclear envelope sets problems which are without parallel in a membrane labelling study. The outer nuclear membrane is contiguous with the endoplasmic reticulum (Watson 1955; de Groodt et al. 1958; Parks 1962; Hadek and Swift 1962; Franke and Scheer 1974; but see also chapter 5) and the trauma of isolating nuclei can be expected to result in the loss of a small proportion of the outer nuclear

membrane, with the consequent exposure of proteins on both surfaces of the perinuclear space to any potential label. Moreover, the nuclear membranes are punctuated by pores which appear permeable to even quite large molecules (see chapter 1). The problem of labelling the cytoplasmic surface of the nuclear envelope may therefore be compared to the task of labelling a leaky bag, inside of which is another leaky bag full of protein. As such, it presents a much more difficult problem to that of labelling the erythrocyte membrane whose permeability properties are better understood. Furthermore, whereas the erythrocyte contains a major identifiable protein (haemoglobin), there is no such protein inside nuclei by which the specificity of a labelling procedure may be validated. It could be argued that there is a parallel between haemoglobin inside erythrocytes and histone protein inside nuclei but this would be a dangerous parallel to draw. Histone protein is not freely soluble and may exhibit an association with the nuclear matrix. If histones are associated with the nuclear matrix, then it is possible that they will be exposed at the pore complex and hence be accessible to an external probe. There is therefore a real difficulty in validating a labelling system in this particular instance.

Simple chemical probes and soluble enzyme labelling methods were unlikely to be useful in this

investigation because, at the very least they would result in the labelling of both surfaces of the outer nuclear membrane and the cisternal surface of the inner membrane. More probably, unless the probe could be restricted to the nuclear surface, both surfaces of both membranes, the whole of the pore complex and much of the nucleoplasm would be labelled. If an enzyme labelling method were chosen, passage of the enzyme through the pore complex (and hence into the nucleoplasm) could be reduced if not abolished, by complexing the enzyme with another high molecular weight protein. Ferritin, which has been successfully crosslinked to Con A without loss of the lectins sugar affinity (Nicolson and Singer 1971; Virtanen and Wartiovaara 1976; Virtanen 1977), seemed to be a possible candidate for such crosslinking. However, such a complex would still have access to the perinuclear space at points where the outer nuclear membrane had been stripped away from nuclei during isolation; and even if the enzyme were crosslinked to a giant molecule such as haemocyanin, exclusion of the complex from the perinuclear space could not be guaranteed. One possibility seemed to be to render an enzyme completely insoluble by immobilising it onto giant sepharose beads. Such a probe would by definition be impermeant but might suffer from problems of steric hindrance. Moreover, the kinetics of a system in which only the surface components of one body

would be able to interact only with the surface components of another body are not attractive. Nevertheless, it was decided to attempt to label isolated nuclei with an enzyme immobilised onto sepharose beads, and lactoperoxidase was chosen as the enzyme.

3.2. Lactoperoxidase.

Lactoperoxidase mediated iodination of proteins (performed either under the conditions of Phillips and Morrison 1971 or Hubbard and Cohn 1972) is the most frequently used enzymic membrane probe and it has the following advantages;

1) The large size of the enzyme usually precludes its penetration of the membrane.

2) The reaction may proceed under physiological conditions.

3) The kinetics of the reaction have been extensively studied and the specificity of the probe has been well accredited.

4) Iodide can be obtained carrier free, in two radionuclide forms, allowing for easy detection at low levels and for double labelling experiments.

Iodide (I^-) is itself unreactive, but in the presence of hydrogen peroxide, lactoperoxidase activates iodide to a reactive form which is not freely diffusible through the membrane. This may then react with accessible tyrosine, and to a limited extent histidine, residues. Iodine (I_2), by contrast, is highly reactive and will readily cross lipid bilayers, labelling both protein and lipid double bonds.

The main evidence for the specificity of lactoperoxidase labelling may be summarised as follows:

1) At neutral pH, lactoperoxidase catalyses the iodination of tyrosine without the detectable formation of iodine (Bayse and Morrison 1971).

2) The enzyme shows stereospecificity for D over L Tyrosine and can be competitively inhibited by di-iodotyrosine, which cannot be further iodinated (Morrison and Bayse 1973).

3) Although lactoperoxidase and horseradish peroxidase catalyse iodide to iodine about equally well, lactoperoxidase is much more effective at iodinating tyrosine (Bayse and Morrison 1971).

4) Lactoperoxidase will not catalyse the iodination of tyrosine in the absence of peroxide, even when oxidised forms of iodine are added (Morrison and Bayse 1973).

5) Lactoperoxidase will iodinate only one residue of cytochrome C whereas (I_2) will oxidise two (Morrison and Bayse 1973). The evidence of the enzymes stereospecificity is particularly powerful, and strongly suggests that iodination occurs not by some reactive diffusable moiety but by the binding of tyrosine to lactoperoxidase.

Soluble lactoperoxidase has been used to label nuclear envelope and residual nuclear envelope proteins (Mancini et al. 1973; Harris 1978; Monneron and D'Alayer

1978) but its use does not provide vectoral data. Insoluble lactoperoxidase has been used by David (David 1972; David and Reisfeld 1974) to label soluble proteins, and a preliminary communication describing its use in the labelling of sarcoplasmic reticulum proteins has appeared (King and Louis 1976).

3.3. Experimental.

Nuclei were prepared as described in chapter 2. ^{125}I was obtained from the Radiochemical Centre Amersham. Lactoperoxidase and glucose oxidase (used to generate hydrogen peroxide) came from the Sigma Chemical Company and lactoperoxidase was immobilised onto Sepharose 6MB macro beads (Pharmacia Fine Chemicals).

Immobilisation of Lactoperoxidase.

1g of CNBr - activated Sepharose 6MB was swollen in a beaker and washed for 15 minutes on a glass filter with 1mM HCl (200 ml). Lactoperoxidase, dissolved in 0.1M sodium phosphate buffer (pH 7.2), was mixed with the gel in a test tube, and the mixture rotated end-over-end at 4 r.p.m. overnight at 4°C. Unbound material was washed away with 200 ml phosphate buffer (coupling efficiency was always greater than 99.9%), and any remaining CNBr groups were reacted with 1M Glycine for 2 hours at room temperature. Three washing cycles were used to remove non-covalently adsorbed protein (never detected), each cycle consisting of a wash in 0.2M sodium phosphate buffer (pH 7.2) followed by a wash in 1M

Glycine. Lastly, the beads were washed with 200 ml 10% sucrose, 1mM $MgCl_2$, 0.2mM $NaHCO_3$ (pH 7.4) and stored for up to 4 hours prior to use.

In order to pipette the beads with some accuracy, it was necessary to section the ends of plastic disposable Biopipette tips to ensure a bore diameter of greater than 0.2 cm. Settled beads could then be pipetted to an accuracy within ±20%.

Radioactive Counting.

0.1 ml radioactive samples were precipitated into 1 ml 10% TCA containing 1 mg bovine serum albumen (Sigma) as a co-precipitant. After 18 hours at 4°C, precipitated samples were washed on glass fibre filters (Whatman GF/C glass microfibre paper) with 50 ml 5% TCA and dried on a hot plate.

Initially, samples were counted in a Packard Tricarb Liquid Scintillation Counter (Model 2420) at an efficiency of 30% using 0.5% w/v 2-(4-tert-butylphenyl-5-(4 biphenyl)-1,3,4-oxadiazole) in toluene. Latterly, use of a Nuclear Enterprises gamma counter (Model 8311) became available (efficiency 75%).

Iodination Conditions.

Iodination conditions for the complete system per millilitre of final solution: nuclei from $\frac{1}{3}$ gram liver, 1umol glucose, 33ug LPO (coupled in the ratio of 1.33 mg LPO per ml of settled beads), 0.7ug glucose

oxidase, 33uCi $Na^{125}I$, in 10% sucrose, 0.0001% butylated hydroxytoluene (from a stock of 0.5% in ethanol), 20uM $K^{127}I$, 10 mM Tris/HCl (pH 7.2). Incubation was 12 minutes at 23°C in a test tube rotating end-over-end at 4 r.p.m.

The reaction was stopped by the addition of an equal volume of ice cold stopper buffer (10% sucrose, 0.0001% butylated hydroxytoluene, 20uM 3-amino, 1,2,4 triazole, 20uM sodium sulphite, 10mM Tris/HCl pH 7.2). The mixture was filtered through 80u mesh nylon gauze held in a syringe (to remove the Sepharose beads), underlayed with 1 vol of 20% sucrose, 10mM β mercaptoethanol in stopper buffer, and centrifuged to pellet the nuclei (1000g for 10 mins. and at 4°C in the 6 x 100 ml swing-out rotor of an M.S.E. Mistral 4L centrifuge). The supernatant was discarded, and the nuclei were washed twice in 2 vols 10% sucrose in stopper buffer by pelleting at 700g for 5 mins. in the same rotor and at 4°C.

Table 1. Controls of the Iodination Reaction

Reagent deleted	Radioactivity cpm*/mg protein	% of Standard conditions
none	10^6	100
LPO	34×10^3	3.4
GO	16×10^3	1.6

* (Counting efficiency 75%).

3.4. Validation of the method.

3.41. Kinetic data.

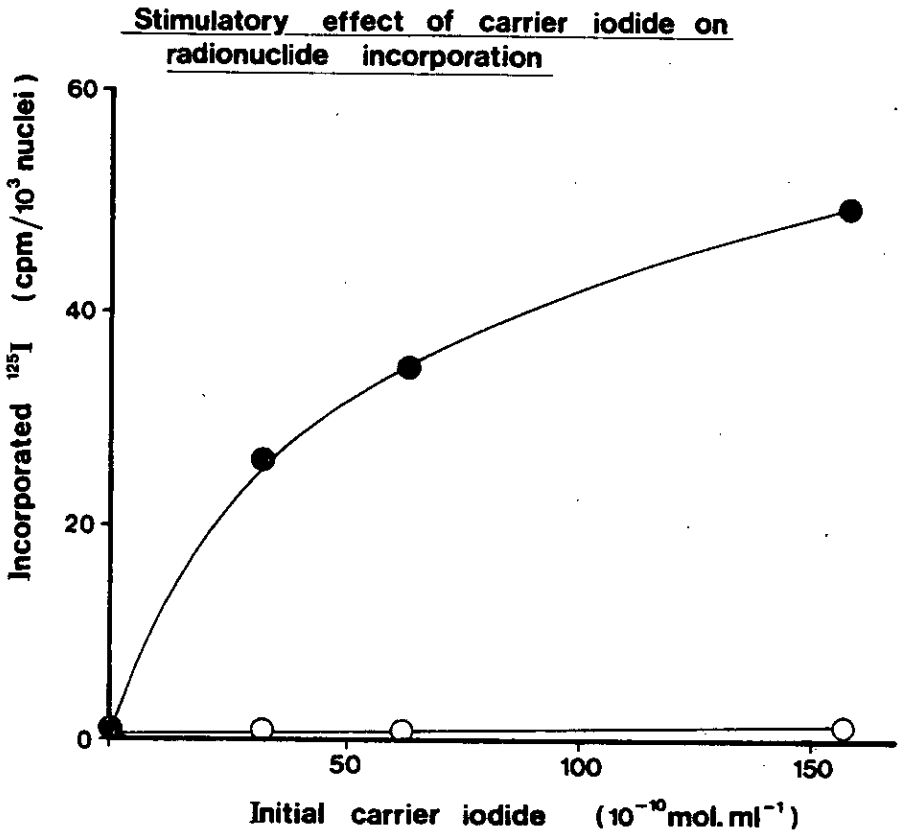


Fig. 1. The Effect of Carrier Iodide on Lactoperoxidase Mediated Iodination of Nuclei.

Black dots represent lactoperoxidase dependent iodination and open circles represent lactoperoxidase independent (non-specific) iodination. (Points are the mean of triplicate samples). 1 ml of reaction mixture contained 7 x 10^7 nuclei, 2.3ug glucose oxidase, 60ug lactoperoxidase (LPO - coupled in the ratio of 0.33 mg of enzyme per ml of sepharose beads), 95uCi $Na^{125}I$ and varying amounts of carrier iodide in 10% Sucrose, 1mM $MgCl_2$, 10mM D-glucose, 10mM Tris/HCl (pH 7.2). The mixture was rotated end-over-end at 4 r.p.m. for 30 minutes and at room temperature. After 30 minutes, the mixtures were diluted with 2 volumes of ice cold buffer. The beads were allowed to settle ($\sim$30 secs) and samples of the supernatant were withdrawn for counting.

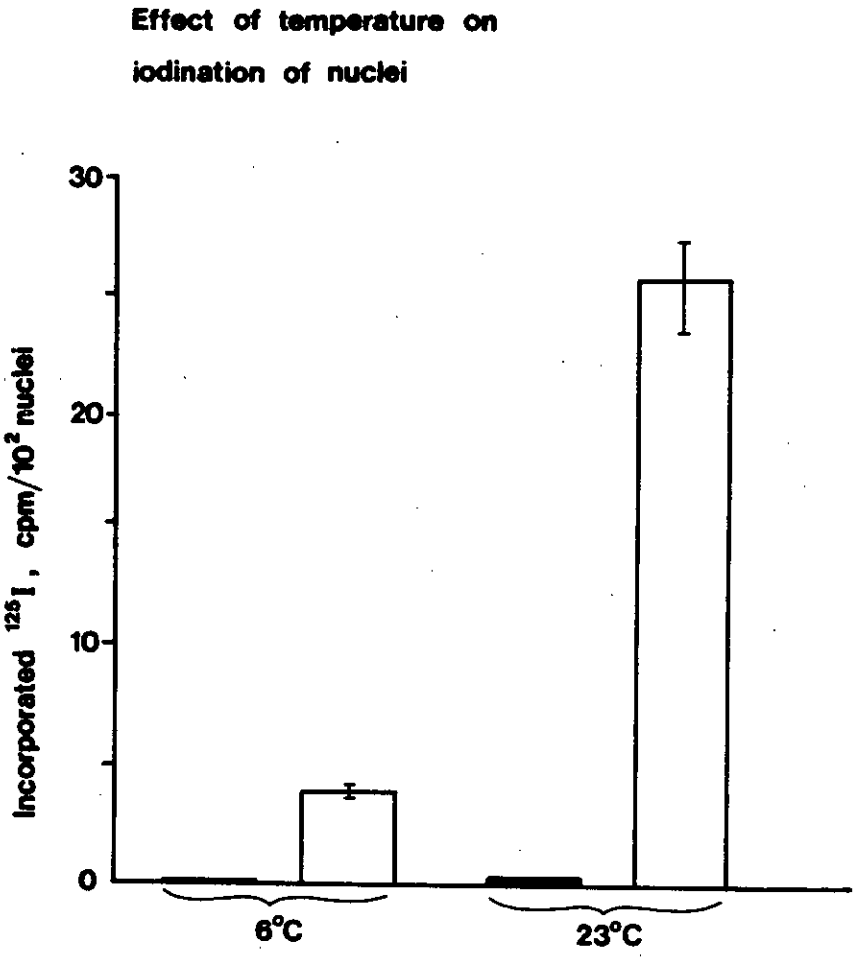


Fig. 2. Effect of Temperature on the Iodination of Nuclei.

Shaded figures represent lactoperoxidase independent, and clear figures lactoperoxidase dependent iodination (error bar ± standard error of the mean, quadruplate samples). 1 ml reaction mixture contained 2.9 x 10^7 nuclei, 0.64ug glucose oxidase (GO), 4ug LPO (coupled in the ratio 170ug of enzyme per ml of settled beads), 160uCi $Na^{125}I$, in 10% sucrose, 1mM $MgCl_2$, 0.8mM D-Glucose, 20uM $K^{127}I$, 10mM Tris/HCl (pH 7.2). After 10 minutes at either 6°C or 23°C while on a rotator, the reaction was stopped by the addition of 2 volumes of ice cold sucrose buffer containing 25mM Na Azide. The beads were allowed to settle (30 secs) and samples of the supernatant were withdrawn for counting.

G.O. conc'n	c.p.m./10^5 nuclei	
	non-specific	LPO dependent
0.1ug/ml	80 (±7)	55 (±4)
1.0ug/ml	271 (±30)	4326 (±466)
3.0ug/ml	331 (±61)	3648 (±289)

Table 2. The Effect of Varying Glucose Oxidase Concentration on the Iodination of Nuclei.

1 ml of reaction mixture contained 5 x 10^7 nuclei, 60ug LPO (coupled in the ratio of 1.25 mg of enzyme per ml of settled beads), 100uCi $Na^{125}I$, and varying amounts of glucose oxidase in 10% sucrose, 1mM $MgCl_2$, 5mM D-glucose, 18uM $K^{127}I$, 10mM Tris/HCl (pH 7.2). After 10 minutes at room temperature, the reaction was stopped by the addition of ice cold buffer containing 25mM Na Azide. Samples taken as before.

ug LPO/ml	c.p.m./10^5 nuclei LPO dependent	LPO independent
44	6042 (±157)	567 (±14)
110	6384 (±516)	660 (±13)
220	6813 (±525)	556 (±16)

Table 3. The Effect of Varying Sepharose Bead Concentration.

1 ml of reaction mixture contained 4.5×10^7 nuclei, 1ug GO, varying amounts of LPO (bound to 6-MB beads in the ratio of 1.3 mg enzyme per ml of settled beads), 80uCi $Na^{125}I$ in 10% sucrose, 1mM $MgCl_2$, 5mM D-glucose, 16uM $K^{127}I$, 10mM Tris/HCl (pH 7.2). After 10 minutes at RT the reaction was stopped by the addition of two volumes of ice-cold buffer containing 25mM Na Azide. Samples taken as before.

Bead enzyme level	c.p.m./10^6 nuclei
0.00 mg/ml beads	186 (±13)
0.17 mg/ml beads	2360 (±139)
0.67 mg/ml beads	6653 (±502)
2.67 mg/ml beads	5763 (±399)

Table 4. The Effect of Bead Enzyme Concentration on Iodination of Nuclei.

1 ml of reaction mixture contained 5×10^7 nuclei, 3ug GO, 40ul 6MB-LPO beads (with varying amounts of enzyme bound), 90uCi $Na^{125}I$ in 10% sucrose, 1mM $MgCl_2$, 10mM D-glucose, 18.5uM $K^{127}I$, 0.9uM sodium sulphite, 10mM Tris/HCl (pH 7.2). After 10 minutes at RT the reaction was stopped by the addition of 2 volumes of ice-cold buffer containing 25mM Na Azide. Samples were taken as before.

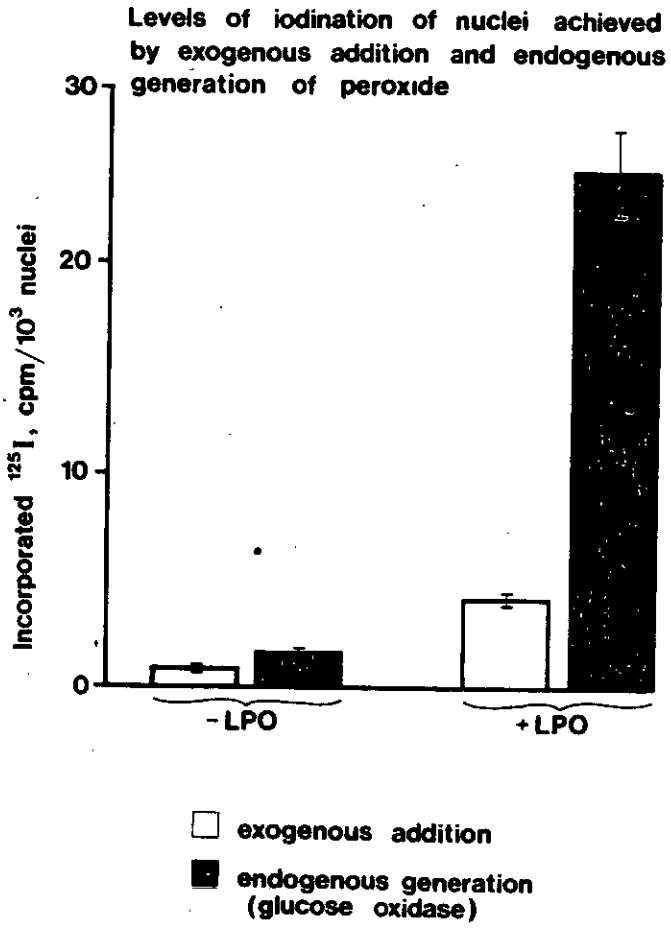


Fig. 3. Comparison of the Levels of Iodination Achieved by Exogenous Addition and Endogenous Generation of Peroxide.

$\frac{1}{4}$ ml. of reaction mixture contained 4.2×10^7 nuclei, either 0.28ug GO or H_2O_2 to 9uM, 12.5ug LPO (bound in the ratio of 0.33mg enzyme per ml of Sepharose beads). 90uCi $Na^{125}I$ in 10% sucrose, 5mM D-glucose, 1mM $MgCl_2$, 18uM $K^{127}I$, 10mM Tris/HCl (pH 7.2). After 10 minutes at RT, the reaction was stopped by the addition of ice-cold buffer containing 25mM Na Azide. Samples were taken as before.

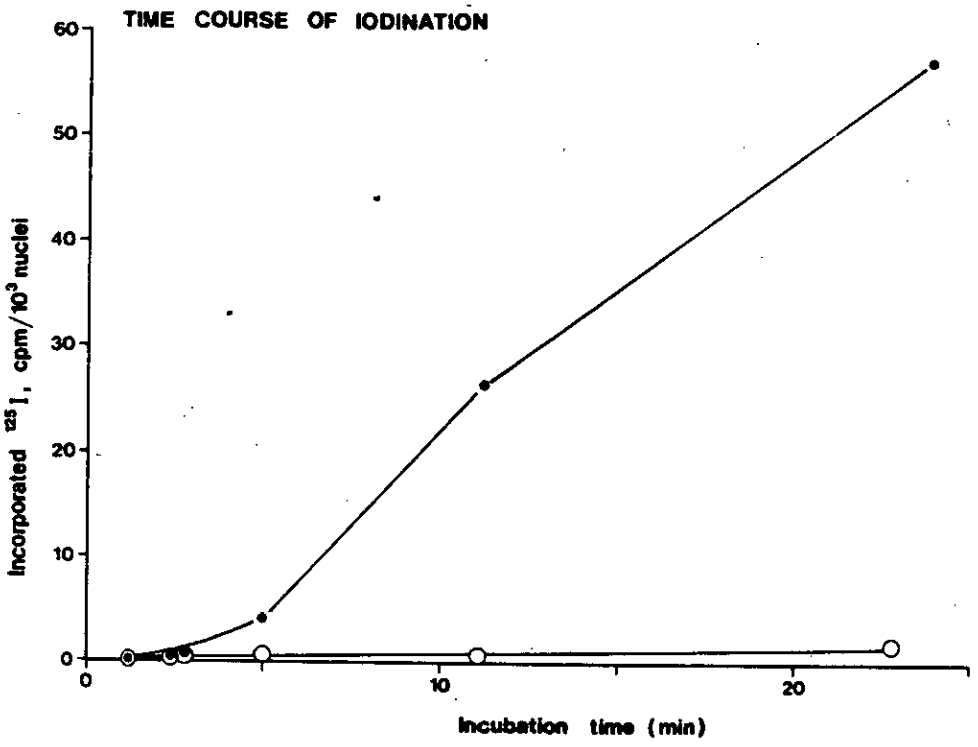


Fig. 4. Time Course of Iodination of Nuclei.

Black dots represent lactoperoxidase dependent iodination and open circles represent lactoperoxidase independent (non-specific) iodination (mean of triplicate samples). 1 ml of reaction mixture contained 4 x 10^7 nuclei, 0.66ug glucose oxidase, 30ug LPO (coupled in the ratio of 1.2mg of enzyme per ml of settled beads, 44uCi $Na^{125}I$, in 10% sucrose, 1mM D-glucose, 1mM $MgCl_2$, 22uM $K^{127}I$, 10mM Tris/HCl (pH 7.2). After a given time at RT, the reaction was stopped by the addition of 2 volumes of ice-cold buffer containing 20uM Na sulphite. Samples were taken as before.

3.43. Lipid Analysis

cpm/10^5 nuclei			
LPO dependent	non specific	extractable with organic solvent	Butylated-OH toluene
7535	161	7	-
7689	137	8	+

Table 5. Determination of the Extent of Lipid Labelling During Lactoperoxidase Mediated Iodination of Nuclei.

1 ml of reaction mixture contained 3 x 10^7 nuclei, 0.66ug glucose oxidase, 36ug LPO (coupled in the ratio of 1.33 mg of enzyme per ml of settled beads), 80uCi $Na^{125}I$ in 10% sucrose, 1mM $MgCl_2$, 0.8mM D-glucose, 16 M $K^{127}I$, 10mM Tris/HCl (pH 7.2), with or without 0.005% w/v butylated hydroxytoluene. After 10 minutes at RT, the reaction was stopped by the addition of 2 volumes of ice-cold buffer containing 20uM Na sulphite.

Nuclei were strained through 80u gauge nylon net to remove the Sepharose beads and pelleted at 1000g for 5 minutes and at 4^oC in the 6 x 50 ml wing-out rotor of an M.S.E. Mistral 4L centrifuge. Nuclei were then washed thoroughly to remove unbound iodide by a further 6 resuspension and pelletings in sucrose buffer. Organic solvent extraction of a wet pellet of nuclei was performed according to Bligh and Dyer (1959).

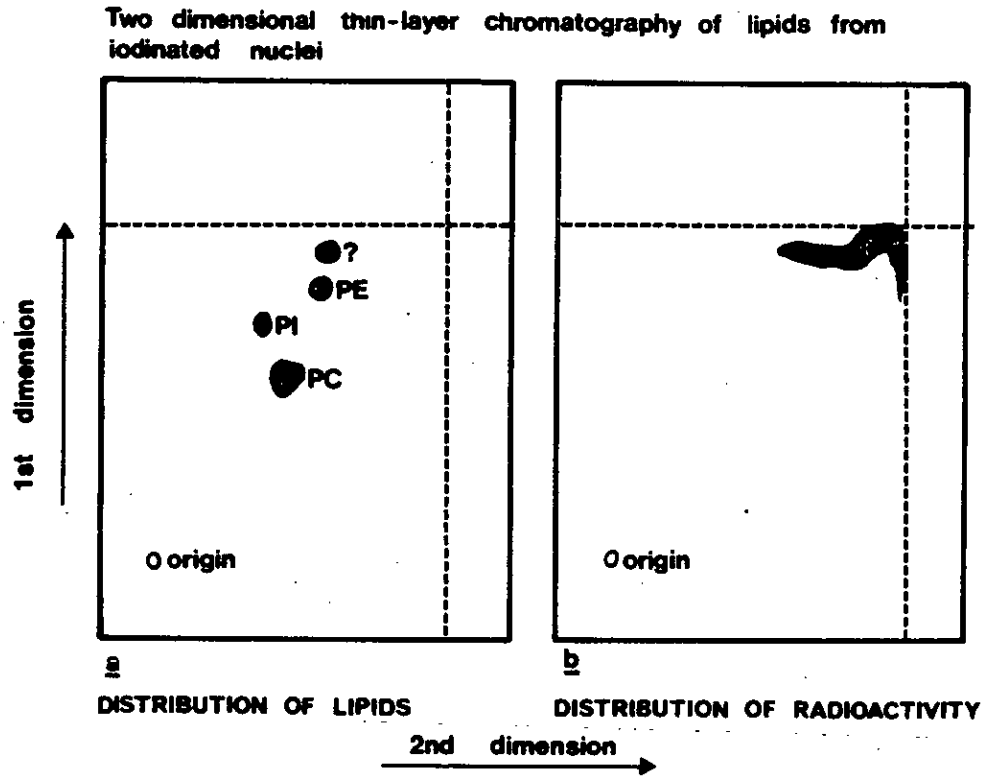


Fig. 5. Chromatography of lipids from iodinated nuclei. PC (phospatidylcholine), PI (phosphatidylinositol), PE (phosphatidylethanolamine). Dashed lines represent solvent fronts.

Lipid extracts were applied onto 8 x 8 cm thin layer chromatography plates (Polygram SIL NHR, ex Camlab.) and run in two dimensions. 1st dimension $CHCl_3$:CH_3OH:NH_3 (25%): H_2O (90:54:5.5:5.5 v/v/v/v). For the second dimension $CHCl_3$:CH_3OH: CH_3COOH: H_2O (90:40:12:2, v/v/v/v). Lipids were identified by co-chromatography with known lipid standards and revealed after spraying a dry plate with 10% sulphuric acid and charring in an oven at 180°C for 10 minutes. The distribution of radioactivity was determined by autoradiography (as described in chapter 2).

3.43. Polypeptide Analysis.

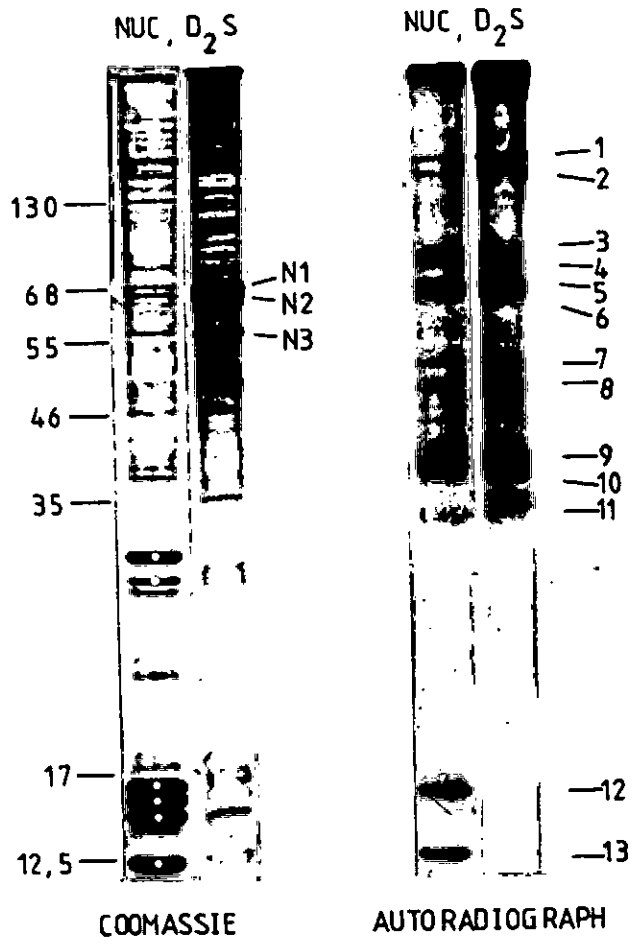


Fig.6. SDS polyacrylamide gel electrophoresis of reduced polypeptides of iodinated nuclei and nuclear membranes (D_2S). For conditions of iodination see Table 6. Numbers to the left of Nucleus slot (coomassie) refer to the mol.wt x 1,000 of standards (see Materials and Methods, Chapter 2). The two left hand slots are coomassie stained gels and the two right hand slots are autoradiographs. Histones are indicated by dots and are identified, in order of increasing mobility as H_1 (pair), H_3, H_2b, H_2a and H_4.

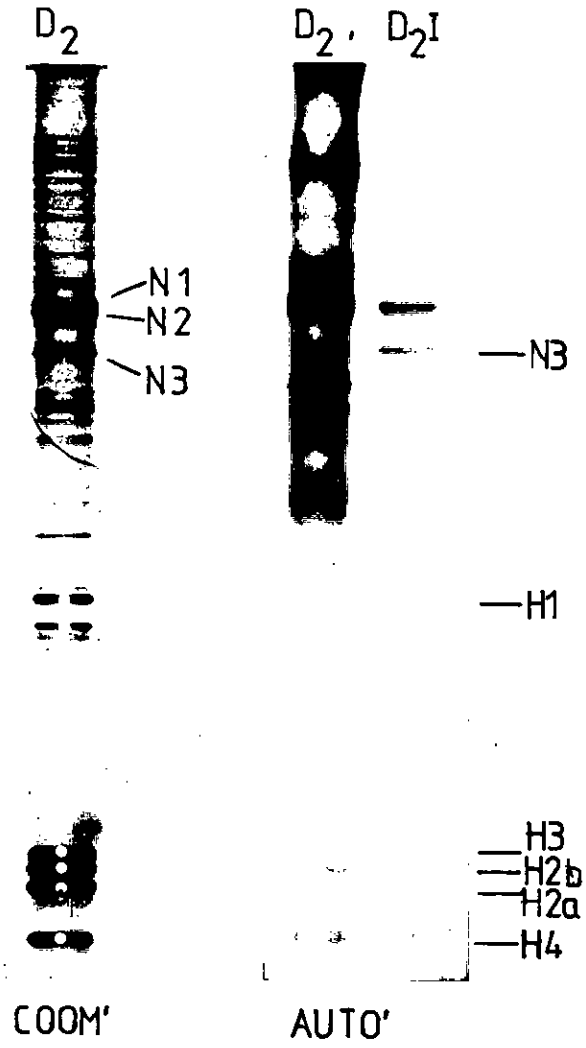


Fig. 7. SDS polyacrylamide gel electrophoresis of reduced polypeptides of crude nuclear envelopes (D_2) iodinated with solid state LPO prior to isolation of envelopes (D_2) and after the isolation of membranes (D_2I). This photograph demonstrates the great difference in labelling found when the permeability of the probe is increased i.e. the inner surface of the inner nuclear membrane is exposed to the probe.

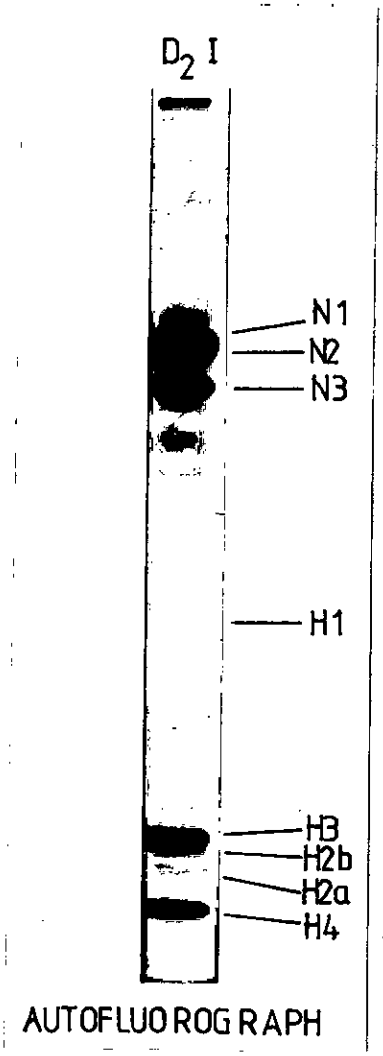


Fig. 8. SDS polyacrylamide gel electrophoresis of reduced polypeptides of crude nuclear envelopes (D_2) iodinated after the isolation of envelopes (D_2I).

3.44. Electronmicroscopy.

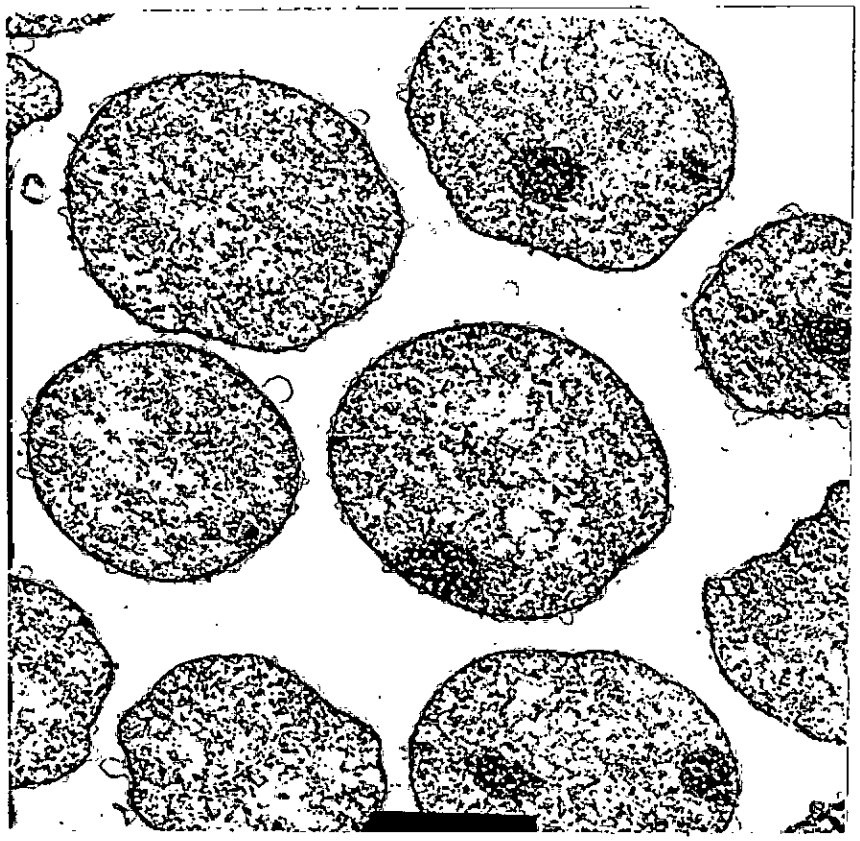


Plate 1. Survey micrograph of iodinated nuclei (x 5,240). Nuclei appear rounded and show a high degree of integrity. Note however, the frequent appearance of outer nuclear membrane blebbing. This phenomenon is not seen prior to iodination and although it cannot be attributed to iodination per se, must represent a chemical trauma during the iodination process (peroxide damage?).

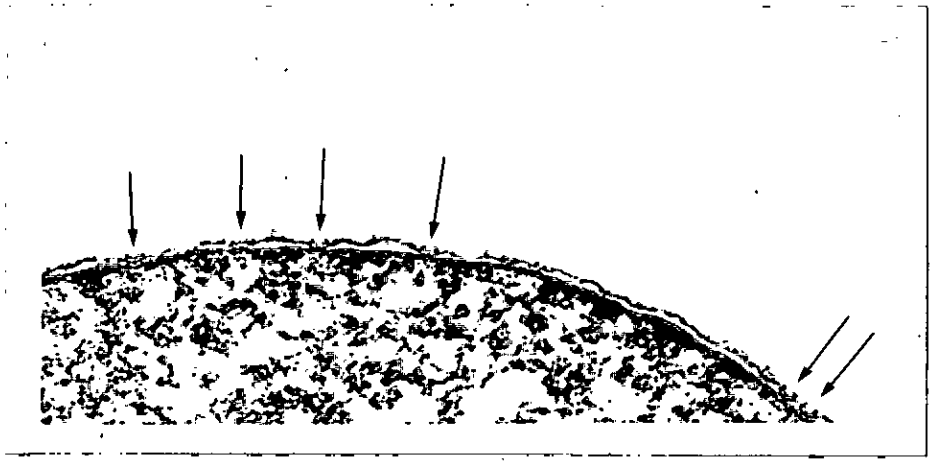


Plate 2. Higher power micrograph (x 28,000) showing detail of the outer nuclear membrane of an iodinated nucleus. Numerous pore complexes are visible, the cytoplasmic surfaces of which are indicated by arrows. Ribosomes can be seen all over the surface of the outer membrane. In this micrograph, the inner and outer membranes show close apposition, but there is a tendency toward a wider, or bleb, separation in the centre of the micrograph. One of the features of iodinated nuclei is that, because the membranes show a wider separation than in un-iodinated nuclei, morphometric determinations of the outer nuclear membrane are much easier to perform.

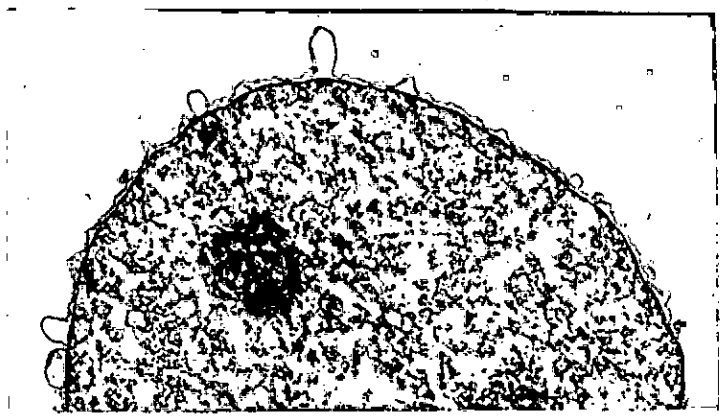


Plate 3. Section of a single nucleus showing gross outer nuclear membrane bleb formation. There is an increased tendency toward bleb formation in iodinated nuclei. (x 9,000).

Preparation of Nuclei.	Nuclear Profile (u)	ONM (u)	ONM/nuclear prof %
1	400	365	91
2	585	510	87
3	714	608	85

Table 6. Morphometric Determination of the Proportion of Outer Nuclear Membrane Present on Iodinated Nuclei. (For technical details of the method of determination, see chapter 2). Nuclei were labelled for 12 minutes at room temperature under the standard conditions given under table 7.

3.5. Discussion

The choice of lactoperoxidase as a labelling enzyme may not at first appear a happy one. Peroxidase activity has been demonstrated cytochemically in the nuclear envelope of rat thyroid cells (Nakai and Fujita 1970), rat parotid gland cells (Herzog and Miller 1970), Human megakaryocytes (Breton-Gorius and Guichard 1972) and the lacrimal gland of the rat (Herzog and Miller 1972). Moreover, there are recent claims (Harris 1978; Stubbs and Harris 1978) of an endogenous peroxidase capable of incorporating ^{125}I into protein in rat liver nuclear envelope and which is inhibited by 3-amino, 1,2-4 triazole. During my early studies, it was certainly the case that lactoperoxidase independent (non-specific) labelling could account for between 25 and 85% of the total. Furthermore, this was not abolished by pre-incubating ^{125}I with sodium sulphite and was dependent on the presence of peroxide. Results suggested that there was truly an endogenous iodinating capacity in fractions of purified nuclei. Of course, this did not necessarily indicate the presence of a peroxidase in nuclei as such, for the activity might well reside in the peroxisomal or mitochondrial contamination present, to varying degrees, in all preparations of nuclei. The non-specific labelling was high only in relation to the lactoperoxidase

dependent labelling which, in comparison to later work (see below), was low. In absolute terms, the background labelling was in fact very low as was lactoperoxidase dependent labelling (less than 1% of total ^{125}I). It seemed, in all probability, that immobilising the enzyme onto Sepharose beads had created enormous steric and kinetic problems - problems which, though they might be trivial when dealing with soluble proteins (see David 1972; David and Reinsfeld 1974), could be insuperable when dealing with a membrane system.

No headway was made, until it was discovered that inclusion of micromolar quantities of carrier iodide in the incubation media provided an enormous stimulation to lactoperoxidase dependent iodination (Fig. 1). This was a surprising finding for, although one might expect an increase in the iodide concentration of the reaction to increase the rate of reaction, the proportion of ^{125}I to ^{127}I falls. Therefore, to increase the amount of ^{125}I reacted, the increased concentration of iodide must produce a proportionately greater stimulation of the enzyme. Hubbard and Cohn (1975) have investigated the effect of carrier iodide concentration on the radioiodination of L cells. At the highest iodide concentration, 12.5×10^{-6}M and at 37°C, 5-10 x 10^6 iodide atoms per cell were incorporated; at 6×10^{-8}M iodide

(carrier free), 10×10^3 to 50×10^3 iodide atoms per cell were incorporated; and at 2×10^{-8}M iodide (carrier free), 2×10^3 atoms were incorporated.

iodide conc^{n} (M)	iodide conc/2 x 10^{-8}	max iodide atoms incorporated/cell	max radioiodide incorporated/cell
2×10^{-8}	1	2×10^3	2×10^3
6×10^{-8}	3	50×10^3	50×10^3
12.5×10^{-6}	6.25×10^2	10×10^6	48×10^3

Table 7. Effect of Iodide Concentration on Iodination of L Cells.

- Derived from Hubbard and Cohn (1975). To effect this, rather crude, comparison, the fact that at 6×10^{-8}M iodide the LPO concentration was doubled has been ignored.

It can be seen from Fig. 7 that raising the iodide concentration increased the incorporation of iodide into L cells. However, if one takes into consideration the proportion of iodide atoms incorporated that are radioactive, (for this, we assume that at 12.5×10^{-6}M iodide, 6×10^{-8}M is ^{125}I) it can be seen that inclusion of carrier iodide does not exert a great stimulatory effect upon the radioiodination of L cells when using soluble lactoperoxidase. In contrast, when using insoluble

lactoperoxidase to iodinate nuclei, increasing the carrier iodide concentration from 0 to 12.5 x 10^{-6}M (at 6 x 10^{-8}M ^{125}I) provided an approximately 50 fold stimulation of ^{125}I incorporation.

The explanation for this phenomenon may lie both in the saturation of nuclear binding sites for iodide and in bringing the iodide concentration closer to the Km, but the reaction kinetics have not been studied sufficiently to establish the cause. However, this single, and indeed simple, finding is the key to labelling the nuclear surface; and one which has eluded other groups that have tried, unsuccessfully, to label the outer nuclear membrane with lactoperoxidase.

The kinetics of labelling with soluble and immobilised lactoperoxidase are clearly rather different and there may be two especial problems arising from the need to include carrier iodide when using immobilised lactoperoxidase. Firstly, Morrison (1974) has suggested that when the iodide to tyrosine (substrate) ratio is high, lactoperoxidase may catalyse the oxidation of iodide to iodine. Since iodine will readily react with both lipids and protein, such a development would abolish the specificity of labelling. Secondly, the higher levels of iodination achieved when the iodide concentration is high may impair membrane function. With regard to this latter point, it is more probable that the higher levels of peroxide, necessary when

iodide is high, would impair the membrane than would the inclusion of iodide perturb a proteins conformation. The first of these points may be answered by extracting the lipids from iodinated nuclei and determining whether the lipids are iodinated (see table 5 and fig. 5). In three separate experiments, less than 10% of the counts associated with washed labelled nuclei could be extracted with organic solvents. When this extract was chromatographed in two dimensions, no significant portion of the radioactivity co-migrated with the three major nuclear lipids (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol) which, combined, account for approximately 93% of nuclear envelope phospholipid (Kleinig 1970). Greater than 95% of the radioactivity ran just behind and, to some extent within, the two solvent fronts (Fig. 5) and probably represented un-bound iodine. The second point, that high levels of iodination may impair membrane function, may be of general validity but is not of specific interest or concern in the present study. Certainly, the iodination reaction does lead to some outer nuclear membrane blebbing (plate 1) but neither the mechanism nor the significance of this phenomenon are at present understood.

Hubbard and Cohn (1975) found that when using carrier-free isotope, the rate of iodination of L cells was parallel over 30 minutes at $4^{\circ}C$ and at $37^{\circ}C$, but

that with the inclusion of carrier iodide an increase in temperature had a marked stimulatory effect upon iodination (a Q_{10} of 3 was obtained). When the iodination of nuclei, using insoluble lactoperoxidase and carrier iodide, was compared at 6°C and at 23°C (Fig. 2), it was confirmed that temperature had a marked stimulatory effect upon iodination. In fact it was apparent that iodination at 4°C would not provide nuclei of a sufficiently high specific activity or lactoperoxidase dependent/non-specific labelling ratio. So that, while it might in principle be preferable to conduct the iodination at 4°C (thereby minimising the potential for proteolysis and for enzyme inactivation), it was regrettably necessary to carry out the procedure at room temperature ($\sim$ 23°C).

Glucose oxidase was used to generate peroxide because it seemed preferable to generate low levels of peroxide, at a rate which approximately equalled its consumption, than to repeatedly add concentrated peroxide to the reaction. Exogenous addition of peroxide to a reaction would create both spatial and temporal gradients of peroxide which under certain circumstances (see Welton and Aust 1972) may result in lipid peroxidation and loss of enzyme activity. The inclusion of butylated hydroxytoluene in iodinating media is reported to reduce this tendency, and may even lead to a stimulation of iodination (Welton and Aust 1972). For these reasons, it was eventually

incorporated into all media, although no stimulation of iodination was observed (Table 5), and it made no difference to the proportion of counts that could be extracted from iodinated nuclei by organic solvents. Levels of iodination achieved by exogenous addition of a high level of peroxide (8uM) and a sub-optimal concentration of glucose oxidase were compared (Fig. 3). The use of glucose oxidase gave a significantly higher degree of iodination than the exogenous addition of peroxide, and the protein iodination patterns were identical (indicating that iodination of glucose oxidase was not spuriously contributing to the iodination pattern of nuclei).

In a series of simple experiments, the iodination conditions were optimised to provide a method that gave both a high degree of labelling and a low level of non-specific labelling. The aim was to produce a method that worked sufficiently well as to provide the answers to questions, rather than to study exhaustively the many parameters involved in the iodination reaction.

The effect of different levels of glucose oxidase in the reaction was studied (Table 2), and the optimal concentration for iodination determined as being between 0.1ug and 1.0ug/ml. The reaction was sensitive to the concentration of lactoperoxidase immobilised onto the beads (Table 4) but was curiously

insensitive to the actual concentration of beads in the reaction (Table 3). The latter phenomenon is an interesting one, and is not in this case to be wholly explained by the concentration of peroxide being rate limiting or by the saturation of available iodination sites on nuclei. The answer almost certainly lies in the fact that lactoperoxidase beads self-iodinate (David 1972; David and Reisfeld 1974). Thus, increasing the bead concentration will both increase the iodinating surface for nuclei but will also increase the capacity for self-iodination. One therefore expects there to be a plateau of iodination, the level of which will be greatly influenced by the surface area/volume ratio of the beads (such a plateau would be elevated by using smaller beads and decreased by using larger beads). Increasing the concentration of beads in the reaction will probably not greatly affect the level of iodination of nuclei (as is seen in Table 3) although the rate at which the plateau is reached should be greater. If this hypothesis is correct, then the choice of 6MB Sepharose beads (with a diameter of $\sim$200u) places an immediate constraint on the degree to which nuclei can be iodinated.

When the time course of the reaction was studied (Fig. 4), it became apparent that little iodination was occurring during the first 3 minutes but that between 5 and 20 minutes the rate of iodinat-

ion was very rapid. The short period of lag is almost certainly the time taken for glucose oxidase to generate sufficient peroxide, from glucose, to stimulate lactoperoxidase, but both during and after the lag the rate of production of peroxide will be somewhat in excess of the capacity of the system to consume it.

Morphometric analysis of nuclei iodinated for 12 minutes (Table 6 see also plate 1) showed little apparent decrease in the proportion of outer nuclear membrane still attached to nuclei. Mechanical damage during the 12 minutes that nuclei mixed with sepharose beads would therefore seem to have been minimal. It is crucial to establish, at this stage, that the iodination process does not damage nuclei. Morphometric analysis of nuclei prior to iodination (chapter 2, Table 6) has shown that approximately 89% of the outer nuclear membrane is retained by nuclei; after iodination, this figure is approximately 88%. Sampling error may have made the margins of error quite wide and the difference between these figures cannot be considered significant (note, the nuclear profiles measured to establish these figures total nearly 3,000 microns). Although it may be wished that nuclei, 100% surrounded by outer nuclear membrane, could be isolated and iodinated, it must be remembered that the outer membrane may be contiguous with the

endoplasmic reticulum and that such perfection does not apparently exist in nature. This is not to say that luminal continuities with the endoplasmic reticulum account for nearly 12% of the outer nuclear membrane, merely that it would be unreasonable to expect to isolate nuclei from rat liver with a much higher degree of outer nuclear membrane integrity.

Under the conditions given in Table 1 solid state lactoperoxidase provided an absolutely reliable method for labelling the external surface of isolated nuclei. The iodination, which was dependent on peroxide, was greater than 96% dependent on the presence of lactoperoxidase. This latter figure could be improved by decreasing the concentration of nuclei in the reaction, and the specific activity of labelling could be increased by increasing the concentration of ^{125}I in the reaction.

It is now possible to arrive at a rough estimate of the proportion of the radioactivity that is incorporated into the cytoplasmic surface of the outer nuclear membrane. The following must be taken into consideration:

1) Morphometry has indicated that 94% of membrane profiles in thin sections of purified nuclei are nuclear membrane profiles and 6% are other membrane profiles (Chapter 2, Table 5). Other

membrane profiles are almost exclusively vesicular and for the present purpose we will consider them all as being vesicular. The nuclear membranes are a double membrane system, approximately half of which may be exposed to the exterior. Thus the proportions of surface area that may be exposed to lactoperoxidase beads are 88% nuclear membranes and 12% other membranes.

2) Morphometric analysis of iodinated nuclei has indicated that 88% of the nuclear surface is covered by outer nuclear membrane (Table 6).

3) 97% of radioactive counts were dependent on the presence of lactoperoxidase.

On the assumption that there is no variation in the capacity of lactoperoxidase beads to iodinate nuclei of high integrity, nuclei of low integrity and other membranous elements present in nuclear preparations, then <u>not more than 75%</u> (88% x 88% x 97%) of radioactive counts may with confidence be ascribed to the cytoplasmic surface of the outer nuclear membrane. The remaining 25% of radioactive counts will be partitioned, in an unknown manner, between the outer nuclear membrane, cytoplasmic membrane contaminants and the outer surface of the inner nuclear membrane; intra-nuclear labelling will be negligible.

Comparison of the coomassie and autoradiographic patterns of the proteins from whole nuclei and of envelopes derived from labelled nuclei (Fig. 6) shows

that the labelling of proteins is very selective. In particular, major iodinated bands co-migrate with two of the major nuclear envelope proteins (N1 and N2) but not with a third (N3). When crude envelopes or disrupted nuclei were iodinated with insoluble lactoperoxidase (Figs. 7 and 8) this latter band became highly labelled and the overall pattern of labelling was substantially changed. The iodination patterns of nuclei and nuclear envelopes prepared from labelled nuclei were almost identical except with regard to bands 12 and 13. These bands co-migrated with histones H_2b and H_4 and their iodination could reflect the iodination of leaky nuclei. However, in crude envelopes, H_2b did not label significantly (Figs. 7 and 8) whereas H_2a and H_4 did. Histones H_1 and H_3 were barely labelled either in whole or disrupted nuclei. Histones do not therefore provide a reliable marker of the labelling methods selectivity. They do not have a uniform capacity for iodination in broken nuclei and the labelling homology with H_2b is unlikely to be histone.

On the basis of the above, it was considered that the iodination pattern was specific for unbroken nuclei and that, with a confidence of 75% represented iodination of externally disposed proteins of the outer nuclear surface. The lines of evidence that

support this conclusion may be summarised as follows:

1) Insoluble lactoperoxidase is absolutely impermeant.
2) Labelling was dependant upon the presence of lactoperoxidase and of a peroxide generating system.
3) Lipid labelling was not detected.
4) Morphometric analysis of iodinated nuclei showed 88% integrity of the outer nuclear membrane.
5) The pattern of labelling was highly selective and dependant upon nuclei being intact. When nuclei were broken open, further proteins were iodinated and the overall iodination pattern was substantially altered.
6) Labelled proteins co-purified with nuclear envelopes and the pattern of labelling of nuclei and isolated envelopes was almost identical.

The method, as developed, was therefore used to study the molecular organisation of the nuclear envelope and the relationship of the outer nuclear membrane to the endoplasmic reticulum.

4. The Identification of Proteins of the Nuclear Pore Complex.

4.1. Introduction

One of the main aims of research into the structure and molecular organisation of the nuclear envelope is an understanding of the mechanism by which message is transferred from the nucleus to the cytoplasm. The main approach to this question has been via studies of the conditions under which RNA may be eluted from isolated nuclei (Ishikawa et al. 1969; Ishikawa et al. 1970a & b; Raskas 1971; Raskas 1973; Schumm and Webb 1972; Yu Ling et al. 1972; Schumm et al. 1973a & b; McNamara et al. 1975; Agutter et al. 1976b; Stuart et al. 1977; see also Agutter et al. 1977). So far, this approach has yielded interesting data, in particular in relation to the nucleoside triphosphatase activity of nuclear membranes and the possible consequences of this activity to RNA transport. There is moderately compelling evidence that nuclear envelope nucleoside triphosphatase activity is an essential component of the mechanism of nucleocytoplasmic translocation of ribonucleoprotein (Agutter et al. 1976) and rather less compelling evidence that release of RNA is independent of exogenous ATP (Stuart et al. 1975; - the evidence is less compelling because no attempt was made to establish whether or not nuclear envelope nucleoside triphosphatase activity survived the isolation of nuclei. If the enzyme, or enzymes, had been inactivated, it would not be greatly surprising

that ATP did not markedly increase RNA elution from nuclei). Further suggested functions for nuclear pore complexes are the attachment of ribonucleoprotein particles and the site of polyribosome assembly (Jacob and Danielli 1972).

The more biochemical approach to the question of ribonucleoprotein transport would be to isolate the pore complex and to study the properties and molecular organisation of this structure directly. Unfortunately, there is as yet no bulk method for isolating the pore complex proper, although some attempts, using Trilon X-100 and conication of nuclear membranes, have been made (Aaonson and Blobel 1975; Dwyer and Blobel 1976; Harris 1977).

Very recently, Krohne et al. (1978) have examined the polypeptides found in the nuclear envelopes of maturing amphibian oocytes. Oocyte nuclear envelopes contain an unusually high number of pore complexes in very close packing. Consequently, their nuclear envelopes provide a remarkable enrichment of nuclear pore complex material relative to membranous or other interporous structures. Extraction of the manually isolated nuclear envelopes with high salt concentrations and detergent provided apparently discrete nuclear pore complexes. The pore complexes showed a very high degree of ultrastructural preservat-

ion, despite the removal of the lipids, and were highly enriched in two polypeptides at 150,000 and 73,000 mol.wt. Similar treatment of isolated rat liver nuclear envelopes showed a further two major components at 78,000 and 66,000 mol.wt. The components at 78,000, 73,000 and 66,000 are clearly recognisable as bands N1, N2 and N3, (see Fig. 2, this chapter) although the molecular weight estimates are rather higher than those of other laboratories. The 66,000 mol.wt. component (N3) was relatively loosely associated and was considered to be a part of the nuclear matrix (presumably from the peripheral aspect of the nuclear matrix known as the fibrous lamina). A further point to note is that the major pore complex proteins were considered to be 'skeletal' proteins (i.e. structural proteins). As will be seen subsequently, these ideas are given substance and confirmed by the present investigation.

In the preceding chapter a method for labelling the cytoplasmic surface of the nuclear envelope was developed. The outer annulus of the nuclear pore complex is a structural feature of this and so the labelling pattern includes both proteins of the outer nuclear membrane proper and proteins of the outer pore complex.

It has been noted that the nuclear pore complex

and its associated lamina are resistant to extraction by the non-ionic detergent Trilon X-100 (Franke and Scheer 1974; Aaronson and Blobel 1975; Dwyer and Blobel 1976; Scheer *et al*. 1976; Shelton 1976; Berezney and Coffey 1977). The procedure removes greater than 95% of membrane phospholipid and the outer nuclear membrane appears almost completely removed. Identification of proteins of the outer annulus of the pore complex would therefore appear to be a simple matter of extracting nuclear envelopes, derived from iodinated nuclei, with Trilon X-100; the labelled proteins remaining after this procedure representing nuclear pore complex proteins. This approach was attempted, on more than 10 occasions, with consistent results.

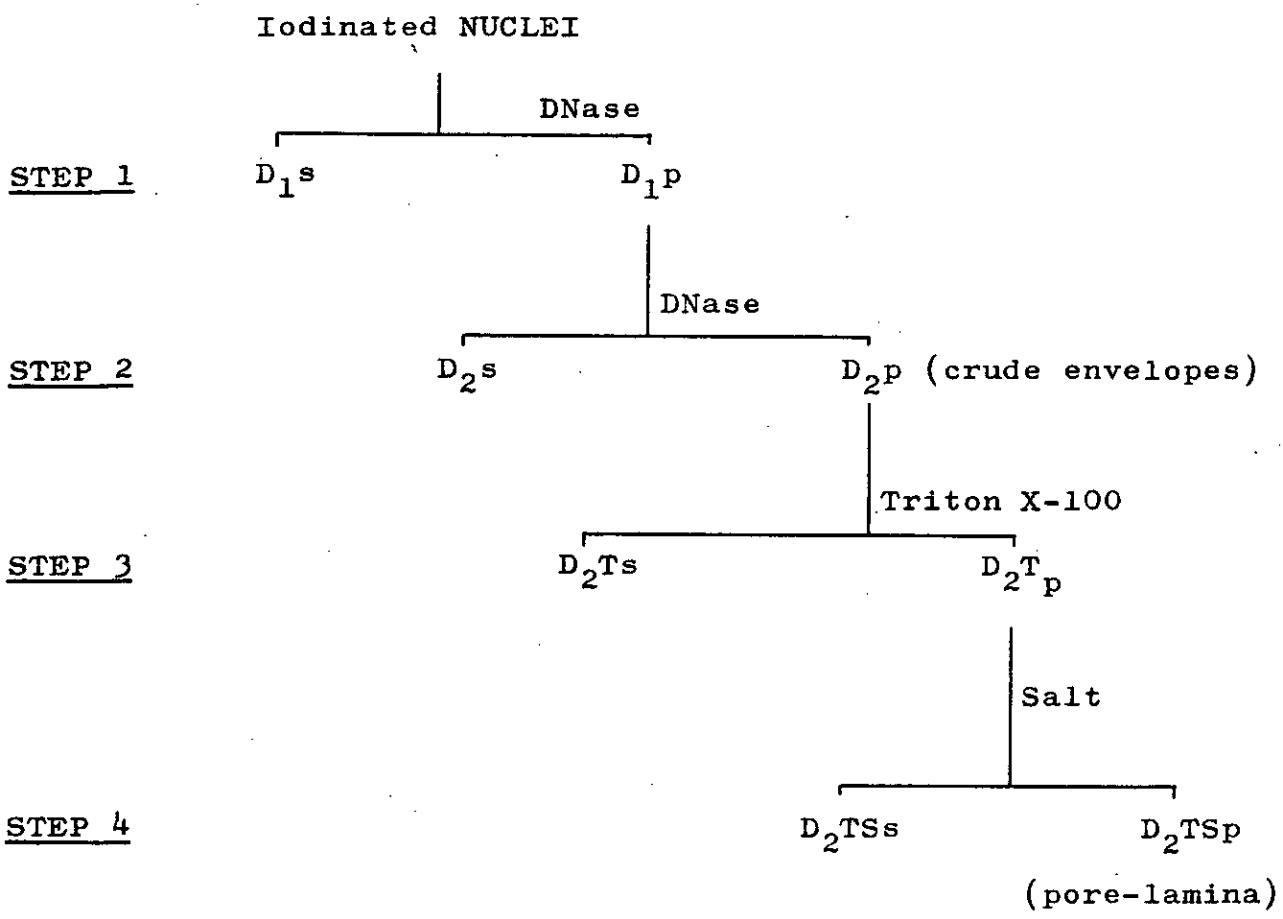


Fig. 1. Flow diagram for preparation of pore complex-lamina fraction (D_2TSp) from surface-labelled nuclei. Small s indicates supernate (to be discarded) and small p indicates pellet after centrifugation. Steps 3 and 4 may be reversed.

4.2. Experimental

Nuclei were isolated as described in chapter 2 and iodinated as described in chapter 3 (legend to table 1).

Isolation of Nuclear Pore Complex-Lamina Fraction

Nuclei were digested twice with DNase (steps 1 and 2 section 2.7) by a slight modification of the method of Dwyer and Blobel (1976).

Step 3. (Triton X-100 wash of crude nuclear membranes):

The DNase digested residue (D_2p) was thoroughly resuspended, using a syringe and fine gauge needle, into 2.5 ml of an ice cold solution of 10% sucrose, 0.1mM $MgCl_2$, 10mM Tris. HCl pH 7.5, to which 0.25 ml 20% (v/v) Triton X-100 (BDH Scintillation grade) was added with vortex mixing. Incubation of the mixture on ice for 10 mins. followed by centrifugation for 10 mins. at $4^{\circ}C$ and at 16,000 rpm (20,000 g. avg) in the 10 x 10 titanium rotor of an MSE Prepspin 50 centrifuge, yielded a supernate (D_2Ts) and a pellet (D_2Tp).

Step. 4. (Salt wash of Triton X-100 extracted nuclear membranes):

The D_2Tp fraction was gently, but thoroughly, re-suspended into 0.2 ml 10% sucrose, 0.1mM $MgCl_2$, 10mM Tris. HCl pH 7.5, using a syringe and fine gauge needle. To this was added 2.5 ml 10% sucrose, 2.0M

NaCl, 0.1mM $MgCl_2$, 100mM Tris. HCl pH 7.5. Incubation of the mixture for 10 mins. on ice, followed by centrifugation as in step 3, yielded a supernate (D_2TSs) and a pellet (D_2TSp). The pellet represents the pore complex-lamina fraction, the outer surface of which is labelled with iodine.

Homogeneous resuspension of the D_2Tp fraction was essential. If the fraction was resuspended directly into the high salt medium, it tended to clump and the preparation remained contaminated with nucleoplasm.

4.3. Results.

4.31. Electron microscopy.

Plate 1. Survey micrograph of nuclear pore complex-lamina fraction (D_2TSp) in thin section (X 48,000). Several pore complexes are seen in tangential section and they appear to contain some internal structure; but this is mostly poorly resolved and diffuse. The fibrous nature of the dense lamina holding the pore complexes together is discernible but not distinct.

Plate 2. High power micrograph (X 130,000) of nuclear pore complex-lamina fraction. A pore complex seen in tangential section clearly shows the annular subunits and fibrils radiating from the internal surface of the annulus to the central element. The central element in this micrograph is unusual in showing a clover-leaf structure.

Plate 3. Survey micrograph of nuclear pore complex-lamina fraction (X 51,000). Several pore complexes are seen in transverse section connected by a fibrous lamina. When seen in transverse section, the pore complexes show little evidence of a residual outer nuclear membrane. The heavy staining of both the pore complex and the fibrous lamina (which are delipidated by Triton extraction) suggests that they contain very high concentrations of protein. If this is so, the pore-lamina fraction will almost certainly account for the bulk of 'nuclear envelope' protein.

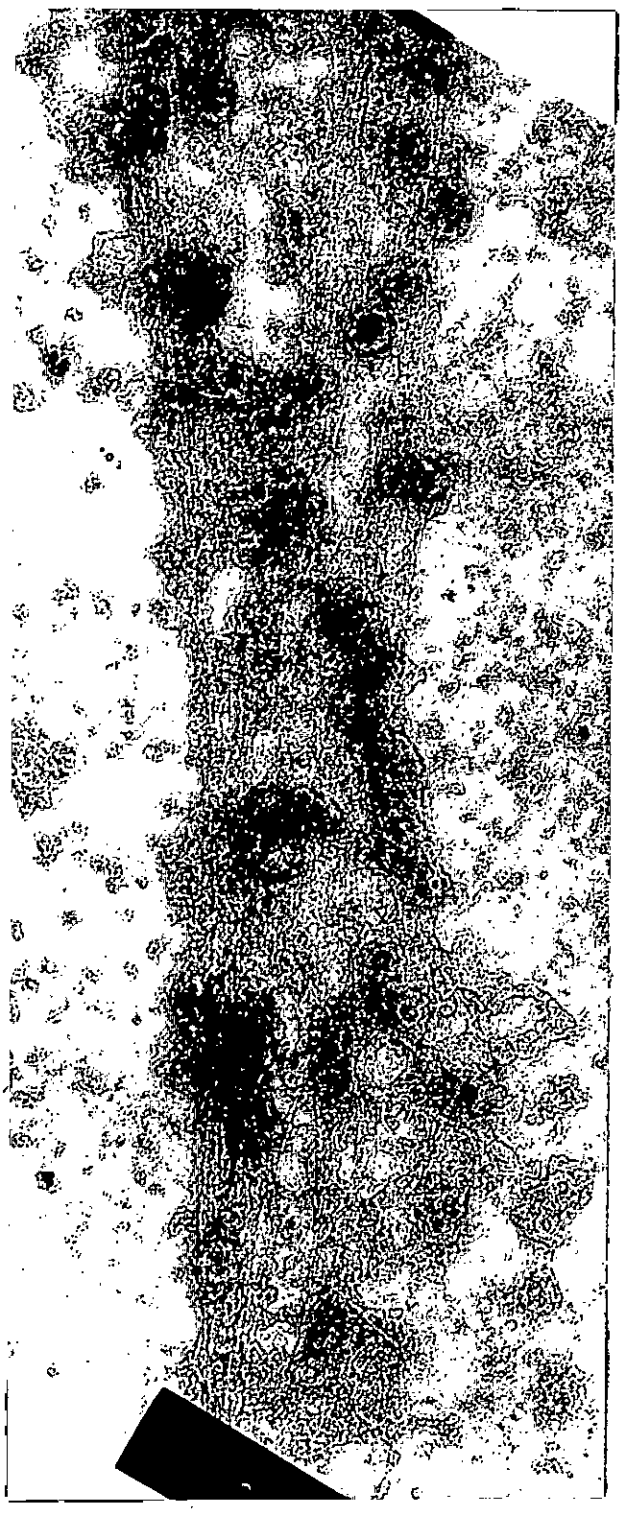


Plate 4. Negatively stained micrograph of nuclear envelopes disrupted under the conditions of staining (X60,000). The very fine fibres connecting the pore complexes and constituting the fibrous lamina are clearly seen. The fibres are continuous between several pore complexes.

4.32. Polypeptide analysis

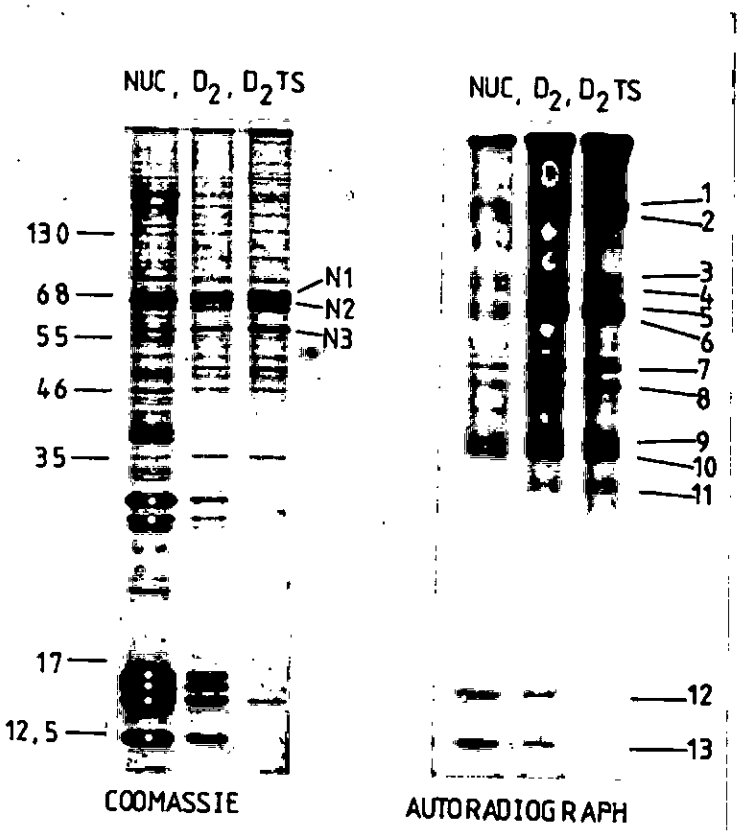


Fig. 2. SDS polyacrylamide gel electrophoresis of reduced polypeptides of iodinated nuclei, nuclear envelopes (D_2) and purified nuclear pore-laminae (D_2TS). Numbers to left of nucleus slot (coomassie) refer to mol.wt. x 1,000 of standard. The three left hand slots are coomassie stained gels and the three right hand slots are auto-radiographs. Histones are indicated by dots. Triplet of bands characterising both highly purified nuclear envelopes and the nuclear pore-lamina fraction is indicated by N_1, N_2 and N_3.

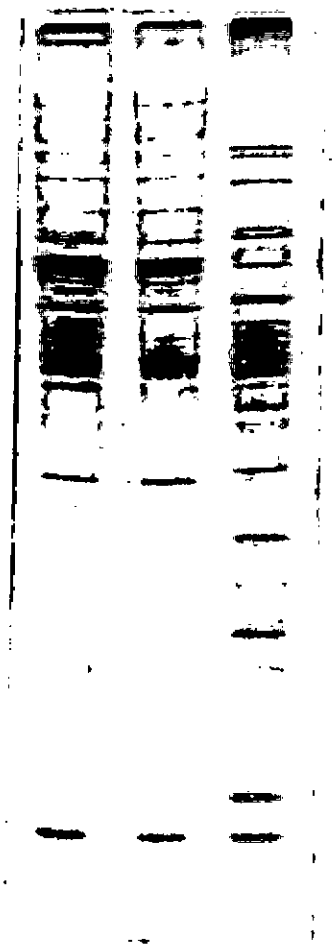


Fig. 3. SDS polyacrylamide gel electrophoresis of reduced polypeptides of (left to right) highly purified nuclear envelopes, Triton extracted nuclear envelopes and the Triton extract. Only a very small proportion of material can be extracted from nuclear membranes with Triton (see also Dwyer and Blobel 1976). Krohne et al. (1978) have estimated that more than 85% of oocyte nuclear envelope dry mass is contained within the pore complexes.

4.33. Chemical Analysis and Specific Activity Measurements

Sample	Protein %	DNA %	RNA %	Phospholipid %
1	93.6	n.d.	6	0.4
2	93.6	n.d.	6	0.4

Table 1. Chemical analysis of purified nuclear pore-lamina fraction. Values based on the approximation that protein + DNA + RNA + phospholipid = 100%. (n.d. - not detectable).

Fraction	Specific activity (c.p.m/mg protein)	Non-specific label % (LPO independent)
Washed nuclei	1,000,000	3.4
pore-lamina	4,200,000	5.2

Table 2. Typical specific activity figures for labelled nuclei and the pore-lamina fraction (at 75% counting efficiency).

4.4. Discussion.

The pore-lamina appears as an extensive meshwork of densely staining pore complexes connected by fine fibrillar threads (plates 1 & 3). Some pore complexes clearly contain an internal structure comprising a central granule and centripetal elements (plate 2), but such detail is not always discernible. Thus although nuclear pore complexes seated on a fibrous lamina are clearly identifiable, they do not exhibit the very high degree of organisation that can be seen in the micrographs of Dwyer and Blobel (1976). It is possible to find some sections which exhibit a very high degree of ultrastructural preservation but they are not representative of the whole. Plates 1 & 3 are considered to be representative of the many preparations of labelled pore-lamina made during this investigation. There are several small differences between the preparative procedures used by Dwyer and Blobel (1976) and in the present study. Aside from the lebelling procedure, perhaps the most important difference is the manner in which pellets, at intermediate stages in the preparation of pore lamina, were resuspended. In this study, pellets were resuspended with some vigour, using a syringe and fine gauge needle, in the belief that this was necessary for the complete removal of nucleoplasm. In relation to this it should

be noted that whereas the material obtained in the present study did not contain detectable amounts of DNA (< 1%), the Dwyer and Blobel preparation contained 3% DNA. The poor ultrastructural preservation of the pore complexes was unrelated to the labelling technique or the inclusion of thiol reagents, for unlabelled pore-lamina prepared in the absence of thiol reagents did not exhibit a higher degree of ultrastructural preservation. High chemical purity was gained at the expense of the integrity of the final preparation but was a necessary compromise in order to allow a confident interpretation of the pattern of iodination. If the order of the Triton extraction and salt extraction was reversed, then there was a further decrease in the integrity, with no measurable gain in the purity, of the preparation.

Chemical analysis of highly purified nuclear pore-lamina (Table 1) has revealed the presence of a significant portion of RNA in the preparation. RNA accounted for 6% of purified pore-lamina (although only 2% in the Dwyer and Blobel study) and this is despite extraction in conditions that completely remove DNA and the outer nuclear membrane with its associated ribosomes. The association between RNA and the nuclear pore complex is already well established (see Scheer 1972, Franke & Scheer 1974) and the strength of this association, combined with the evidence that the

outer annulus is sensitive to RNase (Agutter et al. 1977) suggests that an, as yet unidentified, form of RNA is actually a structural feature of the pore complex.

The iodination pattern of the pore-lamina fraction (Fig. 2) provides striking evidence for the selectivity of the labelling method. The labelling patterns of nuclei and purified pore-lamina are, with the exception of bands 12 and 13, almost identical (the increase in the density of the D_2TSp fraction reflects its increased specific activity over nuclei). The pore-lamina material has been rigorously extracted in low and high salt solutions and with detergent. Label associated with this fraction may therefore be described as 'firmly bound'. Triton extraction removed very little protein-bound label ($\sim$ 10%) although it removed more than 95% of membrane phospholipid. It would appear therefore that the labelling procedure places label predominantly in the nuclear pore complex rather than in the outer nuclear membrane. Such apparent selectivity may be explained by the following:-

1) The pore complex sits well proud of the outer nuclear membrane and its prominence may reduce the accessibility of membrane proteins to LPO beads.
2) The pore complex accounts for a very significant portion of the nuclear surface.

Reference to the negatively stained preparations in chapter 2 shows that the pore complex accounts for greater than 25%, and nearer 50% of envelope surface area.

3) Triton extraction of highly purified nuclear envelopes (Fig. 3) shows that very little protein may be extracted by Triton and that the bulk of nuclear 'envelope' protein is associated with the pore complex and its lamina.

Thus, not only does the pore complex present more protein to the nuclear surface than does the outer nuclear membrane, but it also presents it in a more accessible manner.

From the iodination pattern (Fig. 2), bands N_1 and N_2 of the major triplet may be identified as being externally disposed proteins of the cytoplasmic surface of the nuclear pore complex. As such, these proteins are the first nuclear pore proteins from a mammalian cell to be positively identified. Krohne *et al.* (1978) considered that their 73,000 mol.wt. component (N_2) was a skeletal component of the oocyte and the hepatocyte nuclear pore complex and that the 78,000 mol.wt. (N_1) component was specific to the hepatocyte. They were unable to decide whether the 78,000 component was located in the pore complex or in residual interporous or matrix material. The

above labelling experiments provide compelling evidence that both N_1 and N_2 are major components of the hepatocyte nuclear pore complex.

N_3 might also be a pore complex protein which, buried deep in the more complex, remains inaccessible to lactoperoxidase beads. This cannot be the case however, for N_3 was heavily labelled when lactoperoxidase beads had access to the interior surface of the inner nuclear membrane (see previous chapter). Labelled N_3 almost certainly represents lamina protein rather than intra pore protein, because the very large size of the LPO beads would preclude their gaining access to the interior of the pore complex. This idea is further supported by preliminary experiments that have indicated that soluble lactoperoxidase does not label N_3 in intact nuclei. Although soluble lactoperoxidase may have a rather restricted access to the pore interior, labelling of N_3 might be expected if it was a major intra-pore protein. Krohne et al. (1978) considered that the 66,000 mol.wt. component (N_3) represented an associated intra-nuclear component and found that fibrillar subfractions separated from the nuclear membranes were enriched in this component.

Krohne et al. (1978) also identified a protein of 150,000 mol.wt. as a pore complex component - a major one in amphibian oocyte nuclear pore complexes and a more minor component in hepatocyte nuclear pore

complexes. Although such a component is not immediately evident in the coomassie stained gels above, counterparts do exist as major components of the iodination pattern (bands 1 and 2). The labelled pore complex exhibits 11 major labelled bands and several minor bands. Of the major bands, only 5 and 6 are correspondingly major coomassie bands (N_1 and N_2); the other 9 bands are all very minor coomassie bands. It seems likely therefore that these 9 bands represent the most external proteins on the pore complex and that they are structured into the outer annulus formed by N_1 and N_2; the very high proportion of N_1 and N_2 present in the pore-lamina fraction relative to the minor coomassie bands deems that they must be the major structural elements of the pore complex.

Very recently, Gerace et al. (1978) eluted N1, N2 and N3 (referred to as P70, P67 and P60) of rat liver pore-lamina from SDS polyacrylamide gels and raised antibodies to these polypeptides in chickens. Using immunofluorescence localisation, they found that antibody to N1, N2 and N3 was bound exclusively to the nuclear periphery. Indirect immunoperoxidase staining showed that antibodies to N1, N2 and N3 bound only the fibrous lamina and not the pore complex. From this it was concluded that these polypeptides are not present, or concentrated, in the pore complex in an immunologically reactive form; and it was

suggested that N1, N2 and N3 are the major structural components of the fibrous lamina. There are two important points to be made in relation to this work:

1) Antibodies were raised to SDS solubilised protein with the possible exposure of antigenic sites not normally revealed within the pore complex.

2) Ouchterlony double diffusion analysis showed that N1, N2 and N3 cross-reacted strongly with all three antisera; behaving as immunologically very similar or identical. A single discernible precipitin line was obtained by reaction of anti-N1 or anti-N3 antisera with any of the three polypeptides. There is thus the grave possibility that N1, N2 and N3 possess a common antigen and that antibody has been raised to this antigen in all three cases. If so, then the binding of antibody to N1, N2 or N3 to the fibrous lamina may not be regarded as locating N1, N2 or N3 to the fibrous lamina; but only as detecting exposure of this common antigen in the fibrous lamina. The work may not therefore be regarded as providing firm evidence for the location of N1, N2 or N3 in the fibrous lamina, and certainly does not indicate that these polypeptides are absent from the pore complexes.

The lactoperoxidase labelling studies have indicated that N1 and N2, both major components of the nuclear envelope, are located in the nuclear pore

complex (although not necessarily exclusively so) and that they are exposed at the cytoplasmic surface. It seems improbable, in view of the regular architecture of the envelope and the high proportion of polypeptides N1 and N2 in the envelope, that these polypeptides are other than structural elements, whose gross and dynamic organisation is dependant on other, quantitatively minor, envelope components. The coomassie pattern of the pore lamina fraction reveals approximately 90 bands to the naked eye (rather more than can be seen in Fig. 6), so that there is no shortage of polypeptides whose function might be to organise the structure and provide the active units of the pore complex.

The main vectoral data to have emerged from this study may be summarised as follows:

1) N1 and N2 are major components of the outer annulus of the pore complex and are exposed to the cytoplasmic surface.
2) The outer annulus is composed of at least 14 polypeptides, only two of which are constitutively major or structural components.
3) The fibrous lamina contains N3 as one of its, but not necessarily its only, major constituent.

The above findings are entirely complementary to, and confirmatory of, the recent work that has emerged from Franke's laboratory (see Krohne _et al_. 1978). In particular, the labelling technique has established

details of the molecular organisation of the nuclear envelope which, previously, could only be the subject of conjecture.

Chapter 5.

The Relationship Between the Nuclear Envelope and the Endoplasmic Reticulum.

5.1. Introduction.

It is now an item of dogma that the outer nuclear membrane is continuous with the endoplasmic reticulum and that the nuclear membranes are biochemically similar to the endoplasmic reticulum (see Franke and Scheer 1974; Wunderlich et al. 1976; Fry 1976b; Harris 1978). The view has emerged that the biochemical role of the nuclear membranes is so close to that of the endoplasmic reticulum, that it might only be important in some cells, such as thymocytes, where the nuclear envelope accounts for a significant proportion of the total cytomembrane surface (see Franke and Scheer 1974; Fry 1976b; Franke et al. 1976). Let us evaluate some of the evidence that underlies this belief.

5.11 Membrane continuity.

Watson (1955) first noted the continuity between the endoplasmic reticulum and the outer nuclear membrane, and between the inner and outer nuclear membranes at the pore complex. He considered that all these systems could be regarded as part of the same system and that the nuclear membranes were merely a part of the endoplasmic reticulum specialised for nucleo-cytoplasmic transport. Watsons observations were confirmed by others (de Groodt et al.1958; Whaley et al. 1960; Gibbs 1962; Hadek and Swift 1962; Fawcett 1966) but the early studies, which used only

osmium tetroxide as a fixative, are not especially convincing. However, Franke and Scheer (1974) have provided most elegant micrographs to demonstrate this continuity in the meristematic root tip cell of the onion and in a Xenopus laevis oocyte (lampbrush stage) using both glutaraldehyde and osmium as fixatives; but what is still not clear is the extent to which this continuity is a common phenomenon, and with what frequency this phenomenon may be observed in a given cell type at a specified stage in its development. Such continuities, where they exist, must presumably be transient for the nucleus rotates (Mirsky and Osawa 1961) and, unless the elements attached to the nucleus describe a fixed orbit at the same rate, links must be continually broken and reformed. The functional significance of nuclear rotation is not known but its existence, together with the existence in some cells of links between the endoplasmic reticulum and the outer nuclear membrane, imply an important dynamic relationship between the two membrane systems.

Although a continuity between the outer nuclear membrane and the endoplasmic reticulum has been observed by several laboratories, it is not, apparently, an essential principle of endomembrane organisation (Franke 1977); for such continuities may be missing both in cells in which the endoplasmic reticulum is reduced or absent (Franke 1974a & b) and also in cells with a highly developed endoplasmic reticulum system

(Franke 1974a; Franke *et al*. 1974). On no occasion during this study have I observed a continuity between the outer nuclear membrane and the endoplasmic reticulum in rat liver tissue. Consequently, if such continuities do exist in this tissue, then I believe them to be either extremely unstable or very rare. As a consequence of the presumed infrequency of direct continuities between the two membrane systems, one must conclude that the capacity for exchange diffusion between the two systems, in the plane of the membrane, is very limited.

5.12. Mitosis and membrane repair.

The nuclear envelope is not a permanent structure in most cells of higher organisms. In mammalian cells, transitory disintegration of the nuclear envelope is observed during 'open mitoses'. At the end of prophase the nuclear envelope becomes irregular and breaks down into cisternal pieces or vesicles. Most of the membrane fragments lose their pore complexes and become indistinguishable from endoplasmic reticulum, but some can be observed to remain attached to the chromosomal surfaces and bear normal pore complexes. The nuclear envelope begins to reassemble in late anaphase and early telophase and, although reutilisation of nuclear membrane fragments from the mother cell nucleus may contribute to this, it seems that the major contribution comes from vesicles resembling the cisternae of the endoplasmic reticulum. Bilamellar sheets of

membrane, some already containing pore complexes, coalesce around the chromosome masses in late anaphase and early telophase, and in late telophase enlarge by fusion and recruitment of more cisternal elements to form a continuous nuclear envelope (from Comings 1968; Robbins and Gonatos 1964; Erlandson and de Harven 1971; Bajer and Mole-Bajer 1972; Franke 1974a & b; Franke and Scheer 1974; Kubai 1975; Fry 1976b; Franke 1977).

There does not appear to be a simple precursor-product relationship between the two membrane systems, with newly synthesised outer nuclear membrane displacing older nuclear membrane into the endoplasmic reticulum. In general, the nuclear envelope proteins and lipids incorporate labelled precursors at the same, or even a slower, rate than the ER (Franke *et al*. 1971; Tata *et al*. 1972; Elder and Morre 1974). The reverse would be true if the nuclear membranes contributed substantially to the endoplasmic reticulum.

The endoplasmic reticulum may contribute to the nuclear envelope both during mitosis and after surgical damage to the outer nuclear membrane. Repair of amoeba nuclear membranes that have been damaged microsurgically involves the association of pieces of endoplasmic reticulum with damaged nuclear membranes (Flickinger 1974). Endoplasmic reticulum and nuclear membranes from closely related cell types

can interact during repair of damaged nuclear membranes, and there appears to be a specificity to this interaction since in a combination of relatively dissimilar amoeba cells there was no evidence of repair and the cells died (Flickinger 1978).

5.13. Ribosomes and protein synthesis.

The presence of ribosomes on the cytoplasmic surface is a common feature of both the endoplasmic reticulum and the outer nuclear membrane (Watson 1955; Palade 1955). Moreover, both membrane types exhibit areas that lack ribosomes and which show vesicle and bleb formation (see Franke and Scheer 1974 for refs). Both membrane types almost certainly function in the synthesis of membrane-bound and secretory proteins. It is well established that a single defined protein can be deposited both in the peri-nuclear cisternum and in cisternal spaces of the rough endoplasmic reticulum, as has been demonstrated for the synthesis of ferritin and peroxidase antibodies in plasma cells (De Petris et al. 1963; Avrameas and Bouteille 1968; Leduc et al. 1968, 1969; Avrameas 1970). Protein synthesis may show some regional specialisation. There is some evidence that cytoplasmic messenger RNA's coding for mitoplast proteins are preferentially compartmentalised onto a particular subclass of rough endoplasmic reticulum (Shore and Tata 1977); and so if areas of the endoplasmic reticulum

are differentiated with respect to the type of protein synthesis, then it is reasonable to suppose that the outer nuclear membrane may also be responsible for the synthesis of a particular sub-set of proteins (see Gorovsky 1969). Of course, the role of the nuclear membranes is not restricted to that of protein synthesis and there is ample evidence that storage of proteins may also occur in the perinuclear cisternum (Behnke and Moe 1964; Poux 1969; Perrin 1969, 1970; Narquet and Sobel 1969; Fahimi 1970; Herzog and Miller 1970, 1972; Strum and Karnovsky 1970; Strum et al. 1971).

Although the presence of ribosomes is a common feature of both membrane types, their presence on the outer nuclear membrane could be an indication of the need to synthesise and insert nuclear membrane proteins at the nuclear membranes rather than receive them, by diffusion, from the endoplasmic reticulum (which would require the membranes to be fluid as well as continuous). Thus, the presence of ribosomes on the outer nuclear membrane may also be cited as potential evidence in favour of the differentiation of nuclear membrane proteins from those of the endoplasmic reticulum and of the independance of the two systems.

5.14 Lipids.

Rough endoplasmic reticulum and nuclear

envelopes have similar total lipid, phospholipid and fatty acid patterns (Gurr et al. 1963; Lemarchal and Bornens 1969; Kashnig and Kasper 1969; Franke et al. 1970; Keenan et al. 1970; Kleinig 1970; Stadler and Kleinig 1971). The two systems are characterised by a relatively low cholesterol and sphingomyelin content compared with, for example, the plasma membrane. There are some significant differences - in particular the relatively high content of cholesterol ester in nuclear envelopes (Kleinig 1970; Kleinig et al. 1971; Sato et al. 1972). Furthermore, the phospholipids of nuclear envelopes generally contain more saturated fatty acids (Stadler and Kleinig 1971; Keenan et al. 1972). The differences suggest that the nuclear membranes may have a greater stability than those of the endoplasmic reticulum (Stadler and Kleinig 1971).

5.15. Carbohydrate.

The nuclear envelope does not contain detectable amounts of glycolipid (Keenan et al. 1970, 1972; Kleinig 1970) and carbohydrate found in nuclear membrane preparations, comprising 3-4% of delipidated membrane (Kashnig and Kasper 1969) is presumed to be largely protein bound (Kasper 1974). Electrophoretic analysis of nuclear envelope and endoplasmic reticulum glycoproteins has demonstrated a major homology, between the two membrane types

at 160,000 mol.wt. (Bornens and Kasper 1973; Kasper 1974). Analysis of the sugar residues in both membrane types has shown a very high proportion of mannose and galactose (Kawasaki and Ymashina 1972; Franke et al. 1976) and at similar ratios (although, see also Phillips 1973). The high proportion of mannose present in the carbohydrate moieties correlates well with the high capacity for con A binding in the nuclear envelope (Kaneko et al. 1972; Nicolson et al. 1972; Bretton and Bariety 1974; Keenan et al. 1974; Monneron and Segretain 1974; Monneron 1974; Wood et al. 1974; Virtanen and Wartiovaara 1976; Michaels et al. 1977; Stoddart and Price 1977; Virtanen 1977) and in endoplasmic reticulum (Bretton and Bariety 1974; Hirano et al. 1972; Wood et al. 1974; Boulan et al. 1978 a & b). It is of particular importance to note that, in very careful studies it has been shown that con A is bound exclusively to the cisternal surfaces of the nuclear membranes (Virtanen and Wartiovaara 1976; Virtanen 1977) and of the rough endoplasmic reticulum (Boulan et al. 1978 a & b). Moreover, in rough endoplasmic reticulum, two glycoproteins have been identified as being transmembrane proteins (Boulan et al. 1978a) and it is believed that biogenesis and insertion of glycoproteins into the endoplasmic reticulum does not require processing in the Golgi apparatus.

Thus, the two membrane systems exhibit a major glycoprotein homology, similar sugar compositions and exposure of con A binding sites solely on their luminal surfaces. Such similarities should be placed in the context of the following three points;

1) Exposure of the carbohydrate moiety of membrane glycoproteins solely on the luminal surface may be a general principle of endomembrane organisation rather than a characteristic peculiar to the two membranes under consideration.

2) So far only one distinct homology has been demonstrated between nuclear envelope and endoplasmic reticulum glycoproteins; yet the rough endoplasmic reticulum membrane bears at least 15 con A binding glycoproteins (Boulan et al. 1978a) and the nuclear envelope contains 2 major and 5 minor con A binding glycoproteins (Virtanen 1977).

3) Although analysis of the sugar residues has established which are the major residues in the nuclear envelope and the endoplasmic reticulum, there is a rather poor agreement over the relative proportions of different sugar residues. More weight should, however, be given to Franke's closely defined data (Franke et al. 1976) which indicates a very great similarity in the relative proportion of different sugar residues in the nuclear envelope and endoplasmic reticulum.

5.16. Proteins and Enzyme Activities.

The comparatively close agreement between

different laboratories over the lipid and carbohydrate moieties associated with the nuclear envelope contrasts strongly with the great disparities found in enzyme activity measurements. Such disparities may often result from more than one reason and it is a difficult, and even tendencious, exercise to attempt a critical comparison of the enzyme activites found in endoplasmic reticulum and nuclear envelope preparations from different laboratories. There are two principle reasons for the disagreement;

1) Different preparations of nuclear envelope may be contaminated, to widely differing degrees, by other subcellular organelles. Thus, for example, a preparation containing substantial endoplasmic reticulum contamination may be expected to show more ER 'character' than one that is less so contaminated. Estimates of contamination may occasionally be inaccurate for they are often based on the measurement of, frequently labile, enzyme activities at low levels. Moreover, clear-cut marker enzymes do not always exist.

2) Techniques for preparing nuclear envelopes differ widely in their rigour (see Chapter 2) and thus in the likelihood of their retaining labile enzyme activities. Furthermore, it is more usual for a laboratory to treat preparations of endoplasmic reticulum and nuclear envelopes quite differently

rather than to extract the two membrane types with the same rigour and thence to effect a more reliable comparison.

A comparison of enzyme activities and cytochrome contents of nuclear envelopes and microsomes from rat liver tissue is effected in Table I. The table has been constructed according to the following principles.

a) Where an enzyme activity has been established both histochemically and enzymically as endogenous to a membrane preparation, but is described by a particular laboratory as being absent, then this latter data is excluded. Thus, for example, the negative data of Franke et al. 1970 and Agutter 1972b on glucose-6-phosphatase activity in nuclear envelopes is excluded. This enzyme is without question found at high activities in both the nuclear envelope and endoplasmic reticulum (see Kashnig and Kasper 1969; Kay et al. 1972; Gunderson et al. 1975; Kanamura 1975; Sikstrom et al. 1976; Wilson and Ghytil 1976) and in subsequent papers, these authors have retracted their earlier views (Kartenbeck, Jarasch and Franke 1973; Milne, Agutter, Harris and Stubbs 1978).

b) The data from early studies, in which the purity of envelope preparations was either low or poorly defined, are excluded. The data used are solely those from major groups using well described

techniques of envelope preparation.

c) Enzyme activities more normally associated with mitochondria (e.g. cytochrome oxidase) and whose residual activity in the nuclear envelope is on balance doubtful or at the very least controversial are excluded (for details of the controversy surrounding the presence of cytochrome oxidase in the nuclear envelope, see Berezney et al. 1972; Berezney and Crane 1972; Zbarsky 1972; Franke 1974; Jarasch and Franke 1974; Kasper 1974; Franke et al. 1976; Wunderlich et al. 1976; Jarasch and Franke 1977).

d) Enzyme activities such as DNA polymerase and DNA swivel enzyme, which are more likely to reflect either nucleoplasmic contamination or properties pertaining to the inner nuclear membrane and its associated lamina, are excluded.

Thus, in constructing table 1, some effort has been made to ensure a fair comparison of the endoplasmic reticulum and the nuclear envelope. A more exhaustive survey may be found elsewhere (see Franke 1974 a & b).

TABLE 1. Enzyme Activities* and Cytochrome Contents of Nuclear Envelopes and Microsomes from Rat Liver.

Enzyme	NE	Microsomes (ER)	NE/ER	References
Glucose-6-Pase.	355(h)	520	0.68	(Kashnig & Kasper 1969)
	255 (l)		0.49	(")
	160	176	0.91	(Kay et al. 1972)
	83(h)	130(h)	0.64	(Kartenbeck et al. 1973)
	98(l)	162(l)	0.60	(")
	850	254	3.35	(Sikstrom et al. 1976)
	330-450	120	2.75	(Wilson and Chytil 1976)
	255	-	-	(Milne et al. 1978)
Mannose-6-Pase.	75	112	67	(Kartenbeck et al. 1973)
Mg^{2+}-ATPase.	233(h)	73	3.19	(Zbarsky et al. 1969)
	393(l)		5.38	(")
	39(h)	178	0.22	(Kashnig and Kasper 1969)
	29(l)		0.16	(")
	93	122	0.76	(Franke et al. 1970)
	87	128	0.68	(Kartenbeck et al. 1973)
	273	-	-	(Milne et al. 1978)
5' Nucleotidase	3.3	48	0.07	(Jarasch 1973)
	193	65	2.97	(Sikstrom et al. 1976)
	50	100	0.50	(Wilson and Chytil 1976)
	21.7	-	-	(Milne et al. 1978)
p-nitrophenyl phosphatase.				
pH 4.8	53	685	0.08	(Franke et al. 1970)
pH 4.5	12.5	50	0.25	(Kartenbeck et al. 1973)
pH 10.5	28	153	0.18	(Franke et al. 1970)
pH 9.0	2.5	4.0	0.62	(Kartenbeck et al. 1973)
NADPH cytochrome C reductase.	6.5(h)	18	0.36	(Zbarsky et al. 1969)
	7.7(l)		0.43	(")
	18	49	0.37	(Franke et al. 1970)
	104	332	0.31	(Kasper 1971)
	51	34	1.50	(Kay et al. 1972)
	29	57	0.50	(Jarasch 1973)
NADH cytochrome C reductase.	60(h)	283	0.21	(Zbarsky et al. 1969)
	16(l)		0.76	(")
	379(h)	902	0.42	(Kashnig & Kasper 1969)
	381(l)		0.42	(")
	100	350	0.29	(Franke et al. 1970)
	267	759	0.35	(Kay et al. 1972)
	552	981	0.56	(Kasper 1971)
Glutamate dehyrogenase.	48(h)	4.0	12.00	(Zbarsky et al. 1969)
	27(l)		6.75	(")
	32	40	0.80	(Franke et al. 1970)
Arylsulphatase (A + B)	9.3(h)	1.6	5.81	(Zbarsky et al. 1969)
	1.6(l)		1.00	(")
(A) NADase	2.31	4.44	.52	(Fukushima et al. 1976)
(B) Transylycosidase	1.06	1.64	.576	(")
Ratio B/A	0.46	0.42	-	(")

Table 1 contd.

Enzyme	NE	ER	NE/ER	References
Cytochrome b_5	0.034	0.13	0.26	(Franke et al. 1970
	0.183	0.492	0.37	(Kasper 1971)
Cytochrome P450	0.025	0.18	0.14	(Franke et al. 1970
	0-0.22	0.62	0-0.35	(Kasper 1971)

*Enzyme activities are expressed as nmoles substrate metabolised per minute per mg protein. Content of cytochromes is expressed as nmol/mg protein.

(h) Heavy membranes
(l) light membranes

Two things are immediately apparent from Table 1. Firstly, there is very poor agreement between different laboratories over the actual level of different enzyme activities in the two membrane systems. Secondly, there is such a wide variation in the measured levels of given enzymes and their relative proportions in the two membrane types, that it is not always possible to establish which membrane type has the higher activity. The only general conclusion that may be drawn from the limited detail available in Table 1 is that the two membranes exhibit several activities common to one another but that the relative levels of these activities *in vivo* remains to be established.

Nuclear membrane NAD^+ glycohydrolase activity is identical to the microsomal enzyme in its Km for NAD^+, pH optimum, ratio of transglycosidase activity to NAD^+ase activity, thermal stability and sensitivity to various inhibitors (Fukushima *et al*. 1976). Thus, although the absolute levels of activity are different, the actual enzyme is almost certainly identical. A general point worth making at this stage is that, as mentioned in the preceding chapter, in nuclear envelopes with a high pore-complex frequency (i.e. as in liver tissue), the bulk of the protein in the preparation relates to the pore complexes and their associated lamina; thus, if in comparing the levels of membrane enzymes found in the two systems, we

express enzyme activities in terms of protein, we are not truly comparing like with like. It would be much more satisfactory to compare putative membrane enzyme levels in terms of lipid; but this is rarely done, and in the absence of chemical data on the endoplasmic reticulum preparations used by different laboratories, this cannot be recalculated. However, it should be noted that in cases where an enzyme activity, measured in terms of protein, is found to be substantially higher in the nuclear envelope, then its membrane activity in terms of phospholipid will indeed be very much greater (consider the activities quoted in Table 1. for glucose-6-phosphatase). Sagara et al. (1978) have measured the glucose-6-phosphatase activity present in nuclei and microsomes and expressed their measurements in terms of phospholipid (implying the assumption that phospholipid is restricted to the nuclear membranes of isolated nuclei). The nuclear membranes would appear, by this calculation, to possess more than twice the glucose-6-phosphatase activity of microsomes. Although these authors suggest that non-specific phosphatases present in the nuclear matrix might contribute towards the observed activity, their figures are in general agreement with those of Sikstrom et al. (1976), and Wilson and Chytil (1976).

Electron Transport Enzymes and Respiratory Pigments.

The nuclear envelope and endoplasmic reticulum

exhibit several common electron transport activities and respiratory pigments (for refs. see Franke 1974a & b; Fry 1976b; Wunderlich et al. 1976). Cytochrome P450 (which can act as electron acceptor in the NADPH-cytochrome c reductase system) is present at high levels both in the endoplasmic reticulum and nuclear envelopes of rabbit liver tissue (Ichikawa and Mason 1973) and rat liver tissue (Jarasch and Franke 1974; Sagara et al. 1978). The pigment is apparently liable to extraction in high salt buffers (Fry 1976b), which may explain the very low or variable levels detected by other laboratories (see Franke et al. 1970; Berezney et al. 1972; Kasper 1971). Recently, Matsuura et al. (1978) obtained electron microscopic evidence for the presence of cytochrome P450 on the cytoplasmic surface of the outer nuclear membrane using ferritin-labelled antibody to microsomal cytochrome P450 . It therefore seems likely that closely related, if not identical, cytochrome P450 is present in both the outer nuclear membrane and the endoplasmic reticulum.

Kasper (1971) has reported that the drug-oxidising activities of rat liver nuclear envelopes are distinct from their microsomal counterparts since, in contrast to the significant increase of activity seen in microsomes, NADPH-cytochrome C reductase, Cytochrome P450, Aryl hydrocarbon hydroxylase, and N-demethylase were not induced by phenobarbitol treatment. Aryl hydroxylase

activity, which is associated with the NADPH mixed oxidase system, was inducible in both membrane types by the carcinogen 3-methylcholine (Khandwala and Kasper 1973). In contrast to Kasper's findings, Ichikawa and Mason (1973) have reported that phenobarbitol induced the increase of Cytochrome P450 and NADPH-cytochrome C reductase in rabbit liver nuclei. Sagara et al. (1978) have found a significant increase in Cytochrome P450, Cytochrome b5, O-Deethylase and N-Demethylase in nuclei and microsomes from phenobarbitol-treated rat liver. This evidence, combined with Sagara's immunological data and inhibition studies (Sagara et al. 1978) strongly favours the existence of intrinsic microsomal-type enzymes in rat liver nuclear envelope.

The impression may be gained that the nuclear envelope and endoplasmic reticulum contain a series of enzyme and cytochrome activities common to, but also restricted to, one another. Such a view is manifestly false; not only is cytochrome b5 found in mitochondria, but the presence of NADPH cytochrome C reductase in golgi has been firmly established (Ito and Palade 1978). Moreover, glucose 6-phosphatase is also likely to be a golgi enzyme (Howell et al. 1978); and because glucose 6-phosphatase activity is of rather broad specificity, the position of other sugar 6-phosphatases needs to be considered carefully. The properties of nuclear envelope magnesium dependant nucleoside triphosphatase

activity are distinct from the endoplasmic reticulum counterpart and the enzyme has been cleverly implied in nucleocytoplasmic transport of ribonucleoprotein (Agutter et al. 1976; Agutter et al. 1977). In rat adrenal cortex cells, glutamine-oxaloacetic transaminase reaction product has been found in the perinuclear cisterna but not in the endoplasmic reticulum (Chak and Lee 1971). In rat prostate, the nuclear membranes possess half the cellular activity of an enzyme that reduces testosterone to the active metabolite dihydro-testosterone (Moore and Wilson 1972). Peroxidase activity has been detected in the perinuclear cisterna of many cells including rat liver (for refs. see chapter 3) but appears to be absent from rat liver endoplasmic reticulum (Stubbs and Harris 1978).

The presence of similar electron transport enzymes and pigmets in different membranes should not necessarily be taken to imply a functional similarity in vivo. For example, Cytochrome b5 may be found in the outer mitochondrial membrane as well as in the endoplasmic reticulum and nuclear envelope (see Wunderlich et al. 1976 for refs.) and the significance of this wide distribution is still unknown. It has been suggested that the b5 reductase system may represent an electron transport system with multiple roles and that it should perhaps be considered as a membrane-bound reservoir of reducing power which may be tapped

by a number of systems (Wunderlich et al. 1976). In this latter respect, it has been postulated that the nuclear envelope NADH oxidase system represents a primitive electron transport system which was later replaced by the mitochondrial respiratory chain in the main stream of energy generation (Berezney 1972). Whether Berezney's hypothesis is correct is not at this stage important; but it serves the point that although there are several similarities in enzyme composition between the nuclear envelope and the endoplasmic reticulum, it is their molecular organisation within the membrane and their relationship to other enzymes that determines function. Thus in knowing that these particular enzymes are associated with the nuclear envelope we are better informed; but our understanding of their function will not increase until we can integrate this information. Their presence in the nuclear envelope and endoplasmic reticulum is not immediate evidence of the two membranes functional similarity.

5.17. Polypeptide Analysis

Several comparisons between the electrophoretic profiles of nuclear envelope proteins and those of the endoplasmic reticulum have been made (Franke et al. 1970; Deumling 1972; Matsuura and Ueda 1972; Monneron et al. 1972; Bornens and Kasper 1973; Harris 1978). In general, the resolution of these studies has

been so poor that it has not been possible to establish true homologies between the two membrane systems. In the studies of Matsuura and Ueda (1972) and of Harris (1978) the match between the two membrane systems was very poor. Conversely, in the studies of Franke et al. (1970) and Monneron et al. (1972) there were some, poorly resolved, similarities. The most effective study has been that of Bornens and Kasper (1973) in which it was established that approximately 55% of the total nuclear envelope proteins were divided into the two molecular weight ranges of 64,000 to 74,000 and 47,000 to 60,000, whereas the majority of microsomal membrane proteins had molecular weights only in the latter region. Nearly 22% of the nuclear membrane protein were accounted for by polypeptide chains with molecular weights of 70,000 and 74,000 whereas the microsomal membrane had only a single minor component in this molecular weight range. Both membranes revealed a glycoprotein at 160,000 by periodic acid-Schiff staining.

5.18. Strategy for comparing the composition and organisation of the proteins of the nuclear envelope and rough endoplasmic reticulum.

It is worth recalling the generally held belief that 'the biochemical role of the nuclear envelope is so close to that of the endoplasmic reticulum, that it might only be important in cells where the envelope accounts

for a significant proportion of the total cyto-membrane surface' (see section 5.1). In the face of available evidence (v. supra), such a view is untenable. Certainly, the nuclear envelope demonstrates several enzyme activities common to other sub-cellular fractions, and others which may be highly concentrated in the nuclear envelope; but this is not to establish a close similarity with the endoplasmic reticulum but merely confirm what we already know - that the nuclear envelope is a part of a sub-cellular organelle.

In order to clarify the degree of homology between nuclear envelope and endoplasmic reticulum proteins, it was decided to compare the electrophoretic profile of the two systems in high resolution Laemmli gels, and by incorporating two novel features:-

1) It has been mentioned earlier (chapter 4) that the bulk of rat liver nuclear envelope protein resides in the pore complexes and their associated lamina. Thus, studies which attempt to compare the membrane components of the nuclear envelope and endoplasmic reticulum do not effectively compare like-with-like, unless the pore-lamina fraction is first removed from the envelope. Therefore, in the present study, the electrophoretic profile of rough endoplasmic reticulum proteins was compared with nuclear envelope proteins remaining after the removal of the triton-insoluble pore-lamina fraction (i.e. outer nuclear membrane +

integral inner nuclear membrane proteins).

2) In order to gain vectoral data on the disposition of proteins within the membranes, the cytoplasmic surface of both membrane types was labelled with lactoperoxidase.

Thus, it has been possible, for the first time, not only to compare endoplasmic reticulum proteins with 'true' nuclear membrane proteins, but also to compare proteins in the same membrane plane in both membrane types.

5.2. Experimental.

Nuclear envelopes were iodinated and isolated as previously described. Iodinated envelopes were extracted with Triton X-100 as previously described.

5.21. Preparation of Rough Endoplasmic Reticulum.

Rough endoplasmic reticulum was prepared by minor modification of the caesium chloride method (Berastrand and Dallner 1969; Depierre and Dallner 1976), which makes use of the fact that Cs^+ can selectively aggregate rough microsomes (Dallner 1963; Dallner et al. 1971).

Female Wistar rates (250-300 grams) were starved for 20 hours prior to sacrifice. Livers were excised and placed in ice-cold 0.25M sucrose. The liver was minced and washed with further ice-cold sucrose to remove as much blood as possible. The liver pieces were suspended in an equal volume of ice-cold 0.25M

sucrose and homogenised in a Potter-Elvehjem glass-teflon homogeniser with 4 up and down strokes at 500 revs/min. The homogenate was then diluted with 0.25M sucrose to give a concentration of 0.2 grams original liver wet weight ml^{-1}. All subsequent steps were carried out at $4^{o}C$.

The homogenate was centrifuged for 20 minutes at 10,000 rpm (10,000g) in the 6 x 100 ml rotor of an M.S.E. High Speed 18 centrifuge. The supernatant (containing the microsomes) was carefully decanted, and the pellet discarded. Using the M.S.E. 10 x 10 Ti rotor, 1.5 ml of 0.6M sucrose - 15mM CsCl was layered over 3 ml of 1.3M sucrose - 15mM CsCl. Above the gradient was layered 5 ml of the 10,000g supernatant. Centrifugation was carried out for 90 mins. at 38,000 rpm in the 10 x 10 Ti rotor of an M.S.E. Prepspin 50 centrifuge to pellet the rough microsomes. Adsorbed cytoplasmic proteins were removed by resuspending the rough microsome pellet into 0.15M Tris-HCl (pH 8.0) and pelleting by centrifugation for 60 mins. at 38,000 rpm as above.

Typically, 8 grams original liver gave 96 mgs of purified rough microsomes, which were resuspended into 50mM Tris-HCl, 50mM KCl, 5mM $MgCl_2$, 10mM Glucose, 0.0001% butylatedhydroxytoluene pH 7.5 (low salt buffer - LSB) to a concentration of 20-25 mgm/ml.

5.22. Iodination of Rough Endoplasmic Reticulum.

The cytoplasmic surface of rough microsomes was iodinated according to Kreibrich et al. (1974). 1 ml reaction medium contained 3 mgs of rough ER protein, 0.44ug glucose oxidase, 10ug lactoperoxidase, and 100uCi $Na^{125}I$ in LSB. The reaction proceded at 4°C for 12 mins. and was stopped by the addition of 8 volumes of ice-cold LSB containing 10uM Na sulphite. 50 ml iodination mixture was underlayed with 40 ml 20% sucrose LSB and centrifuged for 60 mins. at 35,000 rpm in the 6 x 100 ml rotor of an M.S.E Prepspin 50 centrifuge. Unbound iodide was mostly removed by this procedure as the labelled rough microsomes pelleted.

5.23. Triton X-100 Extraction of Iodinated Rough Microsomes.

Microsomes were extracted under the same conditions as nuclear envelopes. Iodinated rough microsomes were resuspended into 10% sucrose, 0.1mM $MgCl_2$, 10mM Tris-HCL (pH 7.5) to a concentration of 3 mgs protein/ml. Triton X-100 was added to a concentration of 2.5% with vortex mixing, and the suspension was incubated on ice for 10 mins. Triton residue was pelleted by centrifugation for 90 minutes at 40,000 rpm in the 10 x 10 Ti rotor of an M.S.E. Prepspin 50 centrifuge.

5.3. Results.

5.31 Effectiveness of rough microsome iodination.*

Lactoperoxidase-dependant cpm/mg protein	% lactoperoxidase independent counts (non-specific)	% lactoperoxidase-dependant counts Triton extractable	% lactoperoxidase-independant counts Triton extractable
10^6	1.8	16	0.43

* TCA precipitable counts.

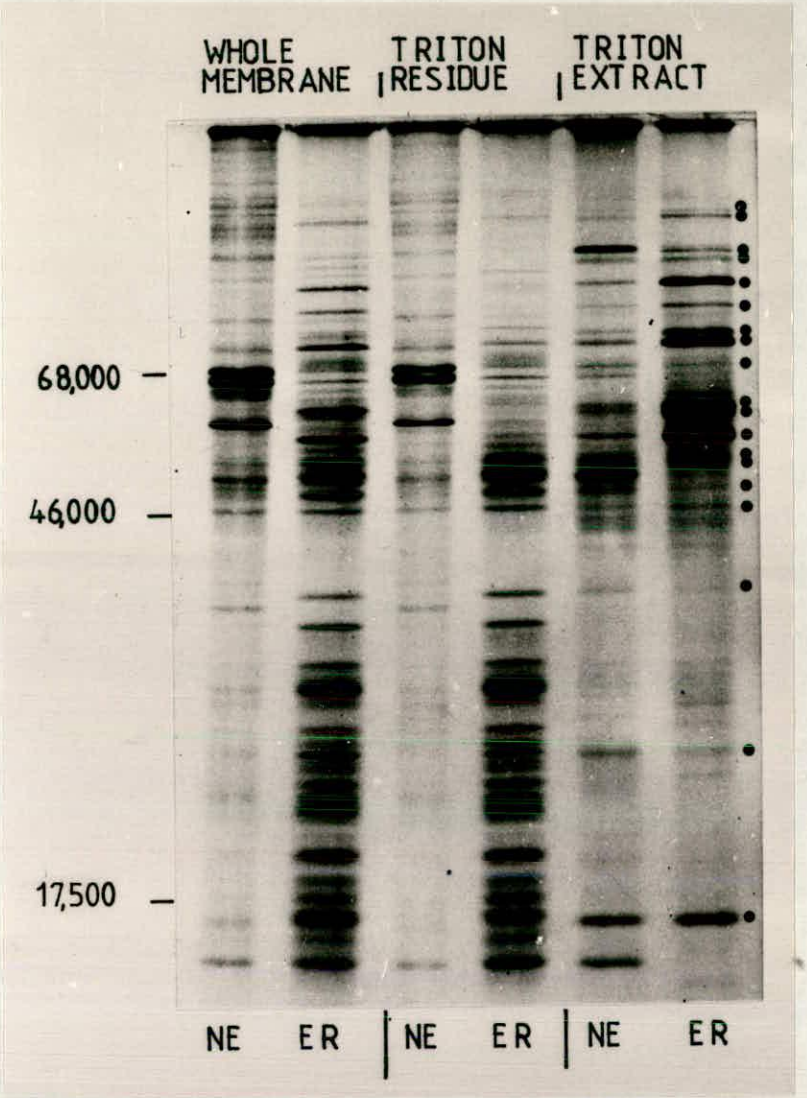


Fig. 1. Polyacrylamide gel electrophoresis of reduced polypeptides from nuclear envelopes and rough endoplasmic reticulum. Coomassie stained. Black dots indicate major homologies between the triton extracts.

Fig. 2. SDS polyacrylamide gel electrophoresis of polypeptides exposed to the cytoplasmic surface in the outer nuclear membrane and rough endoplasmic reticulum, identified by lactoperoxidase-mediated iodination. Left slot, iodinated nuclear envelope Triton extract (outer nuclear membrane + integral inner nuclear membrane proteins). Right slot, iodinated rough endoplasmic reticulum (whole membrane). Autofluorograph.

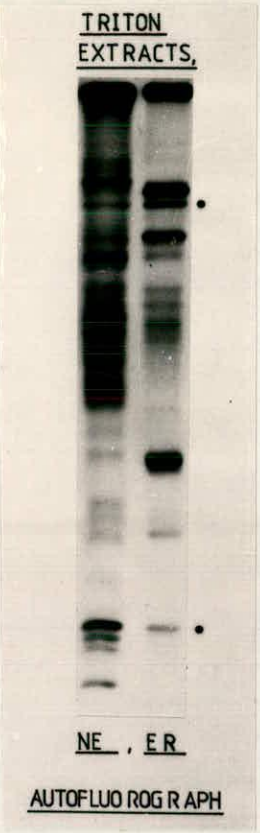


Fig. 3. SDS polyacrylamide gel electrophoresis of polypeptides exposed to the cytoplasmic surface of the outer nuclear membrane compared with the integral proteins of rough endoplasmic reticulum exposed to the cytoplasmic surface. Polypeptides identified by lactoperoxidase-mediated iodination. Autofluorograph. Left slot, nuclear envelope Triton extract (outer nuclear membrane + integral inner nuclear membrane polypeptides). Right slot, rough endoplasmic reticulum Triton extract (integral proteins).

5.4. Discussion.

In Fig. 1. the coomassie pattern of nuclear envelope and rough endoplasmic reticulum fractions are compared. It can be seen immediately that there are very few homologies between the electrophoretic profiles of untreated nuclear envelopes and rough endoplasmic reticulum; indeed they appear quite different. The nuclear envelope pattern is heavily dominated by three bands between 60,000 and 69,000 mol.wt (previously termed N1, N2 and N3) and is predominantly composed of polypeptides of greater than 46,000 mol.wt. The rough endoplasmic reticulum, by contrast, is predominantly composed of polypeptides of less than 55,000 mol.wt.

The nuclear envelope polypeptides may be subdivided into those that are insoluble in Triton X-100 and pellet with the pore lamina fraction, and those which are released from the envelope by Triton. Only a small fraction of the total polypeptides may be removed from the nuclear envelope by Triton and so the polypeptide composition of the pore lamina fraction (Triton residue) is almost identical to that of the whole envelope. There are therefore very few homologies between the Triton residue of the nuclear envelope and whole rough endoplasmic reticulum or between the Triton residues of either membrane system (Fig. 1).

Since the pore complexes are regarded as a specialisation of the endoplasmic reticulum (c.f. Watson 1955), and because the pore lamina fraction comprises

the bulk of nuclear envelope polypeptides, it is necessary to study the Triton extract of the nuclear envelope (largely outer nuclear membrane) in order to truly examine the putative endoplasmic reticulum nature of the nuclear membranes.

If the polypeptide composition of the Triton extract of nuclear envelope is compared with that of untreated rough endoplasmic reticulum (Fig. 1) it can be seen that, although there are many homologies, the relative proportions of which are quite different, nearly 50% of the bands in rough endoplasmic reticulum (particularly those below 46,000 mol.wt) are absent from the nuclear envelope. This is very strong evidence for a high degree of differentiation between the nuclear membranes and rough endoplasmic reticulum. If however, the Triton extracts of both the nuclear envelope (outer nuclear membrane + integral proteins of the inner nuclear membrane) and rough endoplasmic reticulum (integral proteins) are compared (see Fig. 1), we find that all the major integral proteins of rough endoplasmic reticulum exhibit homologies within the Triton extract from nuclear envelopes; but that <u>the relative proportions of these bands are quite different.</u> The homologies cannot be the result of contamination of the nuclear envelope preparation by the rough endoplasmic reticulum for then the relative proportions of the homologies would be the same. Thus it seems that although the nuclear envelope may contain in

its membranes some integral polypeptides common also to the rough endoplasmic reticulum, it is substantially differentiated from the rough endoplasmic reticulum. Of course, the mere establishment of similarities by SDS electrophoresis is poor evidence for polypeptides co-identity and so the differences between the two membrane species may be even greater than has so far been suggested.

This leaves unanswered the question whether proteins shared by both membrane systems are arranged in the same ways in the nuclear membranes and the rough endoplasmic reticulum. A protein located in the cytoplasmic face of the rough endoplasmic reticulum might, for example, be located only on the outer face of the inner nuclear membrane (i.e. assuming a continuity between the outer nuclear membrane and the rough endoplasmic reticulum, the proteins are located in opposite membrane planes) where, although its catalytic activity might be the same, its function and organisation might be quite different.

Fig. 2. compares the proteins of the nuclear envelope Triton extract and the proteins from untreated rough endoplasmic reticulum which are exposed to the cytoplasmic surface (i.e. it identifies the outer nuclear membrane and rough endoplasmic reticulum proteins which are exposed to the cytoplasmic surface). Although the resolution in the middle of the gel is rather poor, the

overall resolution is sufficiently good to establish unequivocally that the proteins on the two surfaces are very different. If those proteins that are both Triton extractable and also exposed to the cytoplasmic surface are compared (Fig. 3) then only two major homologies (indicated by black dots) may be established and the overall pattern is quite different. This means that although homologies in SDS gels exist between integral proteins of the rough endoplasmic reticulum and proteins of the two nuclear membranes (see Fig. 1), either their organisation or their identity is different. Ribosomal proteins may be expected to contribute to the iodination pattern of the two membrane systems and should perhaps be evident as similarities in the iodination patterns. However, ribosomal proteins may be masked by RNA and may even have a low capacity for iodination.

Thus the nuclear envelope is specialised not merely in regard to its pore complexes and their associated lamina, but also in respect of its membranes. Where homologies exist between proteins in the two membrane systems, the relative proportions of these components in the two membranes is quite different, and their organisation within the membrane plane exposed to the cytoplasmic surface is largely different also.

In the light of the above, and taking into consideration what is already known of the biochemistry

of the two membrane systems, it is difficult to see the nuclear membranes as being other than a highly specialised membrane system quite distinct from the rough endoplasmic reticulum. If continuities do exist between the outer nuclear membrane and the rough endoplasmic reticulum in rat liver tissue, they are rare phenomena. The enormous differences in the polypeptide composition and organisation of the two membrane systems argues against lateral diffusion of proteins between the two systems in the plane of the membrane being of significance to the bulk composition of nuclear membranes.

6. Concluding Remarks

The nuclear envelope is a fascinating example of membrane specialization, but its complexity and difficulty of isolation have meant that our understanding of its role in the cell has lagged behind that of other membrane systems. In developing a probe of its molecular organization I have sought to circumvent the problems associated with subfractionating the system into its membranes and pore complexes (see chapter 1), and yet to identify polypeptides of the pore complex, outer nuclear membrane and fibrous lamina.

The confidence with which the vectoral data presented may be accepted rests heavily on the evidence that the probe (insoluble lactoperoxidase) places label predominantly in the cytoplasmic surface of the nucleus. This has been summarised as follows (from chapter 3).

1) Insoluble lactoperoxidase is absolutely impermeant.
2) Labelling was dependant upon the presence of lactoperoxidase and of a peroxide generating system.
3) Lipid labelling was not detected.
4) Morphometric analysis of iodinated nuclei showed 88% integrity of the outer nuclear membrane.

5) The pattern of labelling was highly selective and dependant upon nuclei being intact. When nuclei were broken open, further proteins were iodinated and the overall pattern of iodination was altered.

6) Labelled proteins co-purified with nuclear envelopes and the pattern of labelling of nuclei and of envelopes derived from iodinated nuclei was almost identical.

The absence of lipid labelling established that iodination via I_2 was not a feature of solid-state lactoperoxidase iodination. Moreover, the work of Morrison and co-workers (Phillips and Morrison 1971; Bayse and Morrison 1971; Morrison and Bayse 1973) has indicated that lactoperoxidase iodination of proteins occurs not via some reactive diffusible moeity but by the binding of tyrosine residues to lactoperoxidase. Since lactoperoxidase - mediated iodination requires contact between the enzyme and the protein to be iodinated, and because immobilised lactoperoxidase is absolutely impermeant, it is reasonable to conclude that immobilised lactoperoxidase will only label proteins exposed to the surface of isolated nuclei. It is therefore with some confidence that the labelling studies, described in Chapters 4 and 5, are interpreted.

These studies have indicated that at least 14 polypeptides, only two of which (N1 and N2) are major envelope polypeptides, are exposed on the cytoplasmic surface of the nuclear pore complex. Although N1 and N2 have been located to the pore complex, this is not to suggest that this is their exclusive site in the envelope. The work of Gerace et al. (1978) has pointed to the presence of all three of the major envelope polypeptides (N1, N2 and N3) in the fibrous lamina, but as previously discussed (Chapter 4), the evidence is not absolutely hard. Lactoperoxidase labelling studies have also established that polypeptide N3 (a major envelope polypeptide) is located on the nucleoplasmic face of the inner nuclear membrane, almost certainly in the fibrous lamina. The great predominance of N1, N2 and N3 in relation to other nuclear envelope polypeptides suggests that they are structural elements whose gross and dynamic organisation is dependant on the very large number of minor (but high molecular weight) polypeptides also present in the envelope.

The precise function of the nuclear membranes is unknown. Certainly, the permeability properties of the nuclear envelope appear to be determined by those of the pore complexes rather than those of its membranes. The general view has emerged from other

laboratories, and despite evidence to the contrary, that the biochemical role of the nuclear membranes is so close to that of the rough endoplasmic reticulum that it might only be important in some cells, such as thymocytes, in which the nuclear membranes account for a significant proportion of the total cytomembrane surface (a view that has origins in Watson 1955). Clearly, this is not the case. It has been established that both the composition and organisation of proteins in the two membrane systems are different (Chapter 5) and doubt has been cast on the extent to which the two systems are in fact contiguous. In the light of this evidence and taking into consideration what is already known of the biochemistry of the two systems, the nuclear membranes cannot be regarded as a mere cisternum of the endoplasmic reticulum. Nonetheless, continuities between the two systems have been reported (see Chapter 5 for refs). One feature of the nuclear envelope that is frequently ignored is that the outer nuclear membrane can show local evaginations and form short circuit bridges between neighbouring sites on the nuclear surface (see Franke and Scheer 1974, p. 233 & 234). Thus, reported continuities between the outer nuclear membrane and rough endoplasmic reticulum may frequently be little more than local, contained, specialisations of the outer nuclear membrane.

Although the probe was developed with the specific aim of investigating the organisation of the nuclear envelope, its use could easily be extended to other membrane systems and would be of especial merit in investigations of membrane systems whose permeability properties are ill defined. Its use has so far provided only the very faintest outline of the molecular organisation of the nuclear envelope and an enormous amount yet remains to be done. But if the evidence presented may be accepted as establishing the identity of major proteins of the nuclear pore complex and as finally nailing the lie that the nuclear membranes represent a mere continuum of the rough endoplasmic reticulum, then the probe has fulfilled a most useful function.

REFERENCES.

Aaronson, R.P. and Blobel, G. (1975). J. Cell. Biol. 62, 747-754.

Aaronson, R.P. and Blobel, G. (1975). Proc. Nat. Acad. Sci. 72, 1007-1011.

Aaronson, R.P. (1978). In "Methods in Cell Biology" (D.M. Prescott. ed.). XVI, pp. 337-343.

Abelson, P.H., and Dwyer, W.L. (1949). Biol. Bull. 96, 205-217.

Abelson, P.H. and Smith, G.H. (1970). J. Ultrastruc.Res. 30, 558-588.

Agutter, P.S. (1972a). Ph.D. Thesis. University of Edinburgh.

Agutter, P.S. (1972b). Biochim. Biophys. Acta, 255, 397-401.

Agutter, P.S., McArdle, H.J. and McCaldin, B. (1976). Nature, 263, 165-167.

Agutter, P.S., Harris, J.R. Stevenson, J. (1977). Biochem. J. 162, 671-679.

Agutter, P.S., Birchell, K., Clark, E.J., Holson, J.A., and Porteous, E.R. (1978). Biochem. Soc. Trans. In the press.

Anderson, N.G. and Wilhur, K.M. (1951). J. Gen. Physiol. 34, 647-656.

Avrameas, S. (1970). Int. Rev. Cytol. 27, 349-385.

Avrameas, S., and Bouteille, M. (1968). Exp. Cell. Res. 53, 166-176.

Badr., G.G. (1974). Cytobiologii 10, 117.

Bayer, A. and Mole-Bayer, J. (1972). Int. Rev. Cytol. Suppl. 3, pp. 1-271 (G.A. Boumi and J.F. Danielli, eds.). Academic Press, New York.

Barrack, E.R. and Coffey, D.S. (1974). Fed. Proc. 33, 604 a. 2224.

Baudhuin, P., Evrard, P. and Berthet, J. (1966). J. Cell. Biol. 32, 181-191.

Bayse, G.S. and Morrison, M. (1971). Arch. Biochem. Biophys. 145, 143-148.

Beaulaton, J. (1968). Z. Zellforsch. 89, 453-461.

Behnke, O., and Moe, H. (1964). J. Cell. Biol. 22, 633-652.

Berg, H.C. (1969). Biochim. Biophys. Acta, 183, 65-78.

Barezney, R., Funk, L.A. and Crane, F.L. (1970). Biochim. Biophys. Acta 203, 531-546.

Berezney, R. (1972). J. Cell. Biol. 59, 22a.

Berezney, R. Macaulay, L.K. and Crane, F.L. (1972). J. Biol. Chem. 247, 5549-5561.

Berezney, R. and Crane, F.L. (1972). J. Biol. Chem. 247, 5562-5568.

Berezney, R. (1974). In "Methods in Cell Biology". (D.M. Prescott, ed.) Vol. VIII, pp. 205-228, Academic Press, New York.

Berezney, R. and Coffey, D.S. (1975). Science, 189, 291-293.

Berezney, R., and Coffey, D.S. (1976). Advances in Enzyme Regulation 14, 63-100.

Berezney, R. and Coffey, D.S. (1977). J. Cell. Biol. 73, 616-637.

Berezney, R., and Buchholtz, L.H. (1978), J. Cell. Biol. 79, 130a.

Bergstrand, A. and Dallner, G. (1969). Analyt. Biochem. 29, 351-356.

Bier, K. (1965). Chromosoma, 16, 58-69.

Blanchet, J.P. (1974). Exp. Cell. Res. 84, 159-166.

Bligh, E.G. and Dyer, W.J. (1959). Can. J. Biochem. Physiol. 37, 911-917.

Borisy, G.G. and Taylor, E.W. (1967). J. Cell. Biol. 34, 535-548.

Bornens, M. 1968. C.R. Acad. Sci. Press. Ser. D. 266, 596-599.

Bornens, M. (1973). Nature (Lond.) 244, 28-30.

Bornens, M. and Kasper, C.B. (1973). J. Biol. Chem. 248, 571-579.

Bornens, M. (1977a). In "Methods in Cell. Biology". (D.M. Prescott. ed.) vol. XV, pp. 163-174. Academic Press New York.

Bornens, M. (1977b). Nature (Lond.) 270, 80-83.

Bornens, M. and Courvalin, J.C. (1978). J. Cell. Biol. 76, No. 1. 191-207.

Bonner, W.M. (1975a). J. Cell. Biol. 64, 421-430.

Bonner, W.M. (1975b). J. Cell. Biol. 64, 431-437.

Bosmann, A.B. (1973). J. Cell. Biol. 59, 601-614.

Boulan, E.R., Kreibich, G. and Sabatini, D. (1978a). J. Cell. Biol. 78, 874-893.

Boulan, E.R., Sabatini, D.D., Pereyra, B.N. and Kreibich, G. (1978b). J. Cell. Biol. 78, 894-909.

Breton-Gorius, J. and Guichard, J. (1972). Am. J. Pathol. 66, 277- .

Bretton, R. and Bariety, J. (1974). J. Ultrastruc.Res. 48, 396-403.

Bretscher, M.S. (1971). J. Mol. Biol. 58, 775-781.

Brewer, G.J. and Singer, S.J. (1974). Biochemistry, 13, 3580-3588.

Brunner, M. and Raskas, H.J. (1972). Proc. Natl. Acad. Sci. U.S.A. 69, 3101-3104.

Burton, K. (1956). Biochem. J. 62, 315-323.

Callan, H.G., Randall, J.T. and Tomlin, S.G. (1949). Nature, 163, 280.

Callan, H.G. and Tomlin, S.G. (1950). Proc. R. Soc. Ser. (B), 137, 367-378.

Century, T.J., Fenichel, I.R. and Horowitz, S.B. (1970). J. Cell. Sci. 7, 5-13.

Chak, S.P. and Lee, S.H. (1971). J. Ultrastruc. Res. 35, 265-273.

Chen, P.S. Toribara, T.Y. and Warner, H. (1956). Anal. Chem. 28, 1756-1758.

Clerot, J.C. (1968). J. Microscopie, 7, 973-992.

Coffey, D.S. Barrack, E.R. and Heston, W.D.W. (1974). In "Advances in Enzyme Regulation". (G. Weber. ed), Vol. 12, pp. 219-266. Academic Press, New York.

Cole, M.B. (1969). J. Cell. Biol. 43, 24a-25a.

Comings, D.E. (1968). Amer. J. Human Genet. 20, 440.

Comings, D.E. and Harris, D.C. (1976). J. Cell. Biol. 70, 440-452.

Dallner, G. (1963). Acta. Pathol. Microbiol. Scand. Suppl. 166.

Dallner, G., Ernster, L. and Azzi, A. (1971). Chem. Biol. Interactions, 3, 254.

David, G.S. (1972). Biochem. Biophys. Res. Commun. 48, 464-471.

David, G.S. and Reisfeld, R.A. (1974). Biochemistry, 13, 1014-1021.

de Groodt, M., Derom, F. Lagasse, A. Sebneyns, M. and Thiery, M. (1958). Nature, 182, 1030.

De Petris, S. Karlsbad, G. and Pernis, B. (1963). J. Exp. Med. 117, 849.

Depierre, J. and Dallner, G. (1976). in "Biochemical Analysis of Membranes" (A.H. Maddy ed.). pp. 79-131. Chapman and Hall, London.

De Robertis, E.M., Longthorne, R.F. and Gurdan, J.B. (1978). Nature, 272, 254-256.

De Rousser, D, Mandelkow, E, Silliman, A. (1977). J. Molu. Biol. 113, 679-695.

Deumling, B. (1972). Ph.D. Thesis, 139 pp. Univ. of Freiberg, Freiberg.

Deumling, B. and Franke, W.L. (1972). Hoppe-Seylers. Z. Physiol. Chem. 353, 287-297.

Dwyer, N and Blobel, G. (1976). J. Cell. Biol. 70, 581-591.

Elder, J.N. and Morre, D.J. (1974). J. Cell. Biol. 63, 92a.

Erlandson, R.A. and De Harven, E. (1971). J. Cell. Sci. 8, 353-397.

Evans, W.H. (1978). In "Laboratory Techniques in Biochemistry and Molecular Biology Preparation and Characterisation of Mammalian Plasma Membranes" (J.S. Wark and E. Wark. gen. eds). North-Holland Publishing Co. New York, Oxford.

Fahimi, H.D. (1970). J. Cell. Biol. 47, 247.

Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971). Biochemistry, 10, 2606-

Faberge, A.C. (1974). Cell. Tiss. Res. 151, 403-415.

Fawcett, D.W. (1966). in "The Cell". W.B. Saunders Company, Philadelphia, p. 140.

Feldherr, C.M. (1962). J. Cell. Biol. 12, 159-167.

Feldherr, C.M. (1966). J. Cell. Biol. 31, 199-203.

Feldherr, C.M. (1968). J. Cell. Biol. 39, 49-54.

Feldherr, C.M. (1971). Tissue Cell. 3, 1-5.

Feldherr, C.M. (1972). In "Advances in Cell and Molecular Biology". (E.J. Du Praw, ed.), Vol. 2, pp. 273-307. Academic Press, New York.

Feldherr, C.M. (1975). Exptl. Cell. Res. 93, 411-419.

Feldherr, C.M. (1978). In "Methods in Cell Biology". (D.M. Prescott ed.) Vol. XVI, pp. 167-177. Academic Press, New York.

Flickinger, C.J. (1974). J. Cell. Sci. 14, 421-437.

Flickinger, C.J. (1978). Exp. Cell. Res. 111, 427-436.

Frank, M. and Horowitz, S.B. (1975). J. Cell. Sci. 19, 127-139.

Franke, W.W. (1966). J. Cell. Biol. 31, 619-623.

Franke, W.W. (1967). Z. Zellforsch, 80, 585-593.

Franke, W.W. (1970). Z. Zellforsch. Mikrosk. Anat. 105, 405-429.

Franke, W.W. Deumling, B. Ermen, B. Jarasch, E.D. and Kleinig, H. (1970). J. Cell. Biol. 46, 379-395.

Franke, W.W. and Scheer, U. (1970). J. Ultrastruc. Res. 30, 317-327.

Franke, W.W. Moore, D.J. Deumling, B., Cheetham, R.D. Kartenbeck, J. Jarasch, E.D. and Zentgraf, H. (1971). Z. Naturf. 26b, 1031.

Franke, W.W. Deumling, B. Zentgraf, H. Falk, H. and Rae, P.M.M. (1973). Exptl. Cell. Res. 81, 365-392.

Franke, W.W. (1974a). Phil. Trans. R. Soc. Lond. B. 268, 67-93.

Franke, W.W. (1974b). Int. Rev. Cytol. Suppl. 4, 71-236.

Franke, W.W. and Scheer, U. (1974). In "The Cell Nucleus" Vol. I, pp. 219-347. (H. Busch. Ed.). Academic Press, New York.

Franke, W.W. and Scheer, U. (1974b). Symp. Soc. Exp. Biol. 28, 249-282.

Franke, W.W. Berger, S. Falk, H. Spring, H. Scheer, U. Herth, W. Trendelenberg, M.F. and Schneiger, H.G. (1974). Protoplasma 82, 249-282.

Franke, W.W. Keenan, T.W. Stadier, J. Genz, R. Jarasch, E.D. Kartenbeck, J. (1976). Cytobiologie, 13, 28-56.

Franke, W.W. (1977). Biochem. Soc. Symp. 42, 125-135.

Fry, D.J. (1976a). In "Subnuclear Components" (G.D. Birnic ed.) pp. 59-105 Butterworths. London-Boston.

Fry, D.J. (1976b). In "Mammalian Cell Membranes" Vol. 2. pp. 197-268. (G.A. Jamieson & D.M. Robinson eds). Butterworths, London.

Fukushima, M. Okayama, H. Takakashi, Y. Hayaishi, O. (1976). J. Biochem. 80, 167-176.

Gerace, L. Blum, A. and Blobel, G. (1978). J. Cell. Biol. 79, 546-566.

Gibbs, S.P. (1962). J. Cell. Biol. 14, 433-444.

Giles, K.W. and Myers, A. (1965). Nature, Lond. 206, 93.

Gorkin, V.Z. (1971). Experentic, 27, 30.

Gorovsky, M.A. (1969). J. Cell. Biol. 43, 46a.

Gullasch, J. and Kaufmann, R. (1974). In "Microprobe Analysis as applied to Cells and Tissues" (T. Hall, P. Echlin. and R. Kaufmann, eds). pp. 175-190. Academic Press, New York.

Gunderson, H.M. (1975). J. Biol. Chem. 250, 3552-3559.

Gunderson, H.M. and Nordlie, R.C. (1975). J. Biol. Chem. 238, 3552-3559.

Gurdon, J.B. (1976). Nature, 268, 116.

Gurdon, J.B. (1970). Proc. Roy. Soc. London, Ser. B, 176, 303-311.

Gurr, M.I., Finean, J.B. and Hawthorne, J.N. (1963). Biochim. Biophys. Acta. 70, 406-416.

Hadek, R. and Swift, H. (1962). J. Cell. Biol. 13, 445-451.

Harris, J.R. and Agutter, P.S. (1970). J. Ultrastruc. Res. 33, 219-232.

Harris, J.R. and Milne, J. (1974). Biochem. Soc. Trans. 2, 1251-1253.

Harris, J.R. and Agutter, P. (1976). In "Biochemical Analysis of Membranes" (A.H. Maddy, ed). pp. 132-173. Chapman and Hall Ltd. London.

Harris, J.R. (1977). Methodological Surveys in Biochemistry Vol. 6. Membranous elements and Movements of Molecules. (E. Reid ed.) 245-250.

Harris, J.R. (1978). Biochem. Biophys. Acta, 515, 55-104.

Heinrich, P.C. Gross, V. Northemann, W. and Scheurlen, M. (1978). Rev. Physiol. Biochem. Pharmacol. 81, pp. 102-134.

Herman, R. Weymouth, L. and Penman, S. (1978). J. Cell. Biol. 78, 663-674.

Herzfeld, A. Federman, M. and Greengard, O. (1973). J. Cell. Biol. 57, 475-483.

Herzog, V. and Miller, F. (1970). Z. Zellforsch. Mikrosk. Anat. 107, 403.

Herzog, V. and Miller, F. (1972). J. Cell. Biol. 53, 662-682.

Hewish, D.R. and Burgoyne, L.A. (1973). Biochem. Biophys. Res. Commun. 52, 504-510.

Hildebrand, C.E. and Okinaka, R.T. (1976). Anal. Biochem. 75, 290-300.

Hirano, H. Parkhouse, B. Nicolson, G.L. Lennox, E.S. and Singer, S.J. (1972). Proc. Nat. Acad. Sci. U.S.A. 69, 2945-2949.

Ho, M.K. and Guidotti, G. (1975). J. Biol. Chem. 250, 675-683.

Hogebroom, G.A. Schneider, W.C. and Palade, G.E. (1948). J. Biol. Chem. 172, 619-635.

Hokin, L.E. Mokotoff, M. and Kupchan, S.M. (1966). Proc. Nat. Acad. Sci. U.S.A. 55, 797-804.

Horowitz, S.B. and Fenickel, J.R. (1970). J. Cell. Biol. 47, 120-131.

Horowitz, S.B. (1972). J. Cell. Biol. 54, 609-625.

Horowitz, S.B. and Moore, L.C. (1974). J. Cell. Biol. 60, 405-415.

Howell, K.E. Ito, A. and Palade, G.E. (1978). J. Cell. Biol. 79, 581-589.

Hubbard, A.L. and Cohn, Z.A. (1972). J. Cell. Biol. 55, 390-405.

Hubbard, A.L. and Cohn, Z.A. (1976). In 'Biochemical Analysis of Membranes'. (A.H. Maddy ed.). 427-501. Chapman and Hall, London.

Hynes, R.O. (1976). In "New Techniques in Biophysics and Cell Biology". (R.H. Pain and B.J. Smith eds.) Vol. 3, pp. 147-212. John Wiley & Sons. London.

Ichikawa, Y. and Mason, H.S. (1973). in "Oxidases and Related Redox Systems". (King, T.E. Mason, H.S. & Morrison, M. eds.) Vol. 2, pp. 605-625. University Park Press, Baltimore.

Ishikawa, K., Kuroda, C. and Ogata, K. (1969). Biochim. Biophys. Acta 179, 316-331.

Ishikawa, K., Kuroda, C., Ueki, M. and Ogata, K. (1970a). Biochim. Biophys. Acta 213, 495-504.

Ishikawa, K., Kuroda, C., and Ogata, K. (1970b). Biochim. Biophys. Acta, 213, 505-512.

Ito, A., and Palade, G.E. (1978). J. Cell. Biol. 79, 590-597.

Jackson, R.C. (1976). Biochemistry, 15, No. 25, 5641-5651.

Jacob, J. and Danielli, G.A. (1972). Cell. Differ. 1, 119-125.

Jarasch. E.D. (1973). Doctoral dissertation, University of Freiberg.

Jarasch, E.D. Reilly, E.D. Comes, P. Kartenbeck, J. and Franke, W.W. (1973). Hoppe-Seylers Z. Physiol. Chem. 354, 974-986.

Jarasch, E.D. and Franke, W.W. (1974). J. Biol. Chem. 249, 7245-7254.

Jarasch, E.D. and Franke, W.W. (1977). Exp. Cell. Res. 109, 450-454.

Jenkins, R.E. and Tanner, M.J.A. (1977). Biochem. J. 161, 139-147.

Kanamisra, S. (1975). J. Anat. 119, 499-504.

Kaneko, I., Satoh, H. and Ukita, T. (1972). Biochem. Biophys. Res. Commun. 48, 1504-1510.

Kartenbeck, J., Jarasch, E.D. and Franke, W.W. (1973) Exptl. Cell. Res. 81, 175-194.

Kashnig, D.M. and Kasper, C.B. (1969). J. Biol. Chem. 244, 3786-3792.

Kasper, C.B. (1971). J. Biol. Chem. 246, 577-581.

Kasper, C.B. and Kubinski, H. (1971). Nature (London) 231nb, 124-125.

Kasper, C.B. (1974). In: "The Cell Nucleus" (H. Busch. ed.) Vol. I, pp. 349-383 Academic Press, New York.

Kawasaki, T. and Yamashina, I. (1972). J. Biochem. 72, 1517-1525.

Kay, R.R. Fraser, D. and Johnston, J.R. (1972). Eur. J. Biochem. 30, 145-154.

Kay, R.R. and Johnston, I.R. (1973). Sub-Cell. Biochem. 2, 127-166.

Keenan, T.W. Berezney, R. Funk, L.K. and Crane, F.L. (1970). Biochim. Biophys. Acta. 203, 547-554.

Keenan, T.W. Berezney, R. and Crane, F.L. (1972). Lipids, 7, 212.

Keenan, T.W. Franke, W.W. and Kartenbeck, J. (1974). FEBS Lett. 44, 274-278.

Keshgegian, A.A. and Glick, M.C. (1973). Biochemistry, 12, 1221-1226.

Kessel, R.G. (1973). Progress - Surface and Membrane Science. 6, 243-329.

Khandwala, A.S. and Kasper, C.B. (1973). Biochem. Biophys. Res. Commun. 54, 1241-1246.

King, J.A. and Louis, C.F. (1976). Biochem. Soc. Trans. 4, 245-248.

Kinzel, V. and Muellar, G.C. (1973). Biochim. Biophys. Acta, 322, 337-351.

Kleinig, H. (1970). J. Cell. Biol. 46, 396-402.

Kleinig, H. Zentgraf, H, Comes, P. and Stadler, J. (1971). J. Biol. Chem. 246, 2996-3000.

Kodama, R.M. and Tedischi, H. (1968). J. Cell. Biol. 37, 747-760.

Kohen, E. Sebert, G. and Kohen, C. (1971). Hoppe Seylers Z. Physiol. Chem. 352, 927-937.

Koshiba, K. Smitena, K. and Busch, H. (1970). Exptl. Cell. Res. 60, 199-209.

Kraemer, R.J. and Coffey, D.S. (1970). Biochim. Biophys. Acta, 224, 568-578.

Kreilich, G. Hubbard, A.L. and Sabatina, D.D. (1974). J. Cell. Biol. 60, 616-627.

Krohne, G. Franke, W.W. and Scheer, U. (1978). Exptl. Cell. Res. 116, 85-102.

Kubai, D.F. (1975). Int. Rev. Cytol. 43, 167-227.

Laemmli, U.K. (1970). Nature (Lond.) 227, 680-685.

Langendorf, H. Siebert, G. Keiselberg, K. and Hannova, R. (1966). Nature, 209, 1130-1131.

Ledui, E.H. Avrameas, S. and Bouteille, M. (1968), J. Exp. Med. 127, 109.

Ledui, E.H. Scott, G.B. and Avrameas, S. (1969). J. Histochem. Cytochem. 17, 211-224.

Lemarchal, P. and Bornens, M. (1969). Bull. Soc. Chim. Biol. 51, 1021.

Ling, G.N. (1970). A Physical Theory of the Living State, pp. 257-284, New York, Blaisdell.

Loewenstein, W.R. (1964). In "Protoplasmalogia" (M. Alfert, H. Bauer and C.V. Harding, eds.). Vol. V, pp. 26-34. Springer Verlag, Vienna.

Loewenstein, W.R. Kanno, Y. and Ito, S. (1966). In "Annals of the New York Academy of Sciences" (E.M. Weyr. ed.). Vol. 137, pp. 708-716. Acad. of Sciences Press, New York.

Lowry, O.H., Rosebrough, N.J. Farr, A.K. and Randall, R.J. (1951). J. Biol. Chem. 193, 256-275.

Maddy, A.H. (1964). Biochim. Biophys. Acta 88, 390-399.

Marius, P.J. Salb, J.M. and Schwartz, V.G. (1965). Nature, 208, 1122-1124.

Marquet, E. and Sobel, H.J. (1969). J. Cell. Biol. 41, 774.

Matsuura, S, Fujii-Kuriyama, Y and Tashiro, Y. (1978). J. Cell. Biol. 78, 503-519.

Maul, G.G. Price, J.W. and Lieberman, N.V. (1971). J. Cell. Biol. 51, 405-418.

Matsuura, T. and Ueda, K. (1972). Arch. Biochem. Biophys. 150, 44-450.

Matsuura, S. Fujii-Kuriyama, Y. Tashiro, Y. (1978). J. Cell. Biol. 78, 503-519.

McNamara, D.S. Rauvskis, J. Schumm, D.E. and Webb, T.E. (1975). Biochem. J. 147, 193-197.

Mentre, P. (1966). In "Sixth International Congress for Electron Microscopy". (R. Uyeda, ed.), Vol. 2, pp.347-348. Manezen Co. Ltd. Tokyo.

Mentre, P. (1969). J. Microscopie 8, 51-68.

Merriam, R.W. (1961). J. Biophys. Biochem. Cytol. 11, 559-570.

Merriam, R.W. (1962). J. Cell. Biol. 12, 79-90.

Michaels, G.A. Whittock, S.D. Horowitz, P. and Levin, P. (1977). Biochem. Biophys. Res. Commun. 75, 482-487.

Milne, J.F. Agutter, P.S. Harris, J.R. and Stibbs, G. (1978). Biochem. Soc. Trans. 6, 271-273.

Mirsky, A.E. and Osawa, S. (1961). The Cell, (Brachet, J. and Mirsky, A.E. eds.) Vol. 2, pp.677, Academic Press, New York and London.

Monneron, A. Blobel, G. and Palade, G.E. (1972). J. Cell. Biol. 55, 104-125.

Monneron, A. (1974). Phil. Trans. Roy. Soc. Lond. B. 268, 101-108.

Monneron, A. and Segretain, D. (1974). FEBS Lett. 42, 209-213.

Moore, R.J. and Wilson, J.D. (1972). J. Biol. Chem. 247, 958-967.

Morrison, M. and Bayse, G.S. (1970). Biochemistry, 9, 2995-3000.

Morrison, M. and Bayse, G.S. (1973). In "Oxidases and Related Redox Systems". (T.E. King, H.S. Mason and M. Morrison eds.) pp. 375-388. University Park Press, Baltimore.

Nakai, Y. and Fujita, H. (1970). Z. Zellforsch. Mibrosk. Anat. 107, 104.

Nicolson, G.L. and Singer, S.J. (1971). Proc. Natn. Acad. Sci. U.S.A. 68, 942-945.

Nicolson, G. Lacorbiere, M. and Delmonte, P. (1972). Exp. Cell. Res. 71, 468-473.

Nicolson, G.L. (1974). Int. Rev. Cytol. 39, 89-190.

Nozawa, Y., Fukushima, H. and Jida, H. (1973). Biochim. Biophys. Acta, 318, 335-344.

Osborn, N. and Weber, K. (1976). Proc. Nat. Acad. Sci. 73, 867-871.

Paine, P.L. and Feldherr, C.M. (1972). Exptl. Cell. Res. 74, 81-98.

Paine, P.L. (1975). J. Cell. Biol. 66, 652-657.

Paine, P.L. Moore, L.C. and Horowitz, S.B. (1975). Nature, 254, 109-114.

Palade, G.G. (1955). J. Biophys. Biochem. Cytol. 1, 59-68.

Parks, H.F. (1962). J. Cell. Biol. 14, 221-234.

Perrin, A. (1969). C.R. Acad. Sci. Paris, 269D, 570-576.

Perrin, A. (1970). Protoplasma, 70, 131.

Philipp, E.I. Franke, W.W. Keenan, T.W. Stadler, J. and Jarasch, E.D. (1976). J. Cell. Biol. 68, 11-29.

Phillips, D.R. and Morrison, M. (1971). Biochemistry, 11, 4582-4588.

Phillips, J.L. (1973). Archs. Biochem. Biophys. 156, 377-379.

Potter, V.L. (1955). Methods in Enzymology, Vol. 1. p. 10. Academic Press, London and New York.

Poux, N. (1969). J. Microsc. 8, 855.

Price, M.R. Harris, J.R. and Baldwin, R.W. (1972). J. Ultrastruc. Res. 40, 178-196.

Price, M.R. and Stoddart, R.W. (1977). Brit. J. Cancer. 35, 250.

Raskas, H.J. (1971). Nature new Biol. 233, 134-136.

Raskas, H.J. and Okuba, C.K. (1971). J. Cell. Biol. 49, 438-449.

Raskas, H.J. and Bhaduri, S. (1973). Biochemistry, 12, 920-925.

Reynolds, E.S. (1963). J. Cell. Biol. 17, 208-212.

Riley, D.E. and Keller, J.M. (1976). Biochem. Biophys. Acta, 444, 899-911.

Riley, D.E. and Keller, J.M. (1978). J. Cell. Sci. 29, 129-146.

Robbins, E. and Gonatas, N.K. (1964). J. Cell. Biol. 21, 429-463.

Sagara, Y. Harano, T. and Omura, T. (1978). J. Biochem. 83, 807-812.

Sato, T. Fujii, T. Matsuura, T. and Ueda, K. (1972). J. Biochem. 72, 1049-1051.

Scheer, U. (1972). Z. Zellforsch. 127, 127-148.

Scheer, U. (1973). Devl. Biol. 30, 13-28.

Scheer, U. Kartenbeck, J. Trendelenberg, M.F. Stadler, J. and Franke, W.W. (1976). J. Cell. Biol. 69, 1-18.

Schneider, J.H. (1959). J. Biol. Chem. 234, 2728-2732.

Schneider, W.C. (1957). In "Methods" in Enzymology" (S.P. Colowick and N.O. Kaplan, eds.) Vol. 3, p. 680. Academic Press, London and New York.

Schumm, D.E. and Webb, T.E. (1972). Biochem. Biophys. Res. Commun. 48, 1259-1265.

Schumm, D.E. McNamara, D.S. and Webb, T.E. (1973a). Nature New Biol. 245, 201-203.

Schumm, D.E. Morris, H. and Webb, T.E. (1973b). Cancer Res. 33, 1821-1828.

Schumm, D.E. and Webb, T.E. (1974). Biochem. Biophys. Res. Commun. 58, 354-360.

Shelton, K.R. (1976). Biochim. Biophys. Acta. 455, 973-982.

Shelton, K.R. Cobbs, C.S. Povlishock, J.T. and Burkat, R.S. (1976). Arch. Biochem. Biophys. 174, 177-186.

Shore, G.C. and Tata, J.R. (1977). J. Cell. Biol. 72, 726-743.

Siebert, G. (1972). Sub. Cell. Biochem. 1, 277-292.

Sikstrom, R. Lanoix, J. and Bergeron, J.J.M. (1976). Biochim. Biophys. Acta. 448, 88-102.

Singer, T.P. Kearney, G.B. and Kenney, W.C. (1973). Advances in Enzymology, 37, 189-273.

Smith, S.J. Adams, H.R. Smetena, K. and Busch, H. (1969). Exp. Cell. Res. 55, 185-197.

Smuckler, E.A. Koplitz, M. and Smuckler, J. (1976). In "Subnuclear Components" (G.D. Birnie ed.) pp. 1-57. Butterworths. London-Boston.

Spangler, M. Coetzee, M.L. Katyol, S.L. Morris, H.P. and Ove, P. (1975). Cancer Res. 35, 3131-3145.

Stadler, J. and Kleinig, H. (1971). Biochim. Biophys. Acta, 233, 315-319.

Stadler, J. and Franke, W.W. (1972). Nature New Biol. 237, 237-238.

Stadler, J. and Franke, W.W. (1974). J. Cell. Biol. 60, 297-303.

Stacy, R. Ben-Shaul, Y. and Galun, E. (1973). Biochim. Biophys. Acta, 323, 167-177.

Steck, T.L. and Yu, J. (1973). J. Supramol. Struct. 1, 220-232.

Steck, T.L. and Dawson, G. (1974). J. Biol. Chem. 249, 2135-2142.

Stevens, R.H. and Amos, H. (1972). J. Cell. Biol. 54, 1-7.

Stoddart, R.W. and Price, M.R. (1977). Biochem. Soc. Trans. 5, 121-122.

Strum, J.M. and Karnovsky, M.J. (1970). J. Cell. Biol. 44, 655-666.

Strum, J.M. Wicken, J. Stanbury, J.R. and Karnovsky, M.J. (1971). J. Cell. Biol. 51, 162-175.

Stuart, S.E. Rottmann, F.M. and Patterson, R.J. (1975). Biochem. Biophys. Res. Commun. 62, 439-447.

Stuart, S.E. Clawson, G.A. Rottman, F.M. and Patterson, R.J. (1977). J. Cell. Biol. 72, 57-66.

Stubbs, G. and Harris, J.R. (1978). In 'Methodological Developments in Biochemistry', (E. Reid ed.) in the press.

Studier, F.W. (1973). J. Mol. Biol. 79, 237-248.

Tata, J.R. Hamilton, M.J. and Cole, R.D. (1972). J. Molec. Biol. 67, 231-246.

Taylor, C.W. Yeoman, L.C. and Busch, H. (1975). In "Methods in Cell Biology" Vol. IX. D.M. Prescott (ed.) pp. 349-376. Academic Press, New York.

Tilney, L.G. (1975). J. Cell. Biol. 64, 289-310.

Virtanen, I. and Wartiovaara, J. (1976). J. Cell. Sci. 22, 335-344.

Virtanen, I. Brotherus, J. Renkonen, O. and Wartiovaara, J. (1977). Biochem. Biophys. Res. Commun. 76, 142-149.

Virtanen, I. (1977). Biochem. Biophys. Res. Commun. 78, 1411-1417.

Watson, M.L. (1955). J. Biophys. Biochem. Cytol. 1, 257-270.

Watson, M.L. (1958). J. Biophys. Biochem. Cytol. 41, 475-478.

Weber, K. Pollack, R. and Bibring, T. (1975). Proc. Nat. Acad. Sci. 72, 459-463.

Weisenberg, R.C. Borisy, G.G. and Taylor, E.W. (1968). Biochemistry, 7, 4466-4479.

Weisenberg, R.C. (1972). J. Cell. Biol. 54, 266-278.

Whaley, W.G. Mollenkauer, H.H. & Leech, J.H. (1960). J. Biophys. Biochem. Cytol. 8, 233-245.

Widnell, C.C. and Tata, J.R. (1964). Biochem. J. 92, 313-317.

Widnell, C.C. Hamilton, T.H. and Tata, J.R. (1967). J. Cell. Biol. 32, 766-770.

Wilson, E.M. and Chytil, F. (1976). Biochim. Biophys. Acta, 426, 88-100.

Wilson, L. (1970). Biochemistry, 9, 4999-5007.

Wood, J.G. McLaughlin, P.H. and Robbins, P.W. (1974). J. Cell. Biol. 63, 541-549.

Wunderlich, F. Wallach, D.F.H. Speth, V. and Fischer, E. (1974). Biochim. Biophys. Acta, 373, 34-43.

Wunderlich, F, Miller, R. and Speth, V. (1973). Science, 182, 1136-1138.

Wunderlich, F. Berezney, R. and Kleinig, H. (1976). In "Biological Membranes" (D. Chapman and D.F.H. Wallach eds.) Vol. 3, pp. 241-333. Academic Press, London, New York, San Francisco.

Wunderlich, F. Giese, G. and Bucherer, C. (1978). J. Cell. Biol. 79, 479-490.

Yasuzumi, G. and Tsubo, I. (1966). Exptl. Cell. Res. 43, 281-292.

Yoo, B.Y. and Bayley, S.T. (1967). J. Ultrastruc. Res. 18, 651-660.

Yu Ling, C, Racevskis, J. and Webb, T.E. (1972). Cancer. Res. 32, 2314-2321.

Zbarsky, J.B. Perevoshchikova, K.A. Delecktorskaya, L.N. and Delecktorsky, V.V. (1969). Nature, 221, 257-259.

Zbarsky, J.B. (1972). In "Methods in Cell Physiology" (D.M. Prescott ed.) Vol. V, pp. 167-198. Academic Press, New York.

Zentgraf, H. Deumling, B, Jarasch, E.D. and Franke, W.W. (1971). J. Biol. Chem. 246, 2986-2995.