"THE ISOLATION AND COMPOSITION OF RAT LIVER NUCLEAR ENVELOPES"

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ABSTRACT OF THESIS

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Title of Thesis The Isolation and Composition of Rat Liver Nuclear Envelopes.

The significance of the problem of the nuclear envelope in current cell biology, and the susceptibility of the problem to elucidation by present-day biochemical techniques, are examined in the Introduction. Part I of the thesis consists of a review of the various properties of the envelope as determined by electron microscopy and other techniques, and of attempts which have been made to isolate nuclei, nuclear envelopes, and other subnuclear fractions. From the introduction and the review of properties, it is concluded that four questions are both of particular importance and amenable to biochemical investigation:

1. How can the envelope be isolated, and what structural linkages with other systems are apparent?
2. What are the components of the envelope, and what particular functional properties do they have?
3. How are the components spacially organised in the envelope?
4. Can the overall envelope structure be related to function?

From the review, it is also established that the only acceptable criterion for an isolated envelope preparation is morphological; that is, an isolated preparation is nuclear envelope if and only if it has certain structural features identifiable by electron microscopy. Of the published procedures for the isolation of nuclei, only certain dense sucrose methods are shown to be useful for this project. It is established that, though published methods for the isolation of nuclear envelopes begin with nuclear preparations which are in general acceptable, none of them conforms to the criterion of morphological integrity; hence, none of them (by definition) can be regarded as an acceptable envelope preparation. This finding shifts the emphasis of the first of the above questions to the structural linkages with other systems that confer stability on the envelope; this in turn is closely linked with the possibility of explaining certain other properties of the system - e.g. blebbing activity, disappearance during mitosis, contiguous chromatin organisation - which seem to be related to overall stability.

Part II presents the results of experiments directed towards the

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establishment of an acceptable isolation procedure for the envelope, the determination of factors increasing and decreasing stability, and hence the explanation of the inadequacy of published methods for isolating the envelope. In this work, hypotheses relating to the involvement of the system in mitosis, blebbing and chromatin organisation are generated. In chapter 4, the "purity" of the initial nuclear preparation is established; a range of techniques is investigated for the breaking of the nuclei and the separation of chromatin from the envelope; and only two of these techniques - low temperature incubation in EDTA and sonication in the presence of magnesium - promise to give an acceptable envelope preparation. It is shown that the latter procedure is preferable. DNase, potassium ions and high ionic strength are all shown to render isolation of an acceptable envelope impossible. Loss of morphological integrity - i.e. destruction of pore complexes, is shown to be concommitant with a reduction of envelope DNA content and with collapse of the remnants of the system into minute single-membrane vesicles. Chapter 5 is devoted to a discussion of the "standard procedure" for the isolation of the envelope - sonication in the presence of magnesium followed by differential centrifugation and centrifugation to equilibrium on a sorbitol gradient - and the effects of varying all possible conditions in this procedure on yield, composition and morphological integrity of the preparation are examined. The inadequacy of published isolation procedures is discussed in the light of these findings. Chapter 6 presents a thorough investigation of the stability properties of the system in isolation; the possibility of a molecular interpretation of the findings is discussed but rejected. The physiological significance of the findings of this chapter and of chapters 4 and 5 is discussed.

Part III is concerned with the second of the general questions - that is, with the identification of the components of the isolated envelope. Since there is already a considerable literature on the subject of nuclear and nuclear envelope lipids, lipid analysis is not considered here. Chapter 7 concerns the DNA component of the system. It is found that DNA (using preparations from mouse liver and L-cells in culture) is entirely of the main band type in the case of liver, but contains both main band and satellite in the case of L-cells. It is also demonstrated that nuclei adsorb as much added bulk DNA after sonication as before, reducing the probability that the envelope DNA is an artifact of preparation. Chapter 8 considers the sodium, potassium, calcium and magnesium contents of both disrupted and intact envelopes, prepared from nuclei suspended in buffers containing either calcium or magnesium. The significance of this study lies in the importance of electrostatic interactions in the nuclear envelope and in the variety of ionic effects on the morphology and composition of the system. In chapter 9, the enzymology of the system is investigated. No glucose-6-phosphatase, protease or succinoxidase activity was apparent. High levels of ATPase, NADH-cytochrome c oxido-reductase and magnesium independent alkaline phosphatase were, however, found.

In Part IV, the significance of the results of this study of the nuclear envelope in cell biology is considered, along with the general applicability and potential solubility of the problems raised and the prospects for future research.

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In accordance with the regulations laid down by the University, the author hereby declares that some sections of the work described in this thesis have already been published:-

1. J. R. Harris and P. S. Agutter, J. Ultrastr. Res., 33 (1970) 219.
2. P. S. Agutter, Biochim. Biophys. Acta, 255 (1972) 397.

Reprints of these papers are duly sewn into the binding.

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

CONTENTS

SUMMARY

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centrifugation to equilibrium on a sorbitol gradient - and the effects of varying all possible conditions in this procedure on yield, composition and morphological integrity of the preparation are examined. The inadequacy of published isolation procedures is discussed in the light of these findings. Chapter 6 presents a thorough investigation of the stability properties of the system in isolation; the possibility of a molecular interpretation of the findings is discussed but rejected. The physiological significance of the findings of this chapter and of chapters 4 and 5 is discussed.

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INTRODUCTION

At the present time, cell biology shows two major lines of development. The first is a concern with the structure and function of nucleic acids; the second with the interpretation of cellular ultrastructure. This latter concerns a large range of problems, but the study of membrane systems figures very significantly in this range.

The problem of the nuclear envelope presents, in several senses, a point of intersection between these two lines of development. It is, on electron microscopic evidence, clearly a membrane system; as such it invites such techniques of examination, isolation and analysis as have hitherto been applied to other membrane systems. At the same time, it presents in more or less all living eukaryotic cells a barrier to the passage of nucleic acids and ribosomes, and possibly other macromolecular assemblies (of one or more units), between nucleus and cytoplasm; moreover, it appears to constitute a site of attachment of chromatin, and in all probability a point of initiation of DNA synthesis. The system has in addition a number of properties, the biological significance of which is at present largely mysterious. It shows, in many species, "blebbing" activity; it is continuous with other membrane systems; usually, it disappears during cell division only to be reassembled around the daughter nuclei during telophase; it has unusual permeability properties; its outer surface often, if not usually, bears ribosomes; and so on. Most of all, it is (so far as can be determined) universally a double-membrane system, and studded with highly organised structural units known as "pore complexes".

How the problem of the nuclear envelope is to be handled, what methods are to be brought to bear upon it, depend partly on the

precise way in which the problem is formulated and partly on the techniques available. The formalisation of this (or any other) problem in biology requires two major steps: first, the analysis of the question, apparently teleological, of "Why does X occur?" - where X is either an observed (essentially time-independent) structure or an observed pattern or time-course of behaviour (or "apparent function"); and second, an examination of the observed properties or nature of X, the isolation of biologically meaningful questions from this examination, and some attempt at cross-correlation between these questions and the products of analysis of the first step. (In the expression of the generalised initial question given here, the use of the qualifier "why?" is justified by saying that the answer then given must be in the class of explanatory science, which itself requires and hence includes an adequate descriptive science. Therefore, more immediately obvious, descriptive-science, questions such as "what is X?", "how does X come to appear as it does?" etc. are not only less general than, but are also <u>included in</u>, the question "why does X occur?". This point will be clarified when the analysis of the question is attempted.) After the cross-correlation and establishment of all biologically meaningful questions about X, attempts can be made to answer each of them provided the techniques required for investigation are available. In spite of this formalisation of procedure, however, cell biology remains, as biology has always (with the exception of evolutionary theory) been, a descriptive rather than an explanatory science. Despite the abundance of isolated explanatory statements, a generally applicable explanatory theory of cell biology leading to

an understanding of what accounts for the phenomenon of "living", is still far distant.

When the question "why does X occur?" is asked about any structure, such as the nuclear envelope, the questions actually implied are many and complex. A reasonable analysis might be as follows:-

1. What functions can X perform; that is, in what ways and with what other materials and systems can X, or components thereof, engage in biologically significant behaviour? This question concerns the "purposive" aspect of the observed structure - its "teleology" in a rather narrower sense than the above, more general, question. It will be referred to hereinafter as the purposive question.

2. The questions of origin: generally, by what means was X formed? This can be further analysed:-

   a) The question of genetic origin: What is the genetic background of X - that is, of what portion of the genotype is it the phenotypic expression?

   b) The question of biochemical origin: What synthetic processes are involved in producing each component of X, and how, if at all, are these processes controlled?

   c) The question of environmental origin: What environmental factors (in this case, the environment will in general be the rest of the cell interior) can modify X as a whole or any of its components, both during and after synthesis?

   d) The question of ontogeny: What is the ontogeny of X - that is, what are the details of its developmental history?

3. The question of phylogeny: What is the phylogenetic origin of X?

4. The question of organisation: How are the components organised with respect to one another in space so as to constitute X? In addition, we may ask to what extent the presence and relative position of all actual and potential components is necessary, and what combination of components is sufficient, to constitute X. This corollary to the question of organisation necessarily alters the approach to a fifth question, implied by more or less all the foregoing:-

5. The question of composition: What are the components of X? It cannot, in view of the above corollary, be assumed *a priori* that any set of components found empirically is both necessary and sufficient; the question is, however, not meaningless, some answer being required before questions of origin and organisation can be dealt with. An absolute answer would first require a fully adequate theoretical model of X, which in turn would require answers to the purposive question and the question of organisation.

It is interesting to note that the question of composition generates a whole new set of questions of the type "Why does X' occur?" Here, X' refers to any one of the components of X. Logically, this gives rise to an infinite regression; as long as there are structures, other structures can (in principle) be isolated from them and subjected to the same inquiries. This sweepingly dismisses any search for "fundamental particles"; the problem, however, quickly leaves the field of biology. The understanding of a component does not lead to the understanding of a system, and at each new level of analysis a new set of concepts, new methodologies, and new and more specific types of question are encountered. In short, the progression from level to level is the progression from one science

to another. The same phenomenon can be seen in reverse when the first of the above questions - the purposive question - is considered. The functions in which a group of structures (including the one under observation) are involved necessarily take place at a higher level of organisation than the single structure in question; this higher level will have its own observable structures and should, therefore, by the same argument as above, give rise to a distinguishable science with an essentially irreducible language of its own. The nuclear envelope participates in activities in a higher-level structure, namely the cell. The observation that the sciences pertaining to cells, to organelles, to membranes and to the molecular components of membranes have not as yet clearly separated reflects either upon the immaturity of "cell biology" or upon the general inadequacy of abstract theorising.

Be this as it may, of the above list only the purposive question and the questions of biochemical origin and composition are amenable to current techniques. That of organisation may become so in the not too distant future. Those of genetic and environmental origin, ontogeny and phylogeny are largely grounds for speculation at the moment; the first three are the subject of developmental biology, and their answer must await the fuller development of this field; the question of phylogeny is probably in principle unanswerable. Of the purposive question and the question of composition, only the latter is a suitable problem for such techniques as might be applied to the isolated nuclear envelope; the former is really a question about the observed behaviour of the envelope <u>in vivo</u>. In the hope, however, of forging meaningful links between the studies of adjacent levels of organisation, it is tempting to suggest that answers to the

question of organisation (and, perhaps, that of phylogeny) may have some bearing on the answer to the purposive question. Certainly, it is not reasonable to dismiss the purposive question as irrelevant to the study of the envelope. In the same spirit, it may be very useful to know what other systems are structurally linked, and by what means, to the system under observation. This demands a careful and thorough examination of the isolation techniques used.

How, then, can the question "Why does X occur?" be analysed when X refers to an observed pattern of behaviour, such as nucleo-cytoplasmic exchange or the dissolution of the nuclear envelope during mitosis? A possible analysis is as follows:-

1. What is the purpose of X in the content of the next higher level of organisation from the structures seen to be involved? Again, this is the purposive, "teleological" question, and it ultimately invokes such strictly biological notions as that of survival value.

2. Is X spontaneous, or is it triggered by some environmental change? That is, is there some stimulus to which X is the response, and, if so, what is its nature? In biological systems, some stimulus is generally assumed to be present, X often being regarded as one element of a highly complex set of closely integrated processes.

3. a) With what changes in which systems and materials is X associated? This is the inverse form of the purposive question discussed in the analysis of general investigation of structure X.

   b) By what mechanism does the stimulus (if any) initiate X, and how is the stimulus situation itself modified by X?

4. What is the phylogenetic origin of X?

Of these, all but question 3(a) are intractable by current

techniques. The question of whether or not there is a stimulus, and if so what does the stimulus consist of, is occasionally answerable; more usually, both this and the purposive question (question 1) generate speculative answers on rather insufficient evidence. Such answers may, however, be interesting and may in turn give rise to hypotheses about the general nature of X and its relationship with other processes. Nevertheless, the questions raised by the general analysis of problems of "function" or patterns of behaviour provide a stimulus to develop new techniques and to improve existing ones.

The techniques referred to in the foregoing discussions come from several sources. Initial observations depend upon microscopy, particularly electron microscopy, cell physiology and cytochemistry. Refinements within these fields can lead directly to a number of answers; biochemical and biophysical studies also yield important information. In considering a structure, however, the techniques for isolation and analysis of components are specifically biochemical. Techniques in this field, too, may provide (at least in part) a "critique" of the structural and functional properties of the components, such as is required to obtain an answer to the apparently dominant question - namely, what is the mode of spacial organisation of the components in the structure?

In summary, the questions which may be asked about the nuclear envelope and to which answers can currently be expected may be listed as follows:-

1. a) How can the system be isolated?

   b) From the isolation procedure, can structural links with other systems be deduced?

2. What are the components of the nuclear envelope, and what

structural and functional properties of these components are relevant to the system as a whole?

3. Can any conclusions be drawn about the way in which the components are assembled to constitute the envelope?

4. Is it possible, using available techniques, to relate the structure of the envelope to the known functions in which it participates?

5. What is the purpose of the characteristic patterns of behaviour of the system? The negative converse of this question will perhaps prove more illuminating: Can the consequences for the cell of the system's failing to exhibit its characteristic patterns of behaviour be deduced from experimental findings?

A review of the properties of the envelope will follow. This will generate questions more or less specific to the problem in hand, in contrast to the rather general questions derived above. To the review will be appended, as a separate chapter, an examination of isolation procedures for nuclei and subnuclear fractions; these will pertain specifically to the first of the general questions established above, but will provide a necessary starting-point for subsequent experimental work.

PART I

GENERAL REVIEW

CHAPTER 1

THE PROPERTIES OF THE NUCLEAR ENVELOPE

The existence of the nuclear envelope was first suggested by Brown[1] in 1833. However, only since around 1950 have investigations of the system been made, primarily with the aid of the electron microscope, in a large range of tissues selected from a number of species. Several properties, both structural and functional, have thus been described in or postulated about the envelope. (The term "nuclear envelope" is used throughout this thesis in preference to "nuclear membrane"(cf.Anderson[2]); "membrane" will hereinafter refer specifically either to the outer or to the inner "leaflet" (Abelson and Smith[3]) of the envelope.) Clearly, some of these properties pertain to the system alone, and may be expected to be found in isolated preparations. The rest, however, pertain to the system *in situ*, that is, in interaction with other components of the cell. None of this latter group of properties can wholly be accounted for by an investigation of the isolated nuclear envelope, though information gained in this manner is not necessarily irrelevant.

The properties of the nuclear envelope may, therefore, be sub-classified in two ways:-

a) They may be *structural* or *functional* (in the sense used in the Introduction).

b) They may pertain either to the *envelope alone* or to the envelope in *interaction with other components of the cell*.

The subject will now be reviewed in the framework of this classification scheme.

A. PROPERTIES PERTAINING TO THE ENVELOPE ALONE

I STRUCTURAL

1. The Double Membrane

One of the earliest reports of the double-membrane nature of the nuclear envelope was that of Hartmann[4], in rat nerve tissue. Palay and Palade[5], also investigating sectioned rat nerve nuclei, found the following features: an inner membrane approximately 13 nm across; an apparently granule-free outer membrane some 7.5 nm across; and, separating the two, a "perinuclear space" or cisterna some 20 nm across. From the outer membrane, tubular projections entered the cytoplasm. (This point will be discussed more thoroughly under the heading of "Blebs": v. infra.) In places, the two membranes of the envelope appeared to fuse, giving rise to discontinuities stretching for 28-30 nm tangentially.

These discontinuities were, in all probability, the envelope "pores" (v. infra). However, at the time of the paper by Palay and Palade, it was by no means certain that the "pores" represented true discontinuities in the envelope. Afzelius[6], for example, in a paper which demonstrated the double-membrane nature of the nuclear envelopes of sea urchin and starfish oocytes, claimed that a single thin membrane stretched across the pores. Rebhun[7], investigating snail and clam oocytes, also reported a double-membrane envelope but mentioned no pore diaphragm. Watson[8], too, though he accepted the ubiquity of the double-membrane envelope, denied the existence of a diaphragm across the pore.

Since this time, discussion of the existence of a pore diaphragm has slowly faded from the literature. However, Palay and Palade's original belief that the pores were simple discontinuities is still

not generally accepted. In very thorough reviews of the ultrastructure of the nuclear envelope, Abelson and Smith[3] reported that they could find no evidence for the pore diaphragm, while Franke and Scheer[9] did not discuss the possibility. Both these groups, however, found double-membrane envelopes in their material (i.e. nuclei from amphibian oocytes, African green monkey kidney, and mouse 3T3 culture cells), and both clearly regarded the ubiquity of this structural feature as fully established. In fact, the only case of a single-membrane envelope which appears to have been reported is in nitrogen-starved yeast cells (Koehler[10]). Log phase growth cells, however, have the usual double membrane around the nucleus. Little has been added to the literature about the dimensions of the constituent membranes since the report of Palay and Palade[5]; however, the dimensions of the perinuclear cisterna are not established. Gall[11] suggests a width of 10-30 nm; Feldherr and Harding[12], in the same volume, suggest 15 nm. Given such variations, and given the occurrence of "blebs" in the envelope, it may be suggested that the perinuclear cisterna is not of constant size in any tissue, and that its variability is symptomatic of the dynamic nature (i.e. structural variability with time) of the envelope.

2. <u>The Pore Complex</u>

a) <u>Honeycomb structures</u>

The first "porous nuclear envelope" to be described was that of salamander oocytes (Callan, Randall and Tomlin[13]; Callan and Tomlin[14]). These envelopes were removed from the nuclei by microdissection; on examination they revealed, in the outer membrane at least, discontinuities which on tangential viewing of the system gave a "honeycomb" appearance. Bovey[15] found similar envelopes in locust and cockroach

oocytes, Beirati and Lehman[16] and Harris and James[17] in Amoeba proteus, and Pollister, Gottner and Ward[18] in immature frog oocytes. However, though Callan and Tomlin[14] reported an inner membrane thickness of 15 nm, the corresponding dimension for the outer membrane was 30 nm. Subsequent work on other tissues proved this latter dimension to be anomalously high (v. supra). It was shown that, in addition to the envelope, nuclei with unusually massive outer membranes were surrounded by a cortical layer; it was in this layer that the honeycomb structures could clearly be seen.

Nevertheless, despite some opposition to the belief in "pores" (Sjöstrand and Hanzon[19]; Hartmann[4]; Sjöstrand[20]) it was repeatedly demonstrated that pores did, in fact, exist in a wide range of tissues. It remained for some correlation between these "true pores" and the "honeycomb" structures to be effected. Pappas[21] and Kreidner, Kostir and Frajola[22] established this correlation; the interstices of the honeycombs in Amoeba proteus were found to be continuous with the pores in the envelope. Since this time, the honeycomb structures have been found in other species; Beams et al[23], for example, found a cortical layer within the double membrane of nuclei from Gregarina, a parasite of grasshopper gut.

Honeycomb structures are not, however, widespread, and their significance is at present unknown. Since they may be regarded either as structural components of the nuclear envelope or as structures contiguous to the envelope, an isolated preparation would probably account for their composition and organisation in some detail, if not for their function. However, they are absent from mammalian liver; and therefore the present investigation will not yield any information about them.

b) Pores and Annuli

Discussions of pores, or apparent discontinuities, and their surrounding annuli are inextricably bound together in the literature. Pores have now been reported in all nuclei subjected to ultrastructural examination; size and frequency vary from species to species, possibly from tissue to tissue, and perhaps, at least in some cases, from immature to mature cells in the same tissue. Some examples of pore frequencies are as follows:-

Table 1:1 Interspecific variations of pore frequency

Species	Pore frequency (per μm^2)	Reference
Onion root tip	7-12	Branton and Moor[24]
HeLa cells	13	Fisher and Cooper[25,26]
Frog oocytes:-		
Immature	40-45	Merriam[27]
Mature	25-35	
Echinoderm oocyte	40-80	Afzelius[6]
Simulian salivary gland	80-100	Montré[28]
Tetrahymena	95-135	Franke[29]

Pore frequency may be an indicator of metabolic activity in the cell. Amphibian erythrocytes, for example, have very few pores indeed (Miraky and Chava[30]); such cells show very little protein synthesis and no division. Outer diameters of the annuli are generally around 100 nm (e.g. Wischnitzer[31] gives approximately this value for Triturus oocytes; Afzelius[6] gives the same value for starfish and sea urchin oocytes). Inner diameter measurements are ambiguous; it is not clear in many cases whether the diameter is that of the pore itself or of the inside of the annulus. However, several values for annulus width are available. Afzelius[6] gives 20-25 nm for echinoderm oocytes; Robbins[7] gives the same for molluscan oocytes; Franke[29] gives 7-16 nm for mouse liver and

17.5-20 nm for tetrahymena; and Franke and Scheer[9] give 10-18 nm for six species of amphibian oocytes. In rat liver, the pores appear to occupy some 20-30% of the surface of the nucleus; this is equivalent to a frequency of 26-40/μ^2. The outer annulus diameter is 100 nm and the inner diameter around 50 nm; the pore diameter is uncertain, but is probably in the region 55-60 nm.

Annuli were first found by Bahr and Boorman[32] in Chironomus salivary gland and midgut; here, the nuclei are large and have large pores. The annuli were found in identical positions on both sides of the envelope, and were visible in both tangential and transverse section. Gall[33], who at first believed the annuli to be drying artifacts, became convinced of their reality after studying the nuclei of Triturus, fish, echinoderm and annelid oocytes. Watson's earliest studies[34] on the nuclear envelope revealed, in various orientations of the specimin, pores or discontinuities with which the annuli were always associated. Similar reports confirmed the ubiquity of pores and their associated annuli over the next two years. Afzelius[6] gave a very detailed description of the pores observed in echinoderm oocytes; Kautz and de Marsh[35] investigated chick embryo, and provided the first demonstration of electron-dense material in pores; Dawson, Hossack and Wyburn[36] gave an account of the nuclear envelope of rabbit spinal ganglia; here the 85 nm diameter pores were irregularly distributed, the spacing between them varying from 25 to 180 nm, with a raised annulus surrounding the depression of each pore. The extreme irregularity here may have resulted from distortion during the preparation of the specimins, for example by uneven drying. The structures appeared to be relatively robust, being the only parts of the envelope stable to the fixative used. Gall[37] showed that the

annulus overlaps the pore margin, the pore diameter being greater than the inner but less than the outer diameter of the annulus; Gall[37], Wunderlich and Franke[38], and others have demonstrated a ring within the annulus which presumably corresponded to the pore margin. Franke and Scheer[9] suggest, in opposition to Gall[37], that the shape of the margin is circular rather than octagonally symmetric; some of the photographs published by Abelson and Smith[3] support this view. However, the question of pore margin shape must for the moment be left open; drying artifacts are difficult to avoid for certain, and the envelope is not robust.

Recent work has shown that the annulus is separable from the rest of the envelope in certain tissues. Sonication disrupts pea seedling nuclei leaving the annuli intact (Yoo and Bayley[39]). Monroe, Schidlovsky and Chandra[40] found that negative staining of tissue culture cells using phosphotungstate liberated some annuli with tags of membrane attached. Comings and Okada[41] have more recently demonstrated that the annuli of mammalian and avian interphase nuclei may be stabilised by a stellate array of chromatin fibres firmly attached to them; the annulus-fibre complex is separable from the rest of the envelope. Such findings may have prompted Abelson and Smith[3] to suggest that the "pore complex" be regarded as a distinct, ubiquitous organelle; and, by implication, that it be distinguished from the nuclear envelope. This suggestion will be discussed presently.

c) <u>The Fine Structure of the Annulus</u>

Gall[11] first described the appearance of the annulus as an array of subunits. Rebhun[7] made similar observations but did not comment on the nature of the components. Gall[42] saw them as

ribosome-like entities extending across the annulus. Dawson et al[36] had hinted at a comparable arrangement in the rabbit spinal ganglion, but not until the work of Watson[8] on the subject was it suspected that annulus subdivision might be widespread in life. In this paper, Watson first employed the term "pore complex" to refer to annulus (eight subunits), pore margin and pore lumen contents; following Wischnitzer[31] he regarded the pore complex as a cylinder. Wischnitzer had, in investigating Triturus oocytes, reached the conclusion that the annulus was a tubular structure, 100 nm in diameter and some 150 nm in vertical height. It was not, however, a simple tube; the wall consisted of eight microcylinders each about 150 nm in vertical height and of some 10 nm diameter.

Curiously, the view of the pore complex developed by Wischnitzer and Watson received little or no further attention for about ten years. The view of the annulus as a more or less circular array of eight (or sometimes nine) subunits had become established during this period, but little more was understood about fine structure. Franke and Scheer[9] found in amphibian oocytes "amorphous annular material" - diffuse material extending between neighbouring subannuli, and readily lost unless precautions were taken to stabilise it. Loss of the amorphous material rendered the subannuli more clearly visible. These authors suggested that the immediately surrounding envelope be regarded as a structural part of the pore complex, and hence, presumably, of the annulus. Abelson and Smith[3] found in the mammalian annulus at least two types of structure: the subannulus, which like Witschnitzer[31] they described as a tubule, and which was set within the pore margin; and filamentous extensions of the annulus into both nucleus and cytoplasm, which accounted for the apparent overlapping

of the pore margin by the annulus. This material had a diffuse appearance in tangential section. It is unlikely that these extensions are equivalent to the diffuse material described by Franke and Scheer; the latter authors found many classes of fibrous material (v. infra), and they emphasised the strictly non-fibrous and non-granular appearance of this component of the annulus. The sub-annular tubules ("minitubules", of dimensions and configuration very different from classical microtubules) consisted, according to Abelson and Smith, of 5-6 longitudinally-arranged filaments surrounding a dense central element. They were destroyed by pronase, but, unlike the filamentous extensions, were stable in water.

d) Structures in the Pore Lumen

The most commonly-observed structure in the lumen of the pore is the so-called "central spot" or central tubule. Pollister, Gettner and Ward[18] first observed it in frog oocytes, Watson[34] in a variety of tissues, Gay[43] in Drosophila, and Wischnitzer[31] in Triturus oocytes, but it remained uncertain whether the object was a structure in the pore complex, material passing through the pore, or an artifact. Wischnitzer[31] gave its diameter as 15 nm, but it subsequently became clear that this parameter was variable. In spite of some failures to demonstrate central spots, the structures have become recognised as widespread in life. Monro et al[40] demonstrated them in mammalian tissue culture cells, Wunderlich and Franke[38] in Tetrahymena, Chapman and Wallach[44] in S.Cerevisiae. Merriam[27] noted that the structure was commoner in immature than in mature frog oocytes, and Franke and Scheer[9] confirmed this by showing that 61% of the pore complexes of larval, but only 36% of those of mature, eggs contained central spots. Abelson and Smith[3] perceived the central granule as a

tube with a wall consisting of 6-9 longitudinally-arranged filaments, extending a short distance into nucleus and cytoplasm, and containing a dense granule which may be of ribonucleoprotein material and can often be resolved into smaller granules.

Linking the central granule (or tubule) to the annulus (Abelson and Smith[3]), or to the pore margin (Franke and Scheer[9]), is an array of "struts" or "internal fibres of the lumen". The number of these is large but unknown, and possibly variable; each strut is 3 nm across, and may bear globular particles of 5-9 nm diameter. Similar fibrils often appear to join neighbouring annuli in amphibian oocytes (Franke and Scheer[9]); this observation may, however, be an artifact. It is possible that such an array of struts may have been responsible for earlier reports of a pore diaphragm (v. supra); to disintuigh individual struts demands rather high resolution electron microscopy. Franke and Scheer[9] added to their description of the amphibian pore complex the conical "tip-like projections" passing from the annulus into the lumen. The general applicability of this observation is unknown.

Electron-dense material in the pore has been seen by several workers, and has generally been interpreted as material passing through the system, usually from nucleus to cytoplasm. Kessel[45] observed "streaming" material on both sides of the envelope, apparently passing through the pore, in amphibian oocytes; Stephens and Swift[46], making similar observations on Chironomus salivary gland, concluded that the pore complex might be the site of RNA transport. Rebhun[7] had made similar observations on molluscan oocytes, and Anderson and Beams on Rhodnius[47], but here the streaming appearance might have arisen from a collapse of the extensions of the annulus, either tubular or filamentous (v. supra). The current view on the matter

is that the pore complex is indeed the site of macromolecular transport; the annulus itself may be involved (Franke and Scheer[9]), or the central tubule may structurally represent the transport mechanism. The variation in central tubule frequency with metabolic activity lends circumstantial support to the latter hypothesis. In addition to the material apparently in passage, intra-lumen material has occasionally been observed in the form of granules (Gall[11]) and amorphous material (mentioned briefly by Franke and Scheer[9]).

Not all writers on the subject have, however, described these lumen contents. In part, this may be because the structures are biologically labile; in part, too, because certain fixing and staining procedures destroy them. Abelson and Smith[3] showed that $KMnO_4$, for example, destroys the central tubule and the entire annulus as well. Marinos[48], observing three species of plant cells, and Barnes and Davis[49], observing acidophil and basophil cells in liver, are among those who have asserted that pores are "simple holes".

The dynamic nature of the pore complex (here again, "dynamic" simply means "variable with time in structural organisation") has been emphasised by a number of means. Changes of pore frequency, the lability of ultrastructural components, and the generally high level of protein turnover (Widnell & Siekevitz[50]) except in immature cells (Morrison[27]) have helped establish this point. More recently, Bloom[51] showed that a "halo" appeared round the pore complex of rat heart nuclei when the sarcomere length was minimal (in systole), but disappeared in diastole when the sarcomere was elongated. Bloom suggested that this appearance was not due to a buckling of the envelope; but it presumably reflects a more or less cyclic change in the mutual three-dimensional orientations of the ultrastructural components - a "dynamic" attribute in the sense of this discussion.

3. Ribosomes

Watson[8] first observed ribosomes adhering to the outer nuclear membrane as to a piece of rough endoplasmic reticulum. The observation has since been reported several times, usually incidentally to the main purpose of the research (cf. Parks[52]; Duprow[53]; Fisher and Cooper[26]; Gall[11]). No similar observations have as yet been made on the inner membrane, but it would not be easy to distinguish ribosomes on the nucleoplasmic surface on account of the contiguous chromatin (v. infra). Certainly, ribosomes have not been reported in the perinuclear cisterna. A number of claims, not as yet adequately substantiated, have been made to the effect that the pore complex, or some part thereof, consists of ribosomes or ribosome precursors. Gall[11] believed the subannuli to be ribosomes, because they were in general of similar size and because polysomes can form whorls, roughly similar in appearance to the annulus. Morrison[27] suggested that RNA may be present as a structural component of the pore complexes in annulate lamellae; by implication, this refers also to the nuclear envelope. Many other speculations about the chemical nature of the pore complex have been made, but the view involving RNA, or more specifically ribosomes, has appeared repeatedly and has received elaborate treatment by Franke and Scheer[9]. The fibrillar connections between the inner subannuli and the nucleolus, the apparent migration of dense rodlike configurations of nucleolar material through the central tubule, led once again to the suggestion that the whole complex was a specialised polysome, or perhaps an RNase site for the final cleavage of polycistronic messengers or the ultimate degeneration of r-RNA precursors to the 28S and 18S forms. The fibrillar connections were interpreted as ribonucleoprotein strands, possibly m-RNA bearing 18S ribosomal

subunits or "informofer" protein. However, such interpretations are at present tentative, and an apparent functional connection, as between pore complex and nucleolus, does not necessarily entail compositional similarity. Koshiba, Smetana and Busch[54] showed that the fine filaments in pore lumens of Novikoff hepatoma ascites nucleoi were destroyed rapidly by pepsin, but more slowly by pronase or subtilisin; DNase and, significantly, RNase, had no comparable effect. Trypsin had a destructive effect on the lumen fibres of honeybee embryos (Duprow[53]). The fibres appear, therefore, to be structurally maintained by protein rather than by RNA. The differential protease activity may reflect extremes of "pH" in the micro-environment of the pore complex, variable with species, or may reflect a measure of local steric hinderance to the larger enzyme molecules.

In conclusion, though ribosomes are undoubtedly attached to the outer nuclear membrane, and though ribosomes, ribosomal precursors and presumably other ribonucleoprotein species appear to pass through or become contiguous with the pore complex, it cannot at this stage be conclusively stated that the envelope has any ribonucleoprotein components other than the above-mentioned outer membrane ribosomes. The composition of the pore complex is by no means finally established.

4. Electrokinetic Studies

Electrokinetic properties of the nucleus depend on the distribution of charged groups over the envelope surface, and thus reflect some aspect of structural organisation on the molecular level. Vassar et al[55] found that rat liver nuclei have an apparent isoelectric point of around pH 4, but this can be lowered by DNA which is adsorbed on the surface at low ionic strength. Such adsorbed DNA can be

removed by DNase, and the isoelectric point is thus raised again. At low ionic strength, nuclear contents may leak out and be adsorbed. This phenomenon has been observed in rat liver at pH 6.0 ± 0.2 in this laboratory; at pH 7.5, however, the nuclei were more stable and no leaking occurred; moreover, dications tend to prevent it. Kashimoto and Lieberman[56], making use of the removal of the outer membrane by citric acid (Gurr, Finean and Hawthorne[57]), investigated the effects on the distribution of charged groups on the inner membrane of partial (67%) hepatectomy and a variety of pharmacological agents. 2-6 hours after hepatectomy, a 1.4-fold increase in electrophoretic mobility occurred in the citrate nuclei; the mobility was thereafter constant. (10% hepatectomy produced no detectable change after 6 hours.) These observations were not affected by adrenalectomy (depletion of adrenaline and corticosteroids), but the mobility increase was suppressed by actinomycin D in six times the quantity required to suppress RNA synthesis, and by p-fluorophenylalanine. Noradrenaline, ionising radiation and EDTA had no effect on the increase. It was concluded that the mobility change was independent, at least initially, of RNA synthesis, and independent of the divalent cations involved in DNA synthesis.

Clearly, no far-reaching conclusions about nuclear envelope structure follow from these observations, but the results of such studies may usefully illuminate the problem once relevant information is obtained from other lines of investigation.

II FUNCTIONAL

The subject characteristic of this subclass of properties is that of enzymology. The enzymic components of the nuclear envelope are, however, not well understood, since until recently no isolated

preparations were available, and comparatively few enzymes can be studied in the system *in vivo*. Nevertheless, a small range of activities can be discussed; and enzymes commonly present in membrane systems which have previously been demonstrated in the nucleus will be mentioned in passing, with a view to assaying them in isolated envelopes at some later stage.

1. Enzymes associated with the Nuclear Envelope

a) Electron Transport Enzymes

Evidence has accumulated over the past decade for the presence of some components of an electron transport chain in the nuclear envelope, though it is now clear that no complete chain is to be found. Rees and Rowland[58] showed that incorporation of phosphate, adenine, orotic acid and glycine into nucleic acids and proteins of rat liver nuclei was inhibited by dinitrophenol, anoxia, azide and chlorpromazine. Though no ATP synthesis was detected, NADH-cytochrome c oxidoreductase and cytochrome c oxidase activities were found. Ronniall *et al*[59] showed that the former of these activities was not a contaminant. Succinoxidase was not found. Both cyanide and DNase completely inhibited cytochrome oxidase activity, but the DNase inhibition could be reversed by adding RNA or polyethylene sulphonate. Histones were also found to inhibit. Botel and Klemm[60], using rat thymus, found that 50% of the cellular ATP was to be found in the nuclei; the nuclei manifested aerobic ATP synthesis, inhibited by rotenone, amytal and carbon monoxide; the latter block was photoreversible. Oligomycin block of oxygen uptake was released by dinitrophenol. Arsenite, with or without BAL, gave no inhibition. The P:O ratio had a minimal value in the region 0.6-1.0.

Kuzmina *et al*[61], using a membrane preparation from rat liver nuclei (see chapter 2), recovered respiratory activity, the specific

activity being 4-6 times that in the nuclei. NADH- and NADPH-cytochrome c oxidoreductase, glutamate dehydrogenase, and cytochrome c oxidase were found. Berezney, Funk and Crane[62,63], using an annulus-free membrane preparation from bovine liver nuclei (see chapter 2), found NADH-cytochrome c oxidoreductase, NADH oxidase, andcytochrome c oxidase. The first of these activities was dependent on exogenous cytochrome c, was 40% inhibited by DNase (the mitochondrial enzyme is only 5% DNase inhibited), and was 26% inhibited by the addition of 0.56 mg histone/mg protein, sufficient completely to inhibit the mitochondrial enzyme. The effect of DNase in this case must be interpreted with caution, since DNase was employed in the isolation procedure. The inhibition may be, in reality, considerably greater than 40%.

b) ATPases

Rees and Rowland[58], though they could detect no ATP synthesis, found in nuclei a Mg-dependent ATPase activated by dinitrophenol and inhibited by chlorpromazine. The activation may, in fact, have been due to dinitrophenol inhibition of ATP synthesis. Betel and Klemm[60] found that at least some of the ATPase activity of rat liver nuclei was anaerobic. Using histochemical staining techniques, namely deposition of lead phosphate on and around the site of activity, Yasumumi and Tsubo[64] and Yasumumi et al[65] found an association between envelope ATPase activity and the annuli of the pore complexes, in mouse choroid plexus and mammalian testis respectively.

Using membranous fragments from mammalian liver nuclei, Zbarsky et al[66] and Franko et al[67] found ATPase activity, essentially unaffected by the presence of sodium or potassium ions. ATPase activity detected in membranous material from mouse liver and hepatoma nuclei by Delektorskaya and Porovoshchikova[68] was 3-6 times that in

nuclei, but again $Na^{\diamond}$-$K^{\diamond}$ activation and ouabain inhibition were not observed. This does not, as Zbarsky et al suggest, necessarily imply that there is no active transport of alkali metal ions in the nuclear envelope. Doloktorskaya and Porovoshchikova found the ATPase, like that in mitochondria, to be 50-60% inhibited by oligomycin (5 µg/ml) and gramicidin c (50 µg/ml); this observation contradicts that of Langendorf et al[69] who asserted that these inhibitors have no effect on the nuclear ATPase. However, the histological staining results described above suggest that if workers on membrane preparations from nuclei had recovered a significant number of pore complexes in their material, they might also have recovered higher specific ATPase activity; the sodium-potassium activation properties of the annulus enzyme are at present unknown.

e) DNA Polymerase

Comings and Kakefuda[70] showed that in synchronous cultures of human amnion cells, tritiated thymidine label is initially incorporated into DNA around the surface of the nucleus and the nucleolus. However, synchrony was attained by excess thymidine treatment; it is possible that the "incorporated" label merely reflects exchange diffusion between labelled and unlabelled nucleotide. High intranuclear concentrations of free unlabelled thymidine prevents rapid entry and incorporation of label beyond the periphery. Pavlovsky and Berlowitz[71], investigating male mealy bugs, noted that more chromatin attachment to the envelope (v. infra) constitutes a necessary but not sufficient condition for DNA replication; however, Alfert and Das[72] suggested that the envelope determines the rate of DNA synthesis; and in addition much has been made of the analogy between DNA synthesis in eucaryotes and the conditions favouring the applicability of the "replicon" theory in bacterial systems.

Jacob, Brenner and Cuzin[73] and Jacob, Ryter and Cuzin[74] first suggested that bacterial DNA synthesis is initiated at a point of attachment between DNA and the surface membrane; subsequent investigation as shown (i) that there indeed is such an attachment (v. infra), (ii) that newly-synthesised DNA is associated with the point of attachment[75,76]. Similar theoretical arguments pertaining to eucaryotes[77], combined with findings such as those of Comings and Kakefuda[70] and the empirical observation of envelope-DNA attachment (v. infra), strongly support the view that DNA synthesis is initiated at the nuclear envelope. The demonstration that labelled DNA precursors are incorporated by isolated envelope preparations would, however, not confirm this view; under these conditions it is not easy to distinguish between the initiation of DNA synthesis and the activity, for example, of a DNA repair enzyme.

2. Other Nuclear Enzymes

Several enzymes, which in other systems are or may be associated with membranes, have been found in the nucleus. It may be interesting to consider the possibility that these enzymes are, in fact, associated with the nuclear envelope. So far, no such investigation has been made. These enzymes include:-

a) Proteases

A complex range of nuclear proteases was reported in rat liver by Dounce and Uzuña[78]. Some or all of these may reside in the envelope; protease activities have been reported in other membrane systems (e.g. Morrison and Nourath[79]).

b) Mg-independent Alkaline Phosphatase

This enzyme is considerably purified in nuclear fractions, and appears to have a pH optimum of around 9.5 (cf. Emory and Dounce[80]). Phosphatase activities are commonly found in membrane systems.

c) Aldolase

The activity of aldolase is rather unpredictable (Roodyn[81]); citrate nuclei retain less than do sucrose nuclei (Dounce and Beyer[82]), which may imply either that aldolase is a soluble enzyme readily leached out of the nuclei under acid conditions, or that it is associated with the outer membrane.

To this brief list may be added:-

a) DNA-dependent RNA Polymerase

This enzyme has been said to be soluble (Ramus et al[83]); nevertheless, any association of it with the nuclear envelope would be functionally interesting. It could, for instance, imply that RNA synthesis occurs partly, or even predominantly, at the site of RNA transport.

b) Glucose-6-Phosphatase

Though commonly believed to be absent from nuclei, this enzyme had 50% of the specific activity of microsomal preparations in the membranous material obtained from liver nuclei by Kashnig and Kaspar[84]. Zbarsky et al[66] and Franke et al[67], who appear to have worked with cleaner nuclei, found the activity to be negligible.

III IMPORTANCE OF THESE PROPERTIES IN THE ISOLATION OF THE ENVELOPE

From the brief review of the properties of the nuclear envelope presented above, two sets of concepts can be derived:-

a) Problems: It should be possible, ideally at least, to confirm or refute, to extend, and to explain (i.e. to provide underlying molecular models and mechanisms for) the given list of properties. The investigation of the nuclear envelope described hereinafter constitutes an attempt to deal with a fraction of these problems.

b) Criteria for a nuclear envelope preparation: Clearly,

material isolated from "pure" nuclei (see chapter 2) constitutes an acceptable nuclear envelope preparation if and only if it manifests all the properties which have been associated, reproducibly and certainly, with the nuclear envelope. The enzymic properties do not provide suitable markers here. Some of the activities have been found only in isolated membrane material, while the others (e.g. polymerases, ATPases) are not necessarily associated only with the envelope. This means that the ultrastructural properties must provide the criteria for a satisfactory envelope preparation: a double-membrane system, with 20-30% of its surface occupied by pores, the outer membrane bearing ribosomes, is acceptable as nuclear envelope. The other features of the pore complex may be regarded as a necessary and integral part of the envelope system, or they may be regarded (as by Abelson and Smith[3]) as structurally distinct. In the succeeding account, the former alternative has been selected on the grounds that throughout its history (except perhaps during cell division, v. infra) the envelope and the pore complex bear to each other a very close structural relationship, and the available evidence suggests that they interact functionally. It may, indeed, be reasonable to suggest that without the pore complex, which is its principal distinguishing feature, the nuclear envelope becomes biologically uninteresting and that any discussion of it would be of debatable value. Moreover, arguments will subsequently be presented to show that the integrity of the double-membrane system depends on the presence in it of pore complexes. Therefore, the presence of a pore complex with all associated structurally recognisable features will hereinafter be accepted as a necessary criterion for a nuclear envelope preparation.

B PROPERTIES PERTAINING TO THE ENVELOPE IN INTERACTION WITH OTHER SYSTEMS

The word "interaction" can have a variety of meanings depending on the context in which it is used. Some attempt at rigorous definition must therefore be made at the outset of this discussion. In general, two entities, A and B, are said to interact when the following two criteria are satisfied:-

a) A and B overlap, mix or are contiguous, or exchange matter, energy or information for at least some portion of their existence;

b) If A and B can also exist separately, i.e. there are cases in which criteria (a) is not satisfied, then the properties of A and B are qualitatively different when criterion (a) is satisfied and when it is not (i.e. when A and B are mutually isolated).

If these properties be once again classified as structural (essentially time-independent) and functional (essentially time-dependent), then the interaction may correspondingly be described as structural or functional (Cf. Introduction). In the first case, the structures (i.e. composition, molecular organisation, ultrastructure, etc.) of interacting systems are in part determined by and in part determine the structure of the system under consideration. In the latter case, the system under consideration is in some way involved in the determination of a time-course of change in the structure or composition of the interacting system, or by controlling the supply and distribution of materials directly affects their functioning.

In the case of the nuclear envelope, structural interaction may involve either the cytoplasm or the nucleoplasm. Here, a question may legitimately be raised; to what extent are the structural characteristics of the envelope, discussed in the previous section, dependent upon such interaction? This question can only be answered by an attempt

to isolate the envelope, observing the effect of successive removal of components of the contiguous systems. However, it serves, even when unanswered, to emphasise the point that a distinction between properties of the system in isolation and in interaction is not a true dichotomy; it is merely a separation of the extremes of a continuum. Functional interaction may be taken to include the permeability properties of the system, the "blebs" which appear to pass out into the cytoplasm and may be involved in nucleocytoplasmic exchange, and the apparently passive involvement of the system in mitosis in the majority of organisms.

I STRUCTURAL

1. Cytoplasmic

Continuity between the nuclear envelope and certain cytoplasmic membrane systems has been reported many times in the literature. Its significance is not as yet altogether clear. It may imply some relationship between the permeabilities or other functional properties of the systems; or a generative link between them, one set of membranes giving rise to the other throughout the cell cycle or at discrete points in time; or that the outer nuclear membrane is best regarded as a mere extension of, for example, the endoplasmic reticulum. Evidence for these conjectures can be reviewed here, but it is not at present sufficiently direct to be acceptable.

Watson[34] first noted the continuity between the outer nuclear membrane and the endoplasmic reticulum, and between the outer and inner nuclear membranes at the pores, in a variety of rat tissues. He concluded that all these systems may be regarded as part of "the same membrane system". Since this phrase is often taken to entail compositional similarity, or even identity, to say nothing of functional similarity, the conclusion must be viewed with caution; (tendons and

muscles are often "continuous" with one another in many higher organisms). From further reports of the continuity of the envelope with the endoplasmic reticulum (de Groodt et al[85], Park[52], Hadek and Swift[86] and others), it can safely be regarded as a fairly general phenomenon, at least in higher organisms. Hadek and Swift[86] believed the continuity to have possible implications in transport processes, and visualised nuclear material entering the cisternae of the endoplasmic reticulum, via the perinuclear cisterna, perhaps finally passing into the cytoplasm. Barer, Joseph and Meek[87] made similar observations on locus testis secondary spermatocytes, and suggested that the nuclear envelope might be generated from the endoplasmic reticulum. Hertig[88] believed that the annulate lamellae, which ultrastructurally resemble the nuclear envelope, in human primary oocytes might be derived from interaction between the nuclear envelope and the endoplasmic reticulu .

In other species, continuity between the nuclear envelope and still other membrane systems has been observed. In fungi, for example, the envelope appears to be linked through a double membrane to the plasma membrane; this has been observed in the deuteromycete Stilbum[89] and the ascomycete Mollisia[90]. In Ochromonas and Rhodomonas, Gibbs[91] noted continuity with the outer membrane of the chloroplasts. Such considerations do not, however, seem applicable to mammalian tissue. The organisation of contiguous components of the "soluble" fraction of the cytoplasm by the nuclear envelope remains a possibility which has not, as yet, been investigated.

A point not frequently raised about the continuity of the envelope with other membranes is that the links must in general be continually broken and reformed, since nuclei rotate[30] at an average rate of

1 rev/280 secs. It is improbable that the attached systems describe a fixed orbit at the same rate.

2. Nuclear

a) The Attachment of DNA

In bacteria, the "replicon" theory requires a point of attachment between DNA and the membrane[73,74]. Smith and Hanawalt[92] obtained evidence to suggest that DNA was associated with a lipoprotein complex in some procaryotes, and Tremblay, Daniels and Schaechter[75] showed that the DNA of B. subtilis was associated with phospholipid-rich material of membranous origin. Evidence had previously been obtained by Ganesan and Lederberg[76] that a cell-membrane bound DNA fraction isolated by lysis and gradient centrifugation of B. subtilis contained the newly-synthesised DNA. However, this membrane-DNA complex was stable only in 10 mM Mg^{++}. Such high dication concentrations can give rise to artifactual binding. Arguing largely by analogy with the replicon theory, several groups of workers have suggested that attachment of DNA to the nuclear envelope in eucaryotes may be a prerequisite of DNA synthesis (Cf. Lark, Consigli and Minocha[77], where the discussion centres on various mammalian tissue culture cells, particularly Chinese Hamster). Circumstantial support is lent to this analogy by the isolation of various DNA and lipid containing complexes from nuclei (see chapter 2). More direct evidence for the attachment of DNA to the envelope in a wide range of species is now abundant in the literature.

Bisapultra and Burton[93] demonstrated by electron microscopy DNA-photosynthetic lamella attachment in algal chloroplasts. Duprew[53] showed that chromosome fibrils are attached to the annuli of honey-bee embryo nuclei. Woolam, Ford and Miller[94] showed that the chromosomes

are terminally attached to the envelope in the pachytene stage in mammalian spermatocytes; the same authors[95] showed that the synaptinemal complex of mouse spermatocytes, the only chromosome readily visible in the electron microscope, is linked across the nucleus to the envelope at the centromeric and distal sides; the former side is linked by a heterochromatin-like basal knob. Comings and Okada[41] showed that stellate arrays of chromatin fibres are associated with the annuli of the envelopes of many mammalian and avian interphase nuclei; during cell division, however, despite the "random attachments" between chromosomes and membrane fragments observed in the metaphase and anaphase stages in human amnion cells (Comings and Okada[96]), the nature of the DNA-envelope attachment is far from clear. The problem will be further discussed when mitosis is considered (v. infra).

b) Peripheral Chromatin

It has become clear that, perhaps independent of any definite point of attachment between the envelope and DNA, the presence of the envelope confers upon the chromatin nearby a characteristic and perhaps functionally distinct pattern of organisation. This "granular perinuclear layer" has been observed by electron microscopy in a number of tissues. Farquhar and Palade[97] reported the existence of the layer in renal glomerular cells, and Bruni and Porter[98] in human liver. Patrizi and Poger[99] found the layer to be most readily observed after glutaraldehyde-OsO_4 treatment; it extended for some 40-60 nm into the nucleoplasm and was referred to by these workers as the "zonula nucleum limitans". In all these reports, the layer was found to extend over all the nucleus, except at the sites of the pore complex, being situated immediately below the inner nuclear membrane. This point is made again, implicitly, by Franke and Scheer[9] in their discussion of

the amphibian cocyte envelope; the model presented by these authors shows the granular perinuclear layer extending indefinitely around the nucleus but becoming discontinuous some 10-20 nm from the annulus.

The structure of the layer has been subjected to careful investigation by Davies[100,101] and Davies and Small[102]. The picture arising from this investigation is as follows: the bulk nucleoplasm consists of randomly-distributed structural units, but these same units are arranged in ordered layers at the periphery of the nucleus. The "blebs" which pass into the cytoplasm (v. infra) contain similarly ordered material. The envelope-limited sheets, fragments of envelope with attached chromatin found in and around metabolically active or dividing nuclei, again bear chromatin with a similar morphology. The morphology in question is seen as a succession of electron-transparent and electron-dense bands arranged in parallel to the surface; the transparent bands are crossed, more or less perpendicularly, by a regular sequence of fine electron-dense filaments. However this morphology is to be interpreted in terms of chemical composition, it suggests that the layer has the physical characteristics of a gel. If this is so, then the envelope organises and at the same time rests upon a matrix of gelatinous chromatin. This point may be of significance both in the functional properties of the envelope and in an attempt to obtain an isolated preparation.

However, it is probable that chromatin organisation is determined at least in part by dications present in the nucleoplasm. Calf thymus nuclei, for example, contain 0.115 mg Mg^{++} and 0.024 mg Ca^{++} per 100 g lipid-free dry mass; most of the former ion is apparently bound to the DNA and most of the latter to the protein, though externally added magnesium binds to the protein and externally added calcium to the DNA

(Mirsky and Osawa[30]). This data may again be of significance, not only in the mechanism of structural interaction between envelope and nuclear contents, but also in the separation of envelope from chromatin during an isolation procedure.

II FUNCTIONAL

1. Permeability Properties

The apparent passage of electron-dense material through the envelope has been reported in the bug Rhodnius (Anderson and Beams[47]), the rabbit spinal ganglion (Dawson, Hossack and Wyburn[36]), young amphibian oocytes (Kessel[45]), and a variety of other systems. In the latter two of these observations, the electron-dense material was said to be associated with the pore complex. Such observations illuminate two preconceptions which have been highly important not only in the investigation of permeability properties but also in all interpretation of envelope ultrastructure: first, macromolecules, and even particles of anything up to ribosomal size, must be able to pass through the nuclear envelope; and second, the most important, or even sole, site of such transport is the pore complex or some component thereof. The empirical validation of these preconceptions has been the object of some of the most careful work on envelope permeability.

Permeability to small molecules (inorganic ions, monosaccharides, amino acids, nucleoside bases, etc.) appears to be free. There is no apparent diffusion barrier as in the majority of membrane systems, though as Harding and Feldherr[103] observed (v. infra), there is clearly a diffusion barrier to larger molecules. Anderson and Wilbur[104] showed that isolated liver nuclei are permeable to sodium, potassium, magnesium and calcium ions. Work on the swelling of nuclei in electrolyte solutions will be discussed in the next chapter; in general, such

work implies that the envelope is freely permeable to the electrolytes. The findings of Naora et al[105] that sodium and potassium both concentrate in the nucleus has suggested an active transport system to some workers, but it is difficult to see how active transport could work efficiently in the absence of a diffusion barrier. It seems more probable that the mean activity coefficients of the ions are lowered by the conditions of the nucleoplasm relative to the cytoplasm or bulk solution; such a difference might well arise from the high concentration of charged groups (nucleic acid, histone, etc.) in chromatin. Certainly, the intranuclear sodium forms a separate pool (Langendorf, Siebert and Nitz-Litzow[106]). Entry of more ions into the nucleus by free diffusion may reduce the chemical potential of the intranuclear water, thus leading to water influx and an increase in nuclear volume.

According to Naora et al[105], leucine and alanine are slightly concentrated in the nucleus. The mechanism here may be similar to that postulated above. Certainly, amino-acids in general enter the nucleus freely (Mirsky and Osawa[30]). Many mono-and di-saccharides enter amphibian oocyte nuclei very rapidly, presumably again by free diffusion; e.g. xylose, glucose, sucrose and raffinose (Callan[107]; Goldstein and Harding[108]). Goldstein and Harding[108] state that sucrose, added in 1.5 molal solution in Ca-free Ringer, crosses the envelope more rapidly in young than in old frog oocytes. It is tempting to suggest that this corresponds to the greater pore frequency found in younger oocytes (v. supra). Monné[109] showed that low-molecular weight dyes, both lipid soluble and lipid insoluble, positively or negatively charged, entered the nucleus freely. Feldherr and Harding[12] summed up all these findings by the generalisation that any molecule of less than 500 daltons can enter the nucleus without experiencing a diffusion barrier.

A variety of enzymes also penetrates the envelope. DNase, RNase, trypsin and chymotrypsin entry were described by Anderson[110] in isolated liver nuclei. It is not, however, reasonable to conclude without further evidence that the native envelope is permeable to these molecules; such enzymes may well be entering by degrading the envelope and contiguous chromatin and passing through the channels which they generate. In fact, the entry of any molecule, particularly any macromolecule, can perhaps be seen in terms of a structural modification rather than in terms of permeability properties of the unmodified nuclear envelope. (Such findings may nevertheless be significant when consideration is given to removal of chromatin and nuclear subfractionation.)

Amphibian oocytes take up haemoglobin (Holtfreter[114]); liver nuclei take up heparin (Anderson and Wilbur[112]); and ferritin microinjected into the cytoplasm of amoebae passes into the nucleus (Feldherr[113]). In such cases, however, entry is not immediate. Ferritin particles are absent from the nuclei a few minutes after microinjection, but are present between 1 and 24 hours later. Heparin enters more rapidly, but in view of the marked effects of this molecule on chromatin organisation, this result may be treated as exceptional. Arginine-rich, but not lysine-rich, histones enter the nuclei of Vicia seedlings (Dick[114]). In aqueous media, thymus and liver nuclei lose proteins, particularly histones (Stern and Mirsky[115]); again, protein molecules believed to be of low molecular weight pass, albeit not freely, through the envelope. This result can be related to the finding of Lorch and Danielli[116], that nuclei subjected to aqueous media prove to be inactive when implanted into other cells. Protein loss under such circumstances could perhaps be prevented by increasing the colloid osmotic pressure of the medium used (see chapter 2). Amphibian occytes

seem completely impermeable to very large molecules, e.g. egg albumin, glycogen and acacia (Callan[107]; Goldstein and Harding[108]). Bovine serum albumin appears to be close to the critical size for penetration of nuclear envelopes; it enters Chaetopterus egg nuclei (Merriam[117]) but not those of amphibian oocytes (Battin[118]). It is important to emphasise that molecular size, not weight, appears to be critical in determining nuclear envelope permeability. Stephens and Swift[46] demonstrated the passage of RNA between nucleus and cytoplasm in Chironomus salivary glands - a long-suspected phenomenon; but the molecular weight of the permeating molecule is probably many times greater than that of bovine serum albumin. In cross-sectional area, however, it is if anything slightly smaller (assuming an extended helical configuration).

The site of nucleocytoplasmic exchange of macromolecules has been investigated using colloidal metals. With 4-5 nm colloidal saccharinated Fe_2O_3 particles, Moore et al[119] found that rabbit spleen sinusoidal and Kuppfer cell nuclei were entered at all points; there was no obvious ultrastructural correlate. Feldherr and Harding[12] considered this finding to be an artifact of sectioning of the tissue. Feldherr[120,121] and Feldherr and Marshall[122] used 2.5-5.5 nm colloidal gold particles coated with polyvinylpyrrolidine (final diameter 10-20 nm) in isotonic salt solutions. 1-2 minutes after microinjection into the cytoplasm of amoebae, such particles accumulated round the nuclei and passed into the pore complexes. After 24 hours they appeared to be concentrated in the nuclei; the significance of this concentration effect is unknown. The pore complexes of frog oocytes also bind the particles (Feldherr[123,124]); subsequent reports confirmed that the binding occurred specifically at the pore complexes.

The nucleus also appears to exchange material directly with the extracellular medium. De Bruyn, Robertson and Farr[125] showed that acridine dyes stain the intranuclear material but not the cytoplasm. Epstein[126] provided evidence that fat droplets pass from the extracellular medium to the perinuclear cisterna via the cisternae of the endoplasmic reticulum. Thus, evidence in favour of one of the conjectures raised concerning the continuity of the membrane systems (v. supra) is provided.

2. Electrophysiology

Electrophysiological parameters of the nuclear envelope have been measured in Chironomus and Drosophila salivary glands and in amphibian oocytes. In Drosophila, the potential difference between nucleoplasm and cytoplasm is 15 mV (nucleoplasm being negative relative to cytoplasm), giving a potential gradient of the order of 10^4 volts/cm. The envelope resistance is about 1 megohm/cm^2 and the capacitance about $10^{-2}\mu F$ (Lowenstein and Kanno[127,128]). A porous membrane, permitting free ion flow, would however have a resistance of around 10^{-3} megohms/cm^2 (Lowenstein and Kanno[127]). In frog and newt oocytes, there is no measurable potential difference and the resistance of the envelope is not distinguishable from that of the nucleoplasm and cytoplasm. In neither type does the nucleoplasm of cytoplasm have a measurable resting potential (Kanno and Lowenstein[129]). Attempts have been made[130,131] to correlate these electrophysiological species differences with ultrastructural differences. Whether there is a resting potential or not, material is generally present within the subunits of the pore complexes, and one might expect ion flow always to be thus impeded; however, it has been shown (v. supra) that ion flow across envelopes is always free. The investigators were unable to find ultrastructural differences between high and low resistance envelopes.

Ito and Lowenstein[132] showed that ecdyson reduced the nuclear envelope ion permeability *in situ* in the isolated salivary gland of *Chironomus* to 1/5 its basal value in 1 hour. The same fall, measured as an envelope resistance change, also occurs normally during development. It corresponds to the chromosomal puffing and to increases in the level of DNA and protein. The cell membrane resistance and cytoplasmic ion content, however, remain unchanged. The difference between high and low envelope resistance, therefore, might correspond to a difference in nuclear activity or concentration of protein and nucleic acid around the envelope rather than to any structural difference in the envelope itself. This suggestion, however, does not resolve the contradiction between high resistance and apparently free ion permeability found in many systems.

Such observations as these question the validity of naive attempts at correlation between observed ultrastructure and permeability. Despite the apparent similarity of pore complexes in all systems studied, the freedom of ion permeability as determined by envelope resistance and resting potential is not similar. When this is the case, while free ion flow is assumed in consequence of simple permeability measurements, it must be concluded that the kinetics involved in the two definitions of ion flow are markedly different. Here, a detailed knowledge of the molecular structures involved - presumably the pore complexes - might lead to testable hypotheses explaining the apparent contradiction.

3. Blebs

So far, properties of the nuclear envelope which might be of significance in nucleocytoplasmic exchange mechanisms have been discussed in terms of the continuity of the system with the endoplasmic reticulum, the contiguity and possible functional linkage with chromatin, and the remarkable permeability to many metabolites. There is, however, another

set of properties which falls into the same class, namely the ability of the envelope rapidly to generate outpocketings into the cytoplasm. Often these outpocketings, usually known as "blebs", may be pinched off and subsequently seen as cytoplasmic vesicles. Some writers have speculated that such an event may be an important step in the ontogeny of certain cytoplasmic organelles. Others are content to perceive the mechanism as a possible way of transporting high molecular weight nuclear contents into the cytoplasm.

De Groodt et al[85] believed bleb formation to be associated with mitochondrial activity in neoplastic tissues in mice, on the grounds that blebs appeared only when mitochondria were close to that region of the nucleus. Anderson[2] showed that isolated rat liver nuclei developed blebs when exposed to distilled water or a range of electrolyte solutions, but blebbing was inhibited by the presence of sucrose. This provides a further justification for the inclusion of sucrose in media for the isolation of nuclei (see chapter 2), if blebbing is (and presumably it can be) associated with loss of envelope. This work implies that hydration changes or changes in organisation of the chromatin are responsible for blebbing (see chapter 2). Birdsell and Cota-Robles[133] believed blebbing in E. coli spheroplasts to be due to complex-coil formations from broken membranes. It is improbable that such a mechanism underlies the appearance of eucaryotic nuclear blebs, but the rapidity of formation suggests that either a fairly large pool of envelope precursor can be brought quickly into synthetic action or the envelope is inordinately elastic. Evidence against the former possibility is provided by the observation that a punctured envelope does not reform rapidly, and the nucleus dies (Mirsky and Osawa[30]).

Electron-dense material was observed in blebs of Drosophila salivary gland nuclei by Gay[43,134]. The electron density was reduced by DNase both in the blebs themselves and in the vesicles apparently pinched off from them. The lamellar structure of chromosomes at points of contiguity with the envelope was believed by Gay to constitute a possible abscission layer facilitating the development of out-pocketings. The blebs here were surrounded by double membranes. Blebs have also been observed in many mammalian tissues. Clark[135] described very large double-membrane ones in rat pancreatic acinar cells. Huhn[136] saw them in monocytes, McDuffie[137] in leukaemic and Smith and O'Hara[138] in nonleukaemic leukocytes. In other species, too, blebbing activity appears to be common, though by no means universal[11]. Those described by Bennett[139], McAlear and Edwards[89], Moore and McAlear[90], Gibbs[91], Hadek and Swift[86] and Parks[52], vary in size from minute lumps on the nuclear surface to convoluted masses filling the cell. All these blebs, however, corresponding probably to the first blebs ever reported (Cohen[140]), appear only to have a single membrane; they are outgrowths of the outer membrane of the nucleus. Often, they appear to be contiguous with the endoplasmic reticulum or with the plasma membrane, and may represent a flowing of membrane material from such a system into the nuclear envelope, resulting in the sudden apparent overgrowth of the latter. In isolated liver nuclei, very small blebs have been observed in this laboratory. They are abundant at pH 6.0-6.5, but largely disappear when the pH is raised above 7, so long as dications are present. Such outer membrane blebs, of course, contain no chromatin and must necessarily be free of pore complexes if these structures do indeed link the two membranes, as seems likely. Their significance may lie in the transport of material from the perinuclear cisterna into the cytoplasm. The mechanism of their formation, the subject of speculation above, is at present unknown.

One final way in which nucleocytoplasmic exchange may be effected is by the synthesis of annulate lamellae from the nuclear envelope; there is a certain amount of evidence that such a process may occur[141]. Since the annulate lamellae are predominantly cytoplasmic, and since, if indeed the envelope is their site of origin, they may well carry attached nuclear contents, their synthesis may provide a specialised transport mechanism in some tissues.

4. Mitosis

Although in some species, e.g. ciliates (Anderson[142]) and the stamen hair of Tradescantia (Wada[143]) the nuclear envelope persists throughout cell division, it disperses late in prophase in the majority of cases and is not resynthesised or reassembled until the end of telophase. The mechanism by which the breakdown of the envelope is brought about is uncertain, but its nature has invited speculation. Baud[144], speaking specifically of rat and cat liver, believed the system to be dispersed by some surface-active agent released by other processes at the onset of cell division. Lettré[145] suggested that, as the chromosomes condensed, the surfaces of the envelope no longer protected by nucleic acid were attacked by proteases. Other research, while not explicitly postulating any mechanism, contains information which might be relevant. Meeker[146] for example showed that sea-urchin eggs ceased to divide in potassium depleted media, but this inhibition was removed on the addition of potassium. Mazia[147] showed that 0.75 M mercaptoethanol inhibits mitosis in sea-urchin eggs, with the result that the chromosomes condense more and more on to the envelope and at last become very much shorter than in normal prophase. As yet, however, no widely-accepted or acceptable hypothesis accounting for the phenomenon has been published.

Chromosome condensation is preceded in prophase in rat liver nuclei (Philpot and Stanier[148]) by a coarse granularity of the nucleoplasm, observable in isolated nuclei in the light microscope. The granularity induced by ionic strength increase, reduction of pH to 5.1, addition of histone, protamine or 8 mM Mg^{++}, is significantly finer. Comings and Okada[149] showed the condensed prophase chromosomes of the Indian muntjac to be attached to the inner nuclear membrane, each chromosome having several points of attachment. There was also attachment to the periphery of the nucleolus. As condensation proceeded, the chromosomes remained thus bound but the envelope disintegrated. It is probable, in view of the earlier work by these authors, that the attachment points are the pore complexes in this as in other species. The process by which the envelope is degraded may be any one of, or none of, the above-mentioned speculative mechanisms. It is clear that, by the onset of metaphase, the envelope has wholly disintegrated.

The fate of the degraded envelope has been studied in a number of tissues. In onion and garlic root-tips (Porter and Machado[150]) the envelope and the endoplasmic reticulum with which it is continuous break into fragments in late prophase, and these fragments surround the spindle, excluding mitochondria from the region. It is probable that the envelope resynthesised around the daughter nuclei is built at least in part from these mixed fragments of the membrane systems. The endoplasmic reticulum and nuclear envelope are thus continuous in time as well as in space. In Chaeopterus oocytes, Merriam[117] observed that the envelope becomes vesicular in prophase, breaks apart, and drifts into the nucleoplasm and cytoplasm. In telophase, general cytoplasmic vesicles (from which those originating from the old envelope are indistinguishable in the electron microscope) align themselves on the surface of the daughter nucleus and flatten out into a double-membrane

sheet; pore complexes, with structure characteristics of those in the mature nucleus, can be seen in the nascent envelope. Davies and Tooze[151], observing newt spleen chromosomes during division, described the fate of the envelope. In prometaphase, the envelope collapses at points from which the chromatin is contiguous in interphase has previously withdrawn. Throughout the rest of mitosis, the system survives only as small chromosome-bound fragments, but the endoplasmic reticulum remains intact. Reappearance of a complete envelope begins early in telophase; the membranous material takes the form of linked vesicles which gradually increase in size and, shortly before envelope reformation is complete, have a definitely double-membrane appearance (Mazia[147]).

Though some species differences are apparent from this brief discussion, it is clear that, in general, the nuclear envelope collapses into vesicles - probably single-membrane vesicles - late in prophase and is reassembled from these or similar vesicles in telophase. No adequate trace of the pore complexes in the intervening period, however, has been described. Given the established tubular nature of the spindle fibres, it is conceivable that some components of the pore complexes contribute to the synthesis of the mitotic spindle apparatus. Unfortunately, evidence which lent convincing support to this idea would be difficult to obtain.

III IMPORTANCE OF THESE PROPERTIES IN THE ISOLATION OF THE ENVELOPE

Except in a few special cases, the permeability properties of a membrane system cannot be recovered in isolated preparations. The same is true of electrophysiological properties. However, such properties have an indirect relevance to the problem in hand: any structural model postulated for the nuclear envelope must be consistent with the functional properties discussed above. The breakdown and resynthesis

of the system during cell division raises the problem not only of ontogeny but of maintainance of stability. The former property (see Introduction) is intractable by present techniques; the latter relates to the structural characteristics of the system in interaction. The structural linkages of the nuclear envelope with cytoplasmic membranes and chromatin (including the apparently specific DNA binding at the annuli) thus take on great significance, possibly explaining both the stability of the envelope and its breakdown in mitosis. This renders more specific one of the questions raised in the Introduction, and makes even more important a careful study of the isolation of the envelope and of its attachment to other systems.

SUMMARY

1. The isolated nuclear envelope from rat liver is a double-membrane system bearing structurally recognisable pore complexes at a density of 26-40/μm^2. Once a preparation satisfies this definition, attention can be given to minimisation of its content of, say, DNA, RNA and histone to satisfy arbitrary criteria of "purity". This problem will be discussed more fully in part IV.

2. An examination of the structure of the pore complex, as distinct from the envelope as a whole, may be of value, since the pore complexes appear to confer upon the envelope most of its characteristic properties, notably chromatin binding and permeability.

3. While no inherent functions of the system serve to define a preparation, many are worthy of investigation since they may lead to inferences concerning the purposive aspect, the "functions-in-the-cell", of the envelope. Such functions are outlined above.

4. Special attention must be paid in the isolation procedure to the consequences of removing from the system appended chromatin and

endoplasmic reticulum. Knowledge of structural interaction and of the factors involved in stabilisation and breakdown of the system may be of great significance.

The first and last of these four points of summary greatly increase the relative importance of the first question posed at the end of the Introduction; how can the system be isolated, and what structural links with other systems are apparent? The latter half of the question has been largely answered in the foregoing review, but actual isolation may refine and add details to the available information. The second point directs the next three general questions from the Introduction - (what are the components of the system and what particular properties do they have? How are they organised in space? And can structure be related to function?) - more towards the pore complex than towards the envelope as a whole. Detailed knowledge of the ultrastructure of the complex may be very useful here. However, a thorough investigation of these points does not directly include an approach to the fifth general question; what purposes do the behavioural characteristics of the system serve? This problem belongs to a separate research topic, aimed at elucidating the functions of the system <u>in situ</u> rather than its structure in isolation. This does not minimise its importance, but rather detaches it from the project in hand.

Hence, this thesis presents an empirical examination of the isolation and stability of the envelope under various conditions, together with an examination of the composition of the isolated system. Particular reference will be made throughout to the pore complex. Parts II and III will contain a full account of the experimental findings; part IV will be devoted to a general discussion of the significance and interpretation of the results.

There now follows a review of the techniques described for the isolation and subfractionation of nuclei, with particular reference to the preparation from nuclei of membranous and envelope-like material.

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CHAPTER TWO

METHODS FOR THE ISOLATION OF NUCLEI AND SUBNUCLEAR FRACTIONS

Because of the complex network of membrane systems in mammalian liver cells, it is convenient in the isolation of the nuclear envelope first to isolate nuclei essentially uncontaminated with other subcellular fractions; indeed, it is difficult to conceive of a satisfactory envelope preparation which does not use the isolation of nuclei as a first step. Once nuclei have been prepared, a technique for removing the chromatin and the nucleoli must be devised; this demands some knowledge of the properties of the nucleoplasm and of the susceptibility of its structure to a variety of physical, chemical and enzymatic treatments. In this chapter, therefore, the discussion of this topic will precede the review of attempts to isolate the nuclear envelope. This chapter will consider:-

I) The isolation of nuclei.

II) The behaviour of the nucleoplasm under various conditions.

III) Attempts to isolate the nuclear envelope.

IV) Attempts to isolate other subnuclear fractions.

I) THE ISOLATION OF NUCLEI

In general, methods for isolating nuclei can be discussed under five general headings:-

1. Those employing non-aqueous solvents.
2. Those employing low pH treatment (usually citric acid).
3. Those employing detergents.
4. Sucrose methods:-
   (a) Those using relatively low sucrose concentrations (around isotonic) and low-speed centrifugation.
   (b) Those involving dense sucrose media and high-speed centrifugation.

5. Others; e.g. a few techniques involving glycerol, isotonic saline, etc.

However, this scheme of classification is arbitrary; the medium employed involves parameters other than those suggested in the list, such as ionic strength, pH, presence or absence of dications; and other factors in the preparation are important, e.g. the method by which the tissue is broken up so as to release intact nuclei, the temperature at which operations are performed, etc. Moreover, some techniques are applicable to some tissues but not to others; the source of material is, therefore, an important criterion in choosing a method for the isolation of the nuclei.

1. Nonaqueous solvent methods

Behrens[1] first applied this type of procedure to calf heart and guinea-pig liver tissue, finding it necessary in order to prepare clean nuclei first to starve the animals overnight. Nonaqueous solvent methods were subsequently applied to a variety of mammalian tissues[2,3,4].

Dounce et al[5] claimed that nonaqueous solvent extraction of nuclei prevented the loss of nucleic acid, acid-soluble phosphate and proteins. Clearly, however, lipids will be extracted by such techniques and most proteins will be denatured. Since nuclear envelopes may reasonably be expected, in common with other membrane systems, to consist principally of lipids and a complex mixture of interacting proteins, this means that nonaqueous solvent procedures for isolating nuclei will either dissolve or denature the components of the envelope. This renders such techniques inapplicable to the present problem.

2. Low pH methods

In 1856, F. G. Smith showed that acetic acid appeared to attack skin and tumour cells, liberating nuclei. Not until 1937, however, was the possibility of using low pH treatment in the isolation of

nuclei more fully exploited; Crossmon[6] showed that small numbers of nuclei were liberated when cardiac muscle was teased in 5% citric acid. Stoneburg[7] then isolated nuclei by prolonged exposure of the chopped tissue to 5 volumes 5% citric acid followed by pepsin digestion. The method was, however, not universally applicable. Stoneburg and Harer[8] subsequently modified this procedure. Marshak[9] also used 5% citric acid but thereafter Dounce[10,11] employed much lower citrate concentrations. He found that at pH 6.8-7.0 citrate failed to stabilise the nucleoprotein as adequately as it did at pH 3.8-4.0; Richter and Hullin[12] used citrate at pH 6.0 in the isolation of nuclei from cerebral cortex. Thereafter, citric acid at a concentration of around 1% was employed[13-16].

Though these low pH treatments appear to stabilise the nucleoplasm and enable clean nuclei to be isolated rapidly (only brief low-speed centrifugation is generally used), they have been shown to be undesirable for some purposes, including the isolation of the nuclear envelope. Gurr, Finean and Hawthorne[17] showed that citrate nuclei lack the outer membrane. It is generally believed that nuclear contents of all types are lost to a greater or lesser extent in citric acid as compared to sucrose procedures (Cf. refs. 18, 19, 20). Furthermore, nuclei at low pH develop a granular appearance as their contents are precipitated, and tend to be rather intractable thereafter. Finally, protease treatment of any sort (e.g. the pepsin treatment of Stoneburg) must be avoided when the nuclear envelope is required with all components intact.

Nevertheless, the result obtained by Gurr, Finean and Hawthorne[17] is of great interest. If clean nuclei with intact envelopes can be isolated, then low pH treatment may well provide a method for the isolation of the outer membrane; but attention must be paid in this case to the possibilities that i) the treatment is also extracting intranuclear material and ii) it is removing a large fraction of the

envelope proteins, both because of the low pH and because of the chelating activity of citrate (Cf. refs. 21, 22).

3. Detergent methods

Clean nuclei can also be obtained by the use of a variety of detergents. Frequently, however, detergent extraction is used in combination with other methods (e.g. Allfrey, Littau and Mirsky[23], who found that detergents under hypotonic conditions enabled calf thymus nuclei to be isolated readily using low shearing forces). Bach and Johnson[24] and Ueda et al[25] used media including triton X-100.

Triton X-100, however, removes the outer membrane of the nucleus (Sadowski and Howden[26]); this may account for the purity of the nuclei obtained by its use. Furthermore, it is clear that detergents will interact with hydrophobic regions, and therefore disorganise membranes even at very low concentrations; in the concentrations normally employed they will disperse lipids and denature, disaggregate (or perhaps aggregate) interact with and disperse proteins. Their net effect on any membrane system is therefore completely unpredictable. Hence, despite the apparent usefulness of detergents in lysing otherwise intractable cells and in cleaning nuclei, they must necessarily be avoided when an intact nuclear envelope is subsequently to be isolated.

4. Sucrose methods

(a) Low concentration. Stabilisation of nuclei in isotonic sucrose-containing media was first exploited by Hogeboom, Schneider and Palade[27,28] and Schneider[29]. Liver was homogenised in 0.25 M sucrose containing 1-2 mM $MgCl_2$ (later $CaCl_2$ - Hogeboom, Schneider and Striebich[30]), and nuclei pelleted by low-speed centrifugation.

In subsequent preparations of the same nature, more hypertonic media were found to give more satisfactory results (Cf. Dounce et al[31]);

the stability of the nuclei was enhanced by increasing the dication concentration. Some uncertainty persists as to whether calcium or magnesium is preferable in the homogenising medium; calcium renders the nuclei more mechanically robust, but appears to inhibit DNA-dependent RNA polymerase activity[32]. Under the conditions used in this laboratory, calcium also appears to favour haemoglobin contamination of the "purified" nuclei more than does magnesium.

A short isolation time and buffering of the medium are other desirable features of sucrose preparations of nuclei. This was shown, for example, by Roodyn[33], who found that the distribution of aldolase activity in subcellular fractions was a rather complex function of pH and isolation time. At a pH of less than 6.0, nuclei tend to aggregate; at much lower pH values, the outer membrane is destabilised. A pH of 8 or more destabilises the entire nucleus, and extensive loss of components occurs, accompanied by aggregation. Taking into account the minimal nuclear protease activity around pH 6.0 (Dounce and Umaña[34]), it follows that a pH in this region is most suitable for nuclear preparations in the present work.

(b) Dense sucrose. The low-speed centrifugation and non-aqueous solvent procedures described above implicitly take advantage of the fact that nuclei are denser than whole cells and, in mammalian cells, denser than other subcellular fractions. Dense sucrose methods take advantage of the same property, this time explicitly. There necessarily exists some medium - sucrose or sorbitol solution, organic solvent mixture, etc. - such that, for any tissue homogenate, only free nuclei will pellet through it on centrifugation and all other fractions will float upwards. In practice, the high viscosity of the medium chosen necessitates relatively high centrifugation speeds and times for an adequate preparation to be obtained. Chaveau, Moulé and Rouiller[35,36]

developed the first dense sucrose method; all others have derived, directly or indirectly, from it. The livers were homogenised in 2.2 M sucrose without buffer or dication, and nuclei were pelleted by high speed centrifugation. Despite the emphasis on carefully controlled homogenisation, the work has several weaknesses. Absence of buffer and of dications must tend to make results irreproducible and nuclei unstable; homogenisation in a viscous medium necessarily causes local heating effects; and in the centrifugation, some nuclei would travel a long distance through the sucrose, while others, initially near the bottom of the tube, would travel only a very short distance. Since passage through the viscous sucrose appears to be a very important condition for dragging contaminating materials away from the nuclei, a pad of homogenate-free dense sucrose at the bottom of the tube is required to ensure a finite minimum distance of travel during pelleting.

Subsequent dense sucrose procedures have in general used both buffer and dication; ionic strength control has also been introduced in some variants. Others have, in addition, used homogenisation in a relatively dilute sucrose medium followed by the preparation of a crude nuclear pellet by low-speed centrifugation. Nuclei are then purified from such a pellet. Table 2:1 summarises the principal features of a selection of these variants; other variants are clearly possible.

Table 2:1 Variants of Dense Sucrose Procedure

Reference	Sucrose conc. in homogenising medium	Low speed step	Steps in dense sucrose	pH	Ionic strength (buffer)	Dication
37	0.32 M	+	2.1 M	6.7	1 mM phosphate	1 mM Mg^{++}
38	0.32 M	+	2.2 M	-	Zero	3 mM Mg^{++}
39	130%	-	130%	6.0	1 mM Acetic acid	3 mM Ca^{++}

40	0.32 M	+	2.15 M	6.8	5 mM phosphate	1 mM Mg^{++}
41	0.25 M	+	2.3 M	7.6	0.01 M Tris	3 mM Ca^{++}
42	0.25 M	-	1.02 M / 2.3 M	7.5	25 mM KCl	5 mM Mg^{++}

It is worthy of note that Johnston et al[43] have shown, using sucrose density gradients in a zonal centrifuge, with magnesium ions and bicarbonate buffer pH 7.4, that liver nuclei normally obtained as a single pellet on passage through dense sucrose can be fractionated into several bands. This probably reflects some factor in the nucleoplasm rather than in the state of the nuclear envelope; but heterogeneity in envelopes prepared from a single pellet of nuclei isolated from rat liver remains a possibility.

5. Others

Isotonic saline was a satisfactory medium for the isolation of amphibian oocyte nuclei by microdissection (Callan and Tomlin[44]), but not for large-scale isolation procedures (Hogeboom et al[27,28]). Glycerol was employed at 40% concentration in the homogenising medium by Philpot and Stanier[19], to maintain tonicity and to prevent swelling and aggregation of the nuclei in the absence of dication. Other glycerol procedures have been described (Schneider[45]; Magee and Burrows[46]). Such procedures are, in general, specialised ones valuable only for the isolation of nuclei from relatively intractable material.

Method of Homogenisation is important in the isolation of nuclei. A low range of shearing stresses, the mean value of which is sufficient to liberate but not sufficient to damage the nuclei, is preferable; consequently the Dounce and Potter-Elvehjem homogenisers are most commonly used, the latter with a loose-fitting pestel. The Waring blender, which gives a high range of shearing stresses, is rarely used unless the tissue is very fibrous (Cf. Stern and Mirsky[47]). Emanuel

and Chaikoff[48] introduced a hydraulic homogeniser which, though successfully applied in a number of tissues, has not become widely popular.

In the case of rat liver, the Potter-Elvehjem homogeniser gives satisfactory preparations, and recourse to solvents other than buffered sucrose media containing dication is not necessary. In some laboratories, the Dounce homogeniser has been employed in preference to the Potter-Elvehjem type, which seems to have a rather wider range of shearing forces.

Conclusions

The dense sucrose procedures give reasonably high yields of apparently uncontaminated nuclei with, so far as can be ascertained by electron microscopy, intact envelopes. For reasons discussed above, homogenisation of the rat liver tissue is best carried out in low (isotonic or slightly hypertonic) sucrose media. The total volume of material is usefully reduced by the preparation of a crude nuclear pellet by low-speed centrifugation. The density of sucrose in which this nuclear pellet is resuspended must be high enough not to give a thick interfacial pad when layered over the 2.3 M sucrose necessary finally to clean the nuclei. The choice of a pH of 6.0 or slightly more is justified in the foregoing discussion. Magnesium is preferable to calcium because it tends to cause less contamination of the nuclei with haemoglobin and does not stabilise the nuclei so effectively that subsequent lysis is difficult. Too high an ionic strength, or too high a dication concentration, causes the nucleoplasm to precipitate; the nuclei are thereafter intractable (Cf. Battin[49]). However, the buffering capacity must be sufficient to maintain a stable and reproducible pH, and the dication concentration high enough to prevent chromatin leakage and nuclear swelling and aggregation. 0.03 M sodium phosphate buffer and 3 mM magnesium satisfy both sets of criteria;

potassium ions are avoided for reasons which will be discussed subsequently.

One point which has not been stressed in the literature but which has become clear on observation in this laboratory is that prolonged exposure of nuclei to dense sucrose causes changes of unknown nature in them; they tend to swell, leak, aggregate and become rather intractable. For this reason, 2-3 washes in the low-sucrose homogenising medium are used after pelleting the nuclei through dense sucrose.

II) EFFECTS OF PHYSICAL, CHEMICAL AND ENZYMIC CONDITIONS ON NUCLEI

1. Composition of Nuclei

According to Busch[18], nuclei isolated by the citric acid and sucrose procedures have the following overall compositions:-

Table 2:2. Composition of Nuclei

	Sucrose	Citric acid
DNA	11 pg/nucleus	9 pg/nucleus
RNA	3 " "	2 " "
Protein	44 " "	35 " "

The heterogeneity of these three major components has been the subject of much investigation. The nuclear envelope protein, which probably accounts for only 10% of the total nuclear protein (Berezney, Funk and Crane[50,51]), is itself very heterogeneous (Cf. Franko et al[52]). Classification of the protein into histone and chromosomin (HCl-insoluble nuclear protein), or into 2 M NaCl-soluble, dilute alkali soluble, and residual "envelope" protein (Zbarsky et al[53]), is therefore crude and somewhat arbitrary; no obvious correlation between the presence or absence of particular subclasses and structural or functional properties of the nucleus has been demonstrated.

Other, relatively minor, components of nuclei have been examined in more detail. Chapman and Wallach[54] note that yeast nuclei contain 0.086 mg lipid/mg protein (3-4 pg/nucleus, taking the above value for protein); the same value appears to be applicable in rat liver. Gurr, Finean and Hawthorne[17] calculated that the phospholipid recovered in their nuclei would be sufficient to account for 2.1 monolayers in citric acid preparations, and 5.6 monolayers in sucrose preparations. If membranes are to be regarded as lipid bilayers with protein, these values are roughly consistent with the observed single membrane of citric acid preparations and double membrane of sucrose preparations. Comparing the results of Gurr, Finean and Hawthorne[17] with those of Rouser *et al*[55], species differences in mammalian nuclear phospholipids can be seen; however, such differences are relatively trivial, and the overall composition - approximately 50% phosphatidyl choline, 25% phosphatidyl ethanolamine, and very low sphingomyelin content - is constant. Phospholipids account for 60-70% of total nuclear and nuclear envelope lipid (Gurr, Finean and Hawthorne[17]; Keenan *et al*[56]); this is consistent with the finding in this laboratory that nuclei contain 1 g lipid phosphate/17-18 g total lipid. The neutral lipid appears to be largely free fatty acid and unesterified cholesterol.

Little is to be found in the literature concerning the carbohydrate content of nuclei. Yamashina, Izumi and Naka[57] found traces of sialic acid, but this was probably contamination; earlier, Patterson and Touster[58], carefully purifying their nuclei, had found no sialic acid. Glycolipids appear to be absent (Rouser *et al*[55]).

The ionic content of nuclei has also been discussed. Naora *et al*[58], found in frog oocyte a Na^+/K^+ ratio of 1.1 in the nucleus, as opposed to 0.72 in cytoplasm; Na^+, however, had 3.2 times and K^+ 2.4 times the cytoplasmic concentration in the nucleus. Intranuclear dication

concentration was referred to in chapter 1. Leucine and alanine have also been found to concentrate in the nucleus. It is not known whether these results are to be interpreted as implying active transport in the envelope or lower activity coefficients in the nucleoplasm.

2. Factors causing gross changes in the nucleoplasm

The isolation of the nuclear envelope necessarily involves the disruption of the nuclear contents. In this section, procedures by which the nucleoplasm may be disrupted are discussed.

(a) Enzymic effects

Anderson[60] showed that 1 mg/ml DNase at room temperature caused a significant density decrease in isolated mammalian nuclei in 1-4 minutes. The nucleoli condensed and became granular, exhibiting Brownian motion. There was a slight decrease in nuclear diameter, and the envelope became more sharply defined. RNase did not give this appearance of solating and depleting the nucleoplasm, but the nucleoli collapsed into very small granules within 30 seconds. Proteases caused swelling of the nuclei, and finally rupture and loss of contents.

(b) Ionic effects

Anderson and Wilbur[61] noted that heparin, unless desulphonated, depleted the nucleoplasm of fresh nuclei in 6-8 minutes at room temperature. Other chelating agents have similar effects, but in the case of EDTA the time required is in the order of hours (see chapter 4). Such observations suggest that the structure of chromatin to a large extent maintained by dications. When the pH is lowered, the nuclear volume decreases and the nucleoplasm becomes less responsive to heparin treatment (Anderson and Wilbur[61]; see also chapter 4). A dication concentration of 10mM, however, changes the organisation of the nucleoplasm so as to cause granularisation (Battin[49]).

Increase of ionic strength (sodium chloride, potassium chloride

or phosphate) from 1 to 50 mM decreased the nuclear volume, but no further shrinking was observed when the concentration was further increased. Sodium and potassium effects were indistinguishable at equal concentration (Cf. Hunter and Hunter[62]). More marked shrinking was effected by calcium and magnesium chlorides at concentrations of up to 150 mM. These changes were readily reversed. 0.1 N HCl reduced the nuclear volume, possibly by extracting histones. Higher dication concentrations first caused swelling of the nuclei and finally rupture of the envelope. DNase treatment prevented all subsequent ionic effects of this nature.

(c) Nonelectrolytes

Sucrose has no obvious osmotic stabilising effect on nuclei (Anderson and Wilbur[63]); swelling can be observed in very dense sucrose (see chapter 4). Salts generally reduce the rate of swelling of nuclei in sucrose, the percent reduction being roughly proportional to ionic strength (Battin[49]). Swelling results in loss of contents, perhaps as a consequence of chromatin disorganisation or rupture of the envelope or both. However, Hunter and Hunter[62] found the rate of swelling to be inversely proportional to the external solute concentration, the effects of glucose, glycerol and sucrose being essentially similar.

The lack of traditional "osmotic" properties in nuclei was discussed by Harding and Feldherr[64]; macromolecular species such as bovine serum albumin and polyvinylpyrrolidine, however, caused swelling or shrinkage of nuclei except at concentrations around 3×10^{-4} M, and it was argued that the osmotic pressure of these solutions was such that they could be called "isotonic" with the nuclei. The failure of the nucleus to show conventional osmotic properties, therefore, can be interpreted in part in terms of its permeability to all small molecules.

(d) pH effects

Anderson and Wilbur[63] observed that nuclear volume remains constant between pH 5.1 and 8.9; there is shrinkage below pH 5.1. Observations in this laboratory are consistent with the latter conclusion; as the pH falls below 5, the nucleoplasm becomes granular and the nuclei shrink. At lower pH still, destabilisation and partial loss of the outer membrane is apparent. However, in the absence of dication, the nuclei rupture and the contents gelate at pH values above 8; in the presence of dication, no marked volume changes are seen as the pH is increased.

Conclusions

The above discussion suggests the following approaches to the problem of removing the nuclear contents in attempts to isolate the nuclear envelope:-

(a) RNase may be used to break up the nucleoli.

(b) DNase may be used to disperse the chromatin (Cf. isolation of E. coli cell membrane, Kaback and Stadtman[65]).

(c) Chelating agents may be used, after or instead of DNase, to disperse and "solubilise" the chromatin. However, caution would be required in interpreting the results obtained by the use of chelating agents. Such substances have been shown to dissolve a large fraction of erythrocyte membrane proteins (Marchesi et al[22]), and it is probable that dications are important in maintaining the stability of many membrane systems.

(d) The nuclei may swell and partially empty in water or nonelectrolyte solutions.

(e) Alternatively, a high ionic strength, using either monovalent or divalent cations, may be used to swell and burst the nuclei. In such treatment, care must again be used in the interpretation of results. Callan[66] showed that 0.2 M phosphate disrupted the outer nuclear membrane of amphibian oocytes over a wide pH range. Thus, high ionic strength may lead to loss of envelope integrity.

(f) Exposure of the nuclei to high pH affords a possible means of removing the nucleoplasm and recovering the envelope.

(g) Protease treatment and 0.1 N HCl extraction, though they would disorganise the nucleoplasm, are clearly undesirable in the isolation of the envelope.

In addition to these possible approaches, subjection of the nuclei to mechanical stress by homogenisation, freezing and thawing, sonication, etc. may usefully be employed, either in media in which the nuclei are stable or in combination with one or more of the foregoing techniques.

III) THE ISOLATION OF THE NUCLEAR ENVELOPE

Starting from pure nuclei, the nuclear envelope may possibly be isolated using techniques such as those discussed above. The isolated preparation must, by definition, meet the ultrastructural criteria discussed in chapter 1 if it is to be satisfactory. It may be inferred, albeit tentatively, from the literature that no intranuclear membrane systems exist: no nucleolar membrane is evident (Borysko and Bang[67]), and though intranuclear annulate lamellae have been reported in some species none has been reported in rat liver. This absence of possible contaminating membranes roduces the set of criteria required for an adequate envelope preparation.

The isolation techniques so far published will be reviewed under three headings:-

1. Isolation of lipid-containing complexes of unknown structure from nuclei.
2. Outer membrane preparations.
3. Total envelope preparations.

1. Lipid-containing complexes from nuclei

Wang, Meyer and Thomas[68] extracted nuclei isolated by low pH or

nonaqueous solvent procedures with 1 M NaCl, and isolated from the residue a lipoprotein fraction by thorough extraction at pH 12.5 followed by precipitation at pH 6. Bach and Johnson[24] extracted nuclei isolated in triton X-100 with a medium containing an anionic polymer, such as salmon sperm DNA, and recovered an apparently membranous fraction containing ribosomal aggregates, lipid, and nuclear DNA, leaving the gross morphology of the nuclei unaltered. Magnesium concentration was critical in this procedure; spermidine did not replace the dication. The fraction was apparently of envelope origin, containing very high NADH-cytochrome c oxido-reductase activity, but was highly RNase labile. The finding is consistent with the observation of Vassar et al[69] that DNA binds to the surfaces of nuclei, and suggests that the anionic polymer was behaving as a complicated detergent; the binding of DNA by the envelope is a very interesting property, which may be significant in the function of the envelope in vivo.

Jackson, Earnhardt and Chalkley[70] extracted nuclei of questionable purity (isolated by an isotonic sucrose procedure using tris and EDTA at pH 8) with 1 M NaCl, after which the insoluble residue was lipid-rich and contained traces of DNA and RNA. Ueda et al[25] described a similar preparation, but their procedure included a prolonged low-temperature DNase step. The "heavy" and "light nuclear envelope" fractions obtained after NaCl extraction contained cytochromes and a trace of RNA, and were lipid rich; DNA was not estimated.

If nonionic detergents, used by Bach and Johnson[24] and by Ueda et al[25] fail to remove the electron transport enzymes, then these enzymes presumably exist in some part of the envelope other than the outer membrane; the same is true of ribosomal aggregates. If all or most of the nuclear phospholipid is associated with the envelope, then it would appear from the above results that:-

(a) The envelope is not wholly dispersed by high concentrations of NaCl, a view consistent with that of Zbarsky et al[53].

(b) Some part at least of the envelope is dispersed, or "solubilised", at high pH.

It is interesting to note the application in the procedures discussed above of the techniques suggested for chromatin dispersal, viz. high ionic strength, high pH, DNase and chelating agents.

2. The isolation of the outer membrane

The above discussion suggests three possible methods for the isolation of the outer membranes of clean nuclei:-

(a) Washing in a high ionic strength medium (e.g. 0.2 M phosphate).

(b) Washing at low pH (e.g. 2.5% citric acid, pH 2.3).

(c) Washing in nonionic detergent (e.g. 0.25% triton-X-100).

The first of these procedures, however, must extract many intranuclear components along with the membrane, and probably leads to extensive loss of membrane components (Cf. Dodge, Mitchell and Hanahan[71], on the disintegration of erythrocyte ghosts in high ionic strength).

Low pH extraction, though it undoubtedly solubilises intranuclear components, does not extract bulk chromatin or break up the nuclei. Membrane vesicles appear in the supernatant after low pH washing, and can be pelleted at 100,000 X g for 60 minutes (Smith et al[16]; 30 minutes is in fact sufficient). Though these authors admitted the possibility of cytoplasmic membrane contamination, nuclear contamination and the low pH solubilisation of membrane proteins (Maddy and Kelly[21]) were not considered. The membranous appearance and the presence of ribosomes in the preparation constituted the only evidence that the pellet was an acceptable outer nuclear membrane preparation.

The membrane vesicles in a low pH extract aggregate when the pH is raised to 6 and can be pelleted at low speed. The same criticisms

apply in principle here that applied to the above-discussed procedure of Smith et al, though in practice it is possible that proteins dissolved in the citric acid are precipitated and recombine with the membrane when the pH is raised. In both procedures, the citric acid can be replaced by 1.5% acetic acid with apparently comparable results, implying that it is indeed the low pH, not any ionic strength or chelating effect, that is the significant factor.

Detergent procedures are susceptible to the same criticisms as are such procedures in the isolation of nuclei; the detergent combines with, reorganises, disperses, disaggregates and solubilises various components of the system, rendering it morphologically unrecognisable and structurally and functionally different from the native membrane. Triton X-100 has been used, like citric acid, to prepare outer membrane ribosomes (Sadowski and Howden[26]); tween-80 was used to remove the envelope from amnion cell nuclei and the membranes were often seen to separate; in such cases, the annuli remained attached to the outer membrane (Comings and Okada[72]). The annuli, as previously suspected, appeared from this work to hold the two membranes together.

While none of these procedures promises to give rise to an adequate outer membrane preparation, the confirmation of the presence of attached ribosomes and the curious behaviour with changing pH (dispersal at low pH, reaggregation at pH 6) are useful observations. Moreover, another problem concerning the status of the annuli is raised. Since the structures appear to hold the two membranes together, and since one procedure generates outer membranes with the annuli attached while the others do not, it may be asked: what is the nature of the forces linking the annuli to the two membranes, and how do the two sets of forces differ?

3. The isolation of the total envelope

Previous discussion has suggested that clean isolated nuclei may be disrupted by nuclease, high ionic strength, high pH or chelating agent treatments, with or without the aid of various techniques of mechanical stress. Once separated from other materials, the envelopes may presumably be isolated by differential or gradient centrifugation. It remains to investigate various combinations of such methods, bearing in mind the strict morphological criterion of an acceptable envelope preparation.

After the microdissection technique of Callan, Randall and Tomlin[73], clearly inapplicable to bulk preparation, no isolation procedures for the nuclear envelope were published until that of Franke[74]. Nuclei were briefly sonicated in electrolyte and dication free aqueous media and centrifuged at low speed over dense sucrose. Impure envelopes, which were however suitable for electron microscopy, were collected at the interface. The method constituted an application of low osmolarity, low ionic strength and zero dication techniques, coupled with vigorous mechanical disruption.

Zbarsky et al[75] developed two methods claimed to produce satisfactory envelope preparations from nuclei on sucrose gradients. The resulting material contained electron transport enzymes, monocation independent ATPase, arylsulphatase and glutamate dehydrogenase activities. The material was 27-50% lipid, and contained small quantities of RNA and a trace of DNA. The methods involved treatment of the nuclei with dilute dication-free buffer; the first used a prolonged incubation, the second a brief sonication. After removal of dense chromatin, nucleoli etc., the suspension was layered below a discontinuous sucrose gradient and on centrifugation the material was

collected at densities 1.16-1.19 g/ml. Attempts to repeat the work in this laboratory have not been successful; the first procedure gave an extremely low yield, and both procedures gave only 100 nm diameter vesicles which were pore-free and apparently single-membrane. The isolated material cannot, therefore, justifiably be called nuclear envelope.

Similar material was produced by a very different method published shortly afterwards (Kaspar and Kashnig[76]; Kashnig and Kaspar[77]). It contained 65% protein, 29% lipid, 3% RNA and 5% carbohydrate (largely - hexose; no glucosamine and a small trace of sialic acid, as in the preparation of Zbarsky et al[75]). No DNA was detected. In contrast to the preparation of Zbarsky et al, the glucose-6-phosphatase activity was found to be 50% of that in the microsomes. This, however, may be attributed to the method used for the isolation of the nuclei (Blobel and Potter[42]), which is rapid but permits considerable microsomal contamination. The only mechanical disruption technique found to break open the nuclei was sonication; this procedure was accordingly used in combination with chelating agent and high ionic strength treatments (10% w/v potassium citrate). The envelope material, of density around 1.16 to 1.20 g/ml, was isolated on a sucrose gradient containing the same concentration of potassium citrate. On repetition of this work, pore complexes have been found to be more or less non-existent in the tangled aggregate of vesicles in the gradient band, though they persist in the DNA-rich pellet discarded by Kashnig and Kaspar. The apparently double-membrane nature of a small number of scattered regions in the preparation probably results from a fortuitous contiguity of two membranous fragments.

Berezney, Funk and Crane[50,51] extended a modification of the

method of Ueda et al[25], discussed above, to a bulk preparation of "nuclear envelope". After prolonged incubation of the nuclei with DNase at low temperature, the suspension was dispersed in 0.5 M $MgCl_2$ and the insoluble material pelleted. A combination of nuclease treatment and high ionic strength extraction was applied to the problem of the isolation of envelope from clean (if rather misshapen) nuclei. Pores can be seen in the flat, nonvesicularised regions of the pellet but they are not circular and lack annuli and central tubules. The composition of the preparation was 75% protein, 14% phospholipid (which must imply 22-23% lipid, since phospholipid accounted for only 60-65% of the total lipid; Keenan et al[56]), 9% RNA and 1% DNA. Keenan et al[56] rightly emphasise the close comparability between the detailed lipid composition of the preparation and that of the nuclei. However, their suggestion that the recovery of phosphatidyl inositol is disproportionately high is not justified; Rouser et al[55] showed that bovine liver nuclei, the starting material in the preparation of Borovsky, Funk and Crane, contained much more phosphatidyl inositol than did rat liver nuclei, from the composition of which Keenan et al drew their conclusions. The suggestion of Gurr, Finean and Hawthorne that nuclear envelope lipids contain mainly saturated fatty acids was not corroborated by this investigation.

The lack of pore complexes in the material prepared by Borovsky, Funk and Crane again suggests that this material cannot be regarded as nuclear envelope. One further procedure for the isolation of the envelope employing high ionic strength treatment in combination with sonication, also resulted in a tangled mass of pore-free vesicles (Franke et al[52]). This result, when compared with the foregoing, suggests that high ionic strength treatment in general disrupts the

envelope rather than assisting in its isolation. The final envelope band in the 1.5 M KCl-containing sucrose gradient used by Franke et al had a density of around 1.21 g/ml. Protein and phospholipid accounted for about the same percentage by weight of these vesicles as in the preparation of Berezney, Funk and Crane (v. supra), but DNA accounted for 2.8% and RNA for only 3.6% of the total. Kleinig[78] found most of the neutral lipids of the nucleus to be present in this preparation, and found the neutral lipid and phospholipid compositions to be similar to those reported by Keenan et al[56] in a different species. The similarity between the nuclear and microsomal lipid compositions may imply a measure of similarity between nuclear envelope and endoplasmic reticulum, but the comparison of total proteins attempted by Franke et al[52] is impossible to interpret. The polyacrylamide gel patterns of total microsomal and nuclear envelope proteins were superficially comparable, but their extreme complexity and the presence of large amounts of material at the origin precluded rigorous comparison. No final conclusion about the comparison between the membrane systems is as yet possible.

Of the possible techniques for the isolation of the nuclear envelope discussed previously, no procedure as yet published has applied RNase or high pH treatment. RNase may have been avoided because some workers believe the pore complex to contain RNA (Cf. Franke and Scheer[79]); despite such caution, however, no preparation procedure published to date generates morphologically recognisable envelopes. The pore complexes are always absent.

This review of attempts to isolate the nuclear envelope has generated a number of additional problems:-

(a) Why has the material isolated lost its pore complexes in all cases?

(b) What other combination of techniques can be applied so that the pore complexes will persist throughout the treatment?

(c) Is there any relationship between loss of one of the principal components of the system (protein, lipid, RNA, etc.) and the disappearance of the pore complexes?

(d) What is the significance, if any, of the low osmotic pressures of the media in which all isolation procedures have been attempted?

IV ISOLATION OF OTHER NUCLEAR COMPONENTS

Some insight into the biological significance of a preparation such as those discussed above may be gained by an examination of other techniques of subnuclear fractionation. A brief discussion of methods for isolating nucleoli, chromatin fractions and the mitotic apparatus will be presented, omitting those procedures which employ detergents or organic solvents. Comparability between the properties of these fractions and those of the envelope may be of some interest, particularly when the preparative methods are compared. It is possible that components and properties common to, say, the envelope and some chromatin fraction or the mitotic apparatus may be found.

1. Chromatin fractionation

Frenster, Allfrey and Mirsky[80] isolated chromatin fractions from nuclei by washing them in a calcium-containing medium at neutral pH to remove envelopes and contaminants, and suspending the residue in ion-free isotonic sucrose. The nuclei became swollen (v. supra) and were briefly sonicated, and highly aggregated material was removed. Heterochromatin was pelleted at 1000 X g for ten minutes, intermediate chromatin at 3000 X g for 30 minutes, and euchromatin at 78,000 X g for 60 minutes. From the final supernatant very small chromatin fibrils were precipitated by adding excess calcium. In the last two

fractions the highest rates of nucleic acid and protein precursor incorporation were found.

Other chromatin fractionation procedures have in general been modifications of this. Yasmineh and Yunis[81], for example, used a method which differed only in having a slightly longer sonication time, marginally different centrifugation conditions, and a final precipitation with ethanol instead of calcium.

The intermediate chromatin fraction bears some resemblance, from an operational standpoint, to the early nuclear envelope preparations of Franke[74], though the 3000 X g pellet of Fronster et al[80] was fairly well depleted of envelope material. The preparations of Zbarsky et al are again slightly similar[75], though here the sucrose gradient has taken the fractionation procedure a step further. Methods for nuclear envelope preparation which use DNase or high ionic strength, however, do not resemble the chromatin fractionation in any obvious way.

2. Isolation of the mitotic spindle apparatus

Procedures such as that of Kano[82], using hexanediol to isolate the apparatus in metaphase, depend on the presence of the organic solvent in the isolation medium. Mazia et al[83], however, had previously developed an isolation procedure which did not require organic solvent; dense sucrose at pH 6 was used in the presence of a thiol reagent. The apparatus was dispersed by high pH treatment or isotonic KCl, but dications stabilised it irreversibly even at very low concentration. The basic unit of the apparatus appears to be a 3.5 S protein, which is tentatively identified with the globular 35 Å subunit of each of the 13 fibrils of the microtubular spindle filament (Kiefer et al[84]). Treatment of the 3.5 S protein with thiol reagents converts it to two 2.5 S subunits.

The mitotic apparatus consists largely (90%) of protein, and

accounts for 10% of the total protein of the cell (Mazia[85]). The amino-acid composition resembles that of actin and the filamentous protein of flagellae; the monomer units are probably linked by mainly sulphur-containing bonds. The apparatus has a high Mg-dependent ATPase activity, almost absolutely specific for ATP. Other minor components of the system are reported to be RNA, poly-saccharide, lipid and zinc ions.

It is possible that some part of the mitotic apparatus derives from the nuclear pore complexes. The fate of the latter during mitosis is not adequately understood, and they have in common with the apparatus ATPase activity, attachment to dense chromatin knobs on chromosomes, and a fibrous structure. Moreover, the mitotic apparatus appears, generally speaking, when the pore complexes disappear, and vice-versa, and the condensed chromosomes are attached to each; the stability properties of the two structures are comparable (see Part II); and though its interphase location is not understood, the mitotic apparatus protein is preformed rather than synthesised de novo prior to cell division. Such evidence is circumstantial, but renders the possibility of a relationship in time between the mitotic apparatus and the pore complex worthy of investigation.

3. Isolation of nucleoli

Muramatsu[86] prepared nucleoli by layering clean ion-free sonicated nuclei over fairly dense sucrose solution and collecting the pellet after centrifugation at low speed. The success of this procedure reflects the high density of nucleoli; these potentially contaminating bodies can therefore be removed from nuclear ghosts or envelope fragments by low-speed centrifugation, and their absence from the envelope preparation can be ascertained by monitoring the preparation in the electron microscope.

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PART II

THE ISOLATION OF THE NUCLEAR ENVELOPE

CHAPTER THREE

GENERAL MATERIALS AND METHODS

1. ISOLATION OF NUCLEI

Nuclei were isolated by a dense-sucrose procedure similar to that of Harris and Agutter[1]. Three male albino rats (150-200 g) were killed by a blow on the head and the livers removed as quickly as possible and placed in ice-cold homogenising buffer (0.32 M sucrose - 0.03 M sodium phosphate buffer - 3 mM $MgCl_2$, pH 6.1). All subsequent operations were performed at 0°-5°C. The tissue was minced with scissors for 1-2 minutes, the fluid decanted, and fresh homogenising buffer (4-5 mls/g liver) added. The suspension was homogenised in approximately 30 ml aliquots in a Potter-Elvehjem homogeniser, the pestle having an approximately 0.5 mm clearance and being rotated at 3000 rev/min. 5-6 up-down movements, averaging 20-30 seconds each, were used. The homogenate was filtered through eight thicknesses of cheesecloth and centrifuged at 600 X g (in the 6 x 250 ml. swing-out rotor of an M.S.E. Mistral 4L centrifuge) for 10 minutes. The supernatant was carefully decanted and the pellet thoroughly resuspended in 4 vols. dense sucrose medium (2.3 M sucrose - 0.03 M sodium phosphate buffer - 3 mM $MgCl_2$, pH 6.1). This suspension was layered over 10 mls dense sucrose medium in 37.5 ml capacity centrifuge tubes and centrifuged at 60,000 X g (in the Spinco L centrifuge, no. 30 rotor) for 1 hour. The pellet of clean nuclei was suspended in 5 mls homogenising buffer and centrifuged at 600 X g (as before) for 10 minutes; this final washing was performed 2-3 times until the supernatant was clear and the nuclei completely free of dense sucrose.

2. CONTAMINATION OF NUCLEI

Mitochondrial, microsomal and plasma membrane contamination were estimated in the final nuclear pellet by assays for succinoxidase, glucose-6-phosphatase and 5'-nucleotidase activities respectively.

Plate 3:1

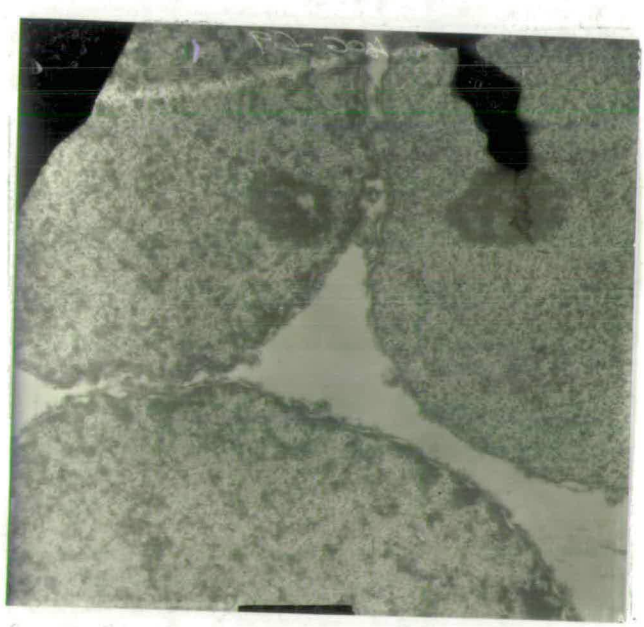
Rat liver ~~nuclear envelopes~~ nuclei, embedded and sectioned as described (see text). Post-stained with uranyl acetate.

a) Magnification X 15,000

b) Magnification X 25,000.

Plate 3:1

a



b

(a) Assay methods

Succinoxidase was assayed by a modification of the method of King[2]. The potassium ferricyanide solution was freshly prepared before each experiment. The assay mixture was as follows, all solutions being preincubated at 37°C:

2 mls	0.2 M sodium phosphate buffer pH 7.8
0.3 mls	0.6 M succinic acid, adjusted to pH 7.8 with NaOH
0.2 mls	0.03 M potassium ferricyanide in water

The mixture was prepared in 4 ml spectrophotometer cells; 0.2 ml water was added to the reference cell, and 0.2 ml sample thoroughly mixed in the sample cell, immediately before recording commenced. The change in optical density at 400 nm was followed continuously at 37°C on a Unicam SP 800 spectrophotometer, using a scale expansion of X5 or X10. Protein was estimated (in Lowry units, v. infra) by the procedure described below, and the enzyme activity expressed as change in E_{400}/Lowry unit/min.

Glucose-6-phosphatase was assayed by a modification of the method of Swanson[3], using a succinic acid buffer. The assay mixture was as follows, all solutions being preincubated at 37°C:

0.3 mls	0.1 M sodium succinate buffer pH 6.5
0.1 ml	0.13 M disodium glucose-6-phosphate, adjusted to pH 6.5
0.1 ml	sample

The sample (nuclei or total homogenate) was thoroughly dialysed against the succinate buffer and adjusted to a concentration of approximately 5 mg protein/ml. The mixture was incubated at 37°C for 15 minutes and the reaction stopped by the addition of 1 ml 10% TCA. The suspension was chilled in ice, 1 ml of water added, and the whole briefly centrifuged. To the clear supernatant was added 0.1 ml 70% PCA, 0.2 mls 5% ammonium molybdate in water, and 0.1 ml freshly-prepared

Fiske-Subbarow reagent (v. infra). Phosphate was estimated in the manner described, and the enzyme activity expressed as µg phosphate released/Lowry unit/15 minutes. The following controls were performed:

(i) Incubation of glucose-6-phosphate and buffer without sample, to determine the extent of spontaneous hydrolysis.

(ii) Incubation of buffer and sample without glucose-6-phosphate, to determine the extent of phosphate release from the sample.

5'-nucleotidase was assayed by the method of Emmelot and Bos[4]. TMK buffer consisted of 0.1 M KCl-5 mM $MgCl_2$-0.05 M tris-HCl, pH 7.2. All solutions were again preincubated at 37°C. The assay mixture was as follows:-

1.8 mls TMK buffer
0.1 mls 0.1 M sodium AMP, adjusted to pH 7.2
0.1 mls sample, exhaustively dialysed against TMK

After incubation at 37°C for 15 minutes, 1 ml 10% TCA was added and the suspension centrifuged briefly after chilling in ice. To the clear supernatant were added 0.35 mls 70% PCA, 4.85 mls water, 0.5 mls 5% ammonium molybdate in water and 0.2 mls Fiske-Subbarow reagent, freshly prepared. Phosphate was estimated as below, making the appropriate correction for dilution, and controls were performed as for the glucose-6-phosphatase assay. Activity was expressed as µg phosphate released/Lowry unit/minute.

(b) Results

The specific activities in the nuclei and in the total liver homogenate were calculated, and thus the recovery of activities in the nuclear fraction was found (Table 3:1). It will be seen that the nuclei were essentially free of the marker enzymes.

Table 3:1

Succinoxidase	Liver homogenate:-		
	1	2	3
Lowry units/0.1 mls	0.21	0.30	0.18
Change in E_{400}/min/0.1 mls	$8.6X10^{-2}$	$12.2X10^{-2}$	$6.7X10^{-2}$
Specific activity (per Lowry)	0.41	0.40	0.37
	Nuclei:-		
Lowry units/0.1 mls	0.048	0.100	0.057
Change in E_{400}/min/0.1 mls	$6.2X10^{-4}$	$14.0X10^{-4}$	$6.3X10^{-4}$
Specific activity (per Lowry)	0.013	0.014	0.011
Recovery in nuclei	3.2%	3.5%	2.9%

Glucose-6-phosphatase	Liver homogenate:-		
	1	2	3
Lowry units/0.1 ml	0.21	0.30	0.18
µg phosphate released/15 minutes:-			
Glucose-6-phosphate + buffer	4.5	4.0	4.5
0.1 mls sample + buffer	16.5	20.0	24.5
Total assay mixture	69	85	74
µg P_i released/15 mins by enzyme:-			
Specific activity/Lowry	228	204	252
	Nuclei:-		
Lowry units/0.1 ml	0.048	0.100	0.057
µg phosphate released/15 minutes by:-			
Glucose-6-phosphate + buffer	4.5	4.0	4.5
0.1 mls sample + buffer	Zero	Zero	Zero
Total assay mixture	4.5	4.5	4.0
µg P_i released/15 mins by enzyme:-	Zero	0.5	Zero
Specific activity/Lowry	≤ 10.5	≤ 5	≤ 8.5
Recovery in nuclei	$\leq 4.6\%$	$\leq 2.5\%$	$\leq 3.5\%$

(The assumption is made that "Zero" implies "less than 0.5 µg")

5'-nucleotidase	Liver homogenate (Lowry as above):-		
	1	2	3
µg phosphate/released/15 minutes by:-			
AMP + buffer	3.0	3.0	2.0

(cont'd on next page)

Table 3:1 (cont'd)

0.1 mls sample + buffer	12.0	24.0	19.0
Total assay mixture	68	93	61
µG phosphate released by enzyme	55	66	40
Specific activity/Lowry	262	220	224
	Nuclei (Lowry as above):-		
µG phosphate released/15 minutes by:-			
AMP + buffer	3.0	3.0	2.0
0.1 mls sample + buffer	Zero	Zero	Zero
Total assay mixture	1.5	2.5	2.0
µG P_i released by enzyme	Zero	Zero	Zero
Specific activity/Lowry	$\leq$ 10.5	$\leq$ 5	$\leq$ 8.5
Recovery in nuclei	$\leq$ 4.1%	$\leq$ 2.3%	$\leq$ 3.7%

3. PROTEIN ASSAY

Throughout the experiments described in this thesis, protein was assayed by a modification (Maddy and Spooner[5]) of the method of Lowry, Rosebrough, Farr and Randall[6].

0.25 mls sample were added to an equal volume of 4% sodium deoxycholate solution in 0.5 M NaOH. This procedure disperses membranous material, nuclei, etc. To 1 volume of the resulting clear liquid 5 volumes copper-alkali solution (50 vols. 0.1 M NaOH-50 vols. 4% Na_2CO_3-1 vol. 2% sodium potassium tartarate-1 vol. 1% copper sulphate) were added, followed by 0.5 volumes 1:3 aqueous Folin-Ciocalteu reagent. After thorough mixing, the colour was developed at room temperature in the dark for 45 minutes, and the optical density was read at 700 nm against a water blank.

Several of the procedures used in the work subsequently described interfere with the development of the Lowry colour. Dications inhibit the dispersal of the material in NaOH-DOC. EDTA itself gives a positive Lowry colour. Caesium gives an insoluble tartarate, so the

assay cannot be performed in the presence of this ion. All such materials were, therefore, removed from the samples by dialysis before the assay. DNA, RNA and nuclear lipids, however, were found to produce no measurable Lowry colour even at high concentration.

4. DNA ASSAY

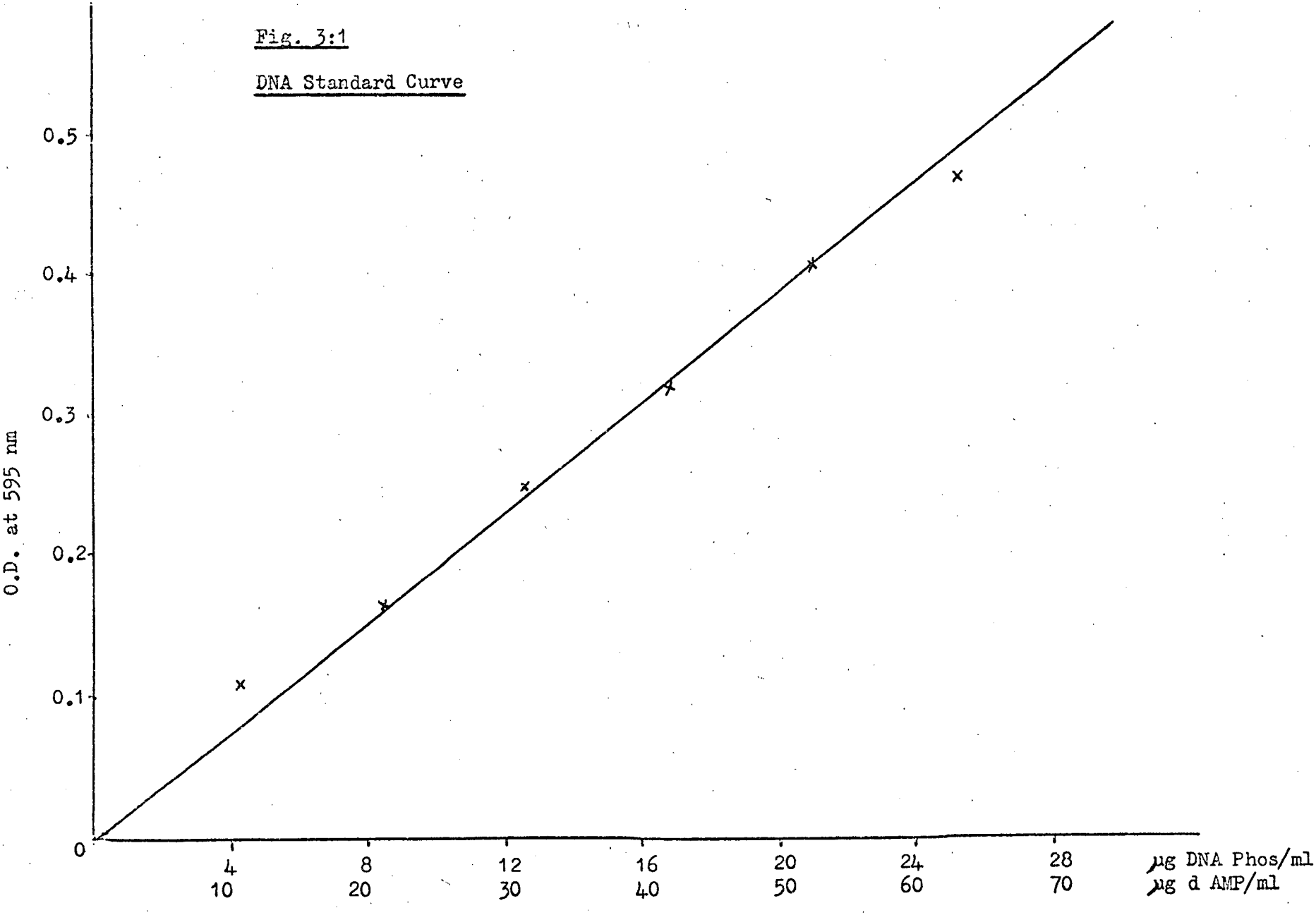
DNA was assayed by a procedure derived from Giles' and Myers' modification[7] of the method of Burton[8]. Analar diphenylamine was recrystallised once from ethanol before use; the diphenylamine reagent (4% recrystallised diphenylamine in glacial acetic acid + 1/40 volume 1.6 mg/ml aqueous acetaldehyde) was freshly-prepared before each assay. The sample, in aqueous solution, was mixed with an equal volume of 1 M perchloric acid and incubated at 70°C for 20 minutes. The incubated mixture was chilled in ice and mixed with an equal volume of glacial acetic acid, then centrifuged to remove any insoluble material. The supernatant was mixed with an equal amount of the diphenylamine reagent and the colour developed for 16-24 hours at 30°C. The optical density at 595 nm was read against a glacial acetic acid blank. Any absorbance at 700 nm was subtracted from this value, thus correcting for light-scatter.

Carbohydrates were found to interfere with the assay. In the presence of sucrose or sorbitol, a pale green colour developed immediately on the addition of the diphenylamine reagent, and no blue colour was formed. Such interfering substances were removed from the samples by dialysis before the assay. Protein and RNA did not interfere.

The concentration of DNA in the original sample was calculated by a method based on that of Schneider[9]:

Fig. 3:1

DNA Standard Curve



$$\mu g \text{ DNA phosphate/ml} = \frac{\text{O.D. at 595 nm}}{0.019}$$

Assuming that in DNA (with G + C content = 50%)

μg DNA/μg DNA phosphate = 325/96,

$$\mu g \text{ DNA/ml} = \frac{\text{O.D. at 595 nm}}{0.019} \times \frac{325}{96}$$

The validity of this procedure is confirmed by the standard curve (fig. 3:1), using d-AMP (Sigma) as the sample material. In DNA of G + C content = 50%, the ratio:-

μg d-AMP/μg DNA = 1/2.6

Thus it can be seen that the DNA concentration in μg/ml is the same whichever method of calculation is employed.

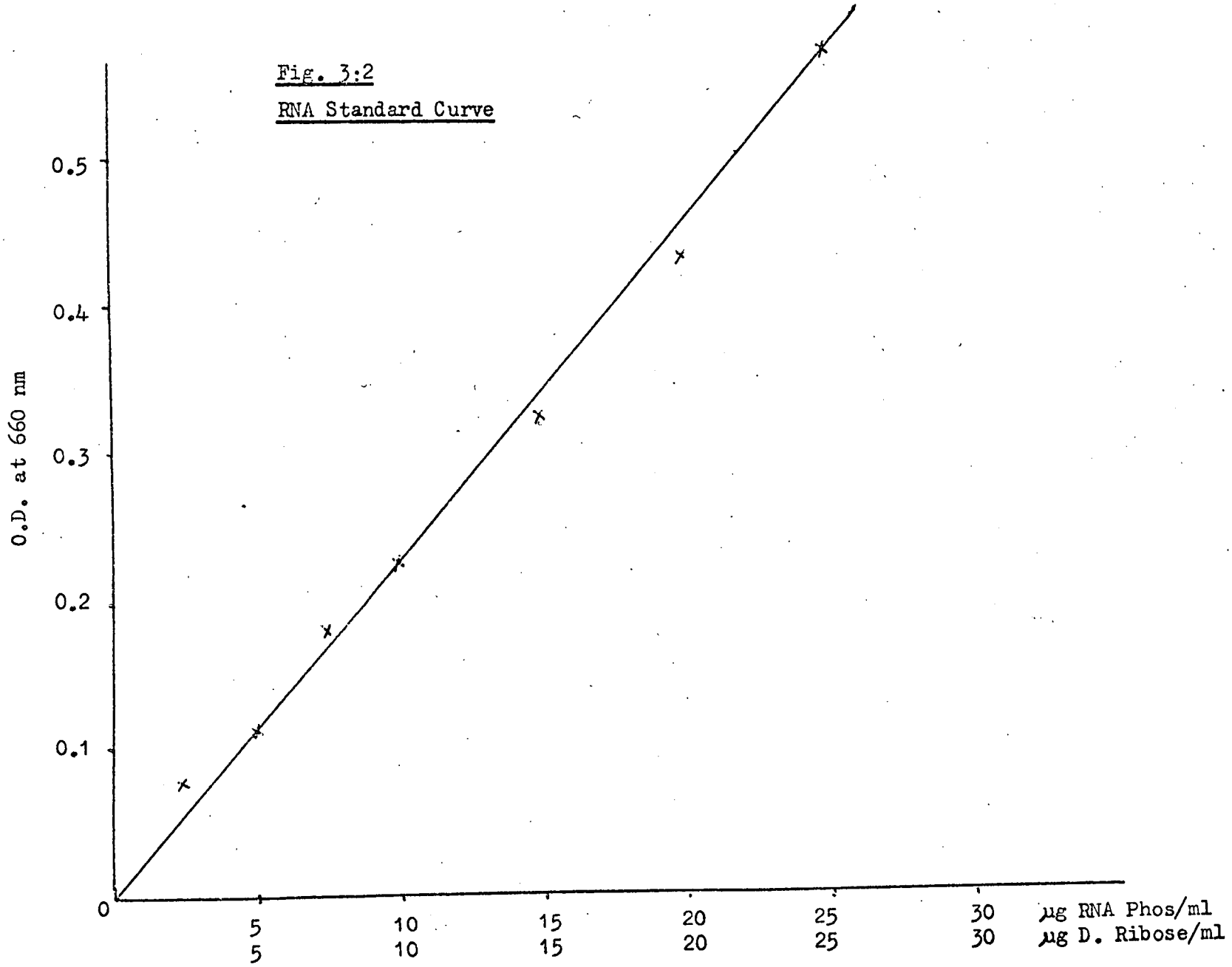
5. RNA ASSAY

RNA was assayed by the method of Schneider[9]. Orcinol (BDH Biochemicals) was recrystallised from toluene before use. The orcinol reagent consisted of 0.5 g orcinol + 0.42 g $FeCl_3$ in 50 mls 12 N HCl. A 70°C perchloric acid extract of the sample was prepared as in the DNA assay, and after chilling in ice and diluting with an equal volume of water was centrifuged briefly to pellet any insoluble material. The supernatant was mixed with an equal volume of orcinol reagent and incubated at 100°C for 20 minutes; it was then cooled in ice-cold water and the optical density at 660 nm read as quickly as possible against a water blank. The blue colour formed in this assay is unstable, and a significant error is introduced if the sample is left for more than 10 minutes before reading. Careful removal of the insoluble material from the PCA digest was important as protein gives a precipitate with the orcinol reagent. Carbohydrates were found to give a slight positive orcinol colour, and were therefore removed from the sample by dialysis before assay.

According to Schneider[9],

Fig. 3:2

RNA Standard Curve



$$\mu g \text{ RNA phosphate}/0.2 \text{ mls} = \frac{\text{O.D. at } 660 + 0.008 - 0.013\ (\mu g \text{ DNA phos.}/0.2 \text{ ml})}{0.116}$$

Assuming that μg RNA/μg RNA phosphate = 322/96,

μg RNA/ml =

$$\frac{E_{660}/\text{ml sample} - 0.013\ (\mu g \text{ DNA phosphate/ml}) + 0.04}{0.116} \times \frac{322}{96}$$

Confirmation of this is obtained by the standard curve (fig. 3:2), using D-ribose as sample, and the assumption that:-

μg D-ribose/μg RNA = 1/2.37

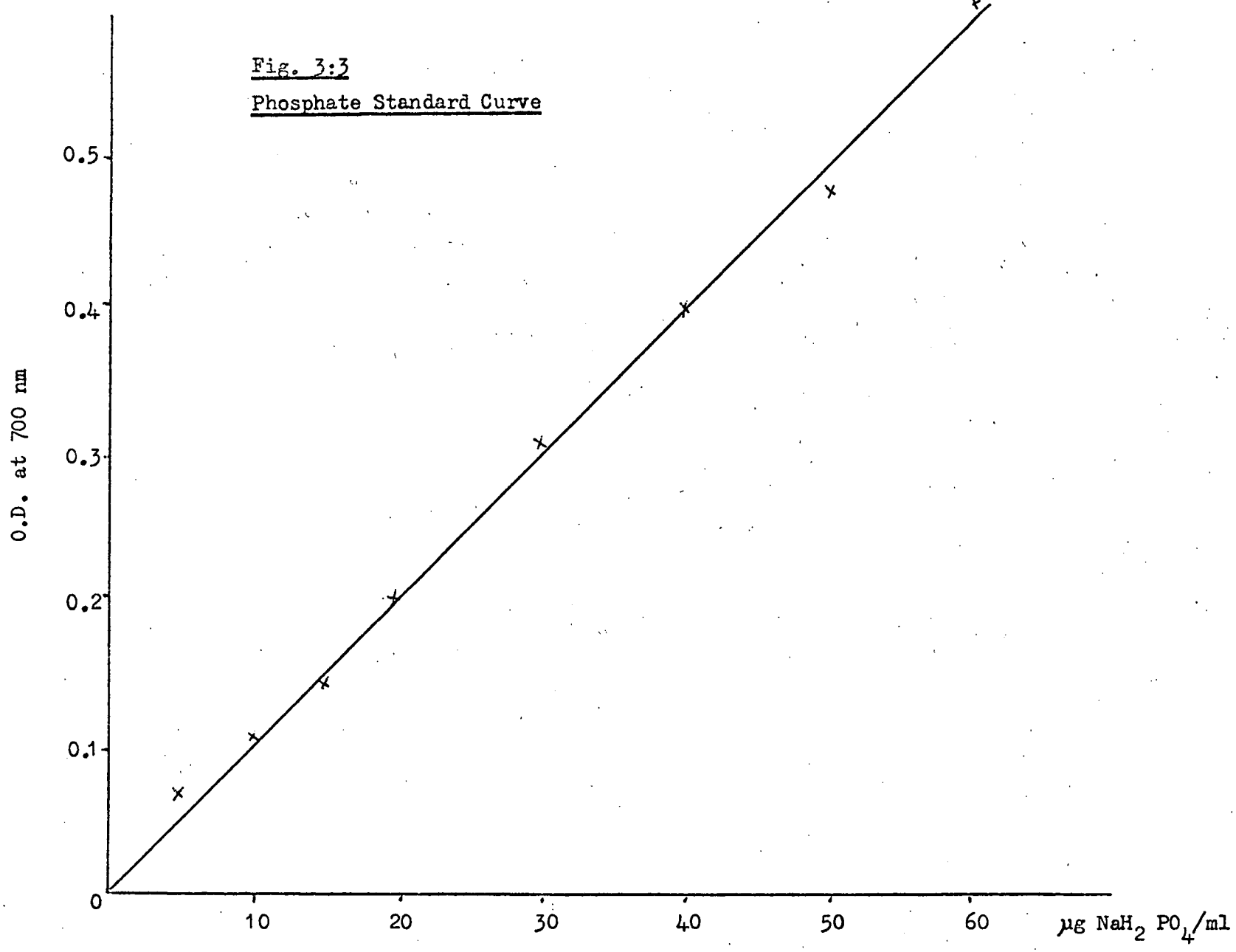
The RNA concentration in μg/ml calculated by the two methods is the same.

6. PHOSPHATE ASSAY

Phosphate was assayed by a procedure based on those of Bartlett[10] and Marinetti[11]. The dry sample was incubated in 0.3 mls 70% PCA for 15 minutes at 145°C. If organic matter persisted after this time, 1 drop 100 volumes Analar H_2O_2 was added and the incubation continued for another 90 minutes, until the solution was cleared. After cooling, 3.75 mls glass distilled water was added and the mixture incubated at 100°C for 20 minutes to cleave any polyphosphates formed during the removal of organic matter. The solution was cooled to room temperature and 0.25 mls 5% ammonium molybdate in water added, followed by 0.1 mls freshly-prepared Fiske-Subbarow reagent. Colour was developed at room temperature for 10-45 minutes and the optical density at 700 nm read against a water blank. The Fiske-Subbarow reagent was prepared as follows:- 80 g sodium metabisulphite was thoroughly mixed by grinding in a mortar with 5.3 g sodium sulphite and 2.64 g aminonaphthosulphonic acid. The mixture was kept dry in a dark glass bottle; under these conditions it was stable. Immediately before use, 0.44 g of the mixture was dissolved in 5 mls water with the aid of gentle warming.

Fig. 3:3

Phosphate Standard Curve



Before each phosphate assay was performed, all the glassware to be used was thoroughly washed in distilled water, to remove all extraneous traces of phosphate. A standard curve using NaH_2PO_3 is shown (fig. 3:3).

7. ESTIMATION OF LIPID

Lipid was extracted by the method of Folch, Lees and Sloane-Stanley[12]. The wet pellet of material was extracted in a blender with 19 volumes 2:1 chloroform-methanol and centrifuged at 3,000 X g for 15 minutes to pellet the non-lipid residue. The upper (aqueous) phase of an 8:4:3 chloroform-methanol-water mixture was used to wash the crude extract, 1 volume of this phase per 5 volumes crude extract being used. This washing was performed twice, then repeated again using the upper phase of an 8:4:3 chloroform-methanol-0.3 M aqueous NaCl mixture. The washed lipid extract was evaporated to dryness. To provide blanks, the same washing procedures were carried out on 2:1 chloroform-methanol.

Total lipid was estimated from a phosphate assay of the dried extract:-

μg phosphate/μg phospholipid = 1/8 (approximately) : M.W. inorganic phosphate = 96-98 ; M.W. phospholipids average about 800.

In nuclei and in membranous material prepared from nuclei, phospholipid accounts for 62-70% of the total lipid (Gurr, Finean and Hawthorne[13]; Keenan et al[14]; Kleinig[15]).

Hence,

$$\mu\text{g phosphate}/\mu\text{g total lipid} = 1/\left(\frac{8}{0.6\text{-}0.7}\right)$$

$$= 1/12 \text{ (approx.)}$$

$\therefore$ μg total lipid = μg phosphate in washed lipid extract X 12.

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CHAPTER FOUR

PRELIMINARY ATTEMPTS TO ISOLATE THE NUCLEAR ENVELOPE

SUMMARY

1. On the basis of the discussion of factors which can modify the state of chromatin (chapter 2) a number of procedures for the disruption of nuclei and the subsequent isolation of the envelope are discussed.

2. It is found that nuclei can be broken by DNase or protease treatment, high ionic strength KCl or $MgCl_2$, dication depletion, sonication in most aqueous media, or high pH ($\geqslant 8.0$) in the absence of free dication.

3. Only two of these procedures, namely sonication in the presence of dication between pH 7.2 and pH 8.0, and low-temperature incubation with EDTA, yield morphologically recognisable envelopes. Reasons for preferring the former procedure, referring largely to criteria of yield, contamination and chemical integrity, are discussed.

4. DNase, potassium and high ionic strength treatments are found to destroy the envelope, causing simultaneous disappearance of the pore complexes, vesicularisation, conversion to the single-membrane form, lowering of DNA content, and a decrease in density.

5. It is suggested that the presence of pore complexes, which determines the morphological integrity of the envelope, depends on the presence of DNA which can be removed by DNase, potassium ion or high ionic strength treatments. It does not primarily depend on either RNA or lipid.

INTRODUCTION

A prerequisite of any method for the isolation of the nuclear envelope is a procedure for disrupting nuclei in such a way that the

morphology of the envelope is not destroyed. Broadly speaking, two approaches to the problem of establishing such a procedure may be adopted:-

(a) The nuclei may be suspended in a medium in which they are stable, and then broken by controlled mechanical shock;

(b) The nuclei may be suspended in a medium in which they are unstable and allowed to disintegrate spontaneously.

The former approach makes use of techniques such as variation in the temperature of the suspension of nuclei, shaking, freezing and thawing, homogenisation, repeated passage through a hypodermic needle, blending and sonication. The latter makes use of procedures by which chromatin can be destabilised (see chapter 2). Various combinations of such techniques can be employed; for each combination, the integrity of any envelope liberated is assessed by electron microscopy. Once the primary criterion of the morphological integrity of the envelope has been satisfied, attention can be given to the separation of the liberated envelope from other nuclear material, and then to problems of yield, contamination, etc. In general, for any procedure attempted, the following points are considered:-

(a) What percentage of the nuclei are broken or emptied, as revealed by phase contrast microscopy?

(b) Can the nuclear envelope be recognised as morphologically intact when liberated from the nuclei?

(c) Can the liberated envelope be recovered, e.g. by differential or density gradient centrifugation and some estimate of the yield be made?

(d) What is the composition of the envelope fraction?

(e) What is the state of the envelope in terms of aggregation, vesicularisation and contamination? (Contamination is defined tentatively as the presence of nucleolar material or organised chromatin fragments as determined by electron

microscopy. Vesicularisation is best avoided as dispersed chromatin may be trapped in pockets of membrane. Aggregation, if extreme, makes contamination as defined above difficult to determine, and prevents reliable determination of the morphological integrity of the bulk of the envelope.)

This chapter summarises an investigation of the range of techniques referred to above, and the results are discussed in the light of the information reviewed in part I.

MICROSCOPY

1. Phase contrast microscopy

Nuclear suspensions after application of some treatment were routinely examined for swelling, breakage and loss of contents under a P20 phase-contrast microscope using a $\frac{1}{12}$ inch oil immersion objective. Concentrations in the region 1-5 mg/ml were used. Liberated nucleoli and dense chromatin fragments appeared as highly refractile bodies. Nuclei appeared to be more or less spherical and were rendered less refractile as their contents were ~~leached~~ leached out or diluted.

2. Electron microscopy

Electron micrographs were taken with an AEI EM 6D, operating at 60 kV with a 50 micron objective aperture. Ilford 3¼ x 3¼ inch plates, Special Lantern Contrasty, were used in the microscope camera.

Negative staining with 2.0% ammonium molybdate at pH 7.2 was carried out as described by Harris and Agutter[1].

Embedding and thin sectioning was carried out according to the method used by Harris and Brown[2], the dehydration of the material involving 1½ h (room temperature) treatment with 10% ethanol, followed by treatments of 5 mins each with 30, 50, 70 and 90% ethanol. The process was completed by 3 X ½ h exposure to absolute ethanol and 2 X 15 mins to epoxypropane prior to embedding in TAAB epoxy-resin.

MECHANICAL DISRUPTION TECHNIQUES

The various procedures for mechanical disruption used in the work described in this chapter were carried out as follows unless otherwise stated:-

Incubation at 5°C was carried out for 16-24 hours in the refrigerator, and at 37°C for 2-3 hours in a water-bath.

Shaking was carried out at 5°C for 16-24 hours.

Freezing and thawing: 1 ml of the suspension was immersed in an acetone-solid CO_2 mixture for 1 minute, after which time it was completely frozen. It was then immersed in a large volume of water at room temperature; 5-10 minutes was required for complete thawing.

Passage through hypodermic needle: 5 mls of suspension were passed into and out of a 10 ml syringe through a no. 6 needle. Six such passages were performed at a mean flow rate of approximately 0.5 mls/sec.

Homogenisation: Vigorous homogenisation was carried out in a Potter-Elvehjem homogeniser with a tight-fitting pestle (0.1 mm clearance) rotated at 3,000 rev/min. Six up-down movements averaging 10 seconds each were used.

Sonication: 5 ml aliquots of the suspension (in 20 ml. capacity tubes) were sonicated for 5 - 90 seconds at 21 kc/sec, 1 μm amplitude, in an M.S.E., ultrasonicator using a 0.5 cm probe placed just below the meniscus.

SUCROSE AND LOW IONIC STRENGTH EFFECTS

In chapter 2, work demonstrating that sucrose and various salts in low concentration had an effect on the swelling of nuclei, and therefore presumably on the stability of chromatin, was reviewed. The work described here was designed to assess the applicability of such effects to the isolation of the nuclear envelope.

Nuclei were suspended at concentrations in the range 1-5 mg protein/ml in the following media:-

(a) Water

(b) Buffers at pH 6.1:-

(i) 0.01 M sodium phosphate
(ii) 0.01 M sodium citrate
(iii) 0.02 M sodium cacodylate
(iv) 0.02 M sodium N-morpholinoethanesulphonate (MES)

The pH of 6.1 was chosen initially because this was the pH of the media used in the isolation of the nuclei.

(c) 0.32 M sucrose in:-

(i) Water
(ii) 0.01 M sodium phosphate buffer, pH 6.1

Various mechanical shock procedures were applied to the nuclei in these media (v. supra).

On low-temperature incubation in these media, the nuclei became somewhat swollen and some partial loss of contents occurred. Both swelling and loss of contents were enhanced by the presence of sucrose but inhibited slightly by the presence of electrolytes. Shaking and increase of incubation temperature did not markedly affect the rate of loss of contents as determined by phase contrast microscopy; nuclei were partially broken but aggregated in all media after freezing and thawing or passage through a hypodermic needle. Vigorous homogenisation and 5 seconds' sonication brought about extensive breakage, but the envelope material released was in the form of an aggregate of minute vesicles, morphologically unrecognisable as envelope and very difficult to separate completely from other nuclear material.

The effects of the buffers on the nuclei could not be distinguished

from each other at constant pH, independent of the mechanical shock procedure applied. As the pH was increased to 7.0-7.5, loss of contents was more marked on prolonged incubation, but envelope fragments were not liberated. Citrate buffer tended at these higher pH values to enhance the release of chromatin material. Sonication, however, for 5 seconds still produced a mass of minute membranous vesicles unrecognisable as nuclear envelope. These findings conflict with those reported by Zbarsky et al[3].

It was concluded that procedures of this type were unsuitable for the isolation of the nuclear envelope.

DICATION EFFECTS

Since dication levels appear to be significant in controlling the stability of chromatin, it was predicted that variation of the dication content of the medium in which the nuclei were suspended might afford possible methods for the isolation of the envelope.

Water, 0.32 M sucrose, 0.01 M sodium phosphate pH 6.1, and 0.32 M sucrose-0.01 M sodium phosphate pH 6.1 were each made:-

(a) 1-5 mM $MgCl_2$,
(b) 2 mM $BaCl_2$,
(c) 1, 10 or 100 mM sodium EDTA, pH 6.1.

higher dication concentrations render the nucleoplasm granular and intractable; very high concentrations appear to disperse nuclei and envelopes. (Cf. p. 160)

Nuclei suspended in these media were then subjected to the range of mechanical shock techniques described above.

Nuclei were found to be stabilised by dications, notably magnesium. No significant loss of contents occurred on incubation at low or high temperature, and considerable mechanical shock was required to produce extensive breakage. Barium did not stabilise the chromatin so effectively as did magnesium, but the loss of contents incurred by barium treatment (Harris and Agutter[1]) was not sufficient to lead to an efficient membrane preparation. Only 5% of the nuclei lost a

Plate 4:1

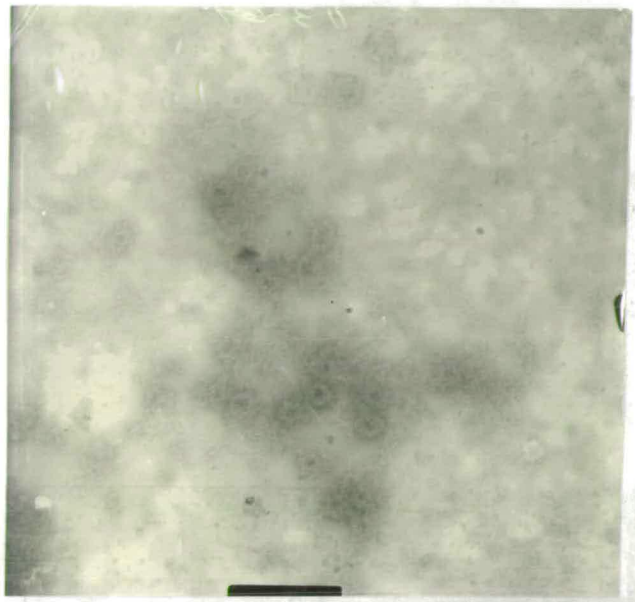
a,b) Envelope fraction from EDTA-lysed nuclei, negatively stained with ammonium molybdate as described in text.

a) Magnification X 25,000

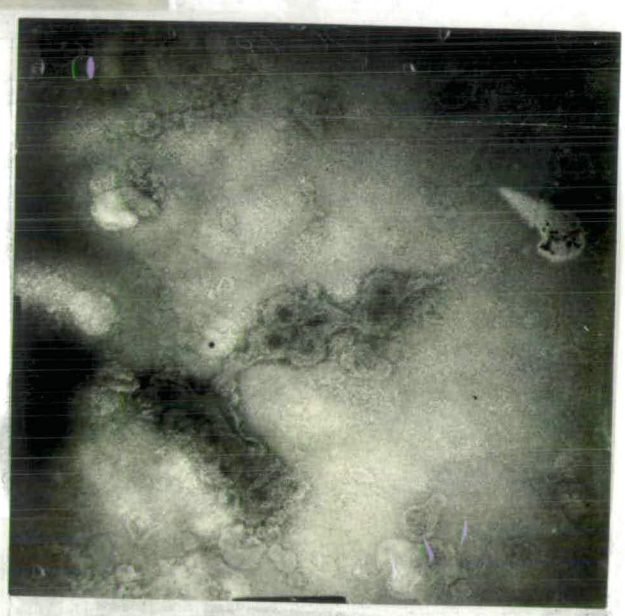
b) Magnification X 40,000

c) EDTA "ghost", embedded and sectioned as described in text. Post-stained with uranyl acetate. Magnification X 10,000.

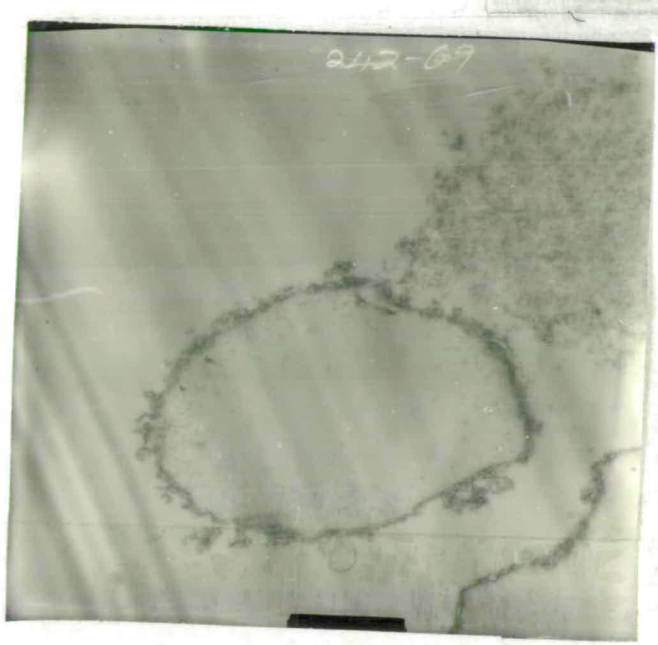
Plate 4:1



a



b



c

significant portion of their contents, and the clarity of the electron micrographs suggests that the barium may have fixed the remaining protein.

Removal of dications by EDTA was sufficient to cause marked swelling and loss of contents of the nuclei in 16-24 hours at 5°C. Homogenisation, passage through a hypodermic needle and even vigorous shaking of the resulting "nuclear ghosts", however, caused fragmentation and extensive aggregation. Recognisable nuclear envelope was visible after treatment with 1mM or 10 mM, but not 100 mM, EDTA. The presence of sucrose enhanced the loss of contents; swelling and loss of contents were inhibited by electrolytes to a small extent. Despite the acceptable appearance of the envelope surrounding the ghosts, two criticisms of the EDTA technique as the basis of an isolation procedure were evident:

(a) Attempts to separate the "ghosts" from other material by density gradient or differential centrifugation caused aggregation of the envelope both with itself and with other solid material of nuclear origin. This aggregation was essentially irreversible. The "ghosts", too, were fragile and fragmented readily.

(b) Despite the morphological integrity of the envelope, the diameter of the "ghosts" was on average some three times that of the nuclei. Gross structural changes must therefore have occurred in the envelope, involving a tenfold increase in surface area.

In addition, EDTA has been shown to solubilise much of the protein of other membrane systems (Marchesi and Steers[4], Rosenthal et al[5]); hence components of the envelope may have been lost in the isolation procedure.

Sonication in the presence of magnesium at pH 6.1 liberated

membrane material in the form of flat sheets, but the material appeared highly contaminated with chromatin and no pore complexes were visible.

The effect of pH variation on these two procedures - low-temperature incubation in the presence of EDTA and sonication in the presence of magnesium - was examined.

pH EFFECTS

The pH of the medium in which the nuclei were suspended was made:-

(a) 2.8-3.0 with 1.5% acetic acid;

(b) 4.0 with 0.02 M sodium acetate buffer;

(c) 7.0, 7.5 or 8.0 with 0.01 M sodium phosphate or 0.02 M tris-HCl buffer.

In each case, the medium was made 1 or 5 mM $MgCl_2$ and sonicated until 90% of the nuclei were broken, or was made 10 mM EDTA at the pH of the buffer and incubated at 5°C for 16-24 hours.

EDTA ghost formation was markedly inhibited by treatment of the nuclei at pH 4.0 (which is close to the isoelectric point of nuclei, cf. Vassar et al[6]) and completely inhibited by pretreatment at pH 2.8-3.0 (which appears to precipitate the chromatin; considerable granularisation of the nucleoplasm is visible under phase-contrast). Even after re-adjustment of the pH to 6.1, EDTA did not induce ghost formation after these treatments.

Increase of pH and increase of temperature appeared to destabilise the ghosts. Instead of ghosts, envelope fragments, still morphologically intact, formed in EDTA at pH 7.0 or 7.5. At 37°C such fragments were formed at pH 6.5. At pH 8.0, independent of the temperature, an immediate rupturing of all nuclei occurred in EDTA, the released chromatin being precipitated as a clear gel which could

not be dispersed without prolonged DNase treatment. Envelope fragments embedded in the gel were of uncertain morphology, since electron microscopy of the gel proved impossible.

Sonication in 1 mM $MgCl_2$ yields envelope fragments. If the pH is below 7.2, however, pore complexes cannot be seen, and marked chromatin contamination is evident (as at pH 6.1; v. supra). At pH 8.5, though no gel of released chromatin forms in the absence of magnesium, no envelope fragments can be identified after sonication and only minute vesicles of membrane remain. This observation suggests that use of pH values greater than 8.0 tends to incur envelope decomposition. In the absence of magnesium, though at higher pH values the minute membranous vesicles released by sonication are less aggregated, they are also very much smaller than at lower pH values.

It is concluded that sonication in the presence of 1 mM $MgCl_2$ at pH 7.2-8.0 releases morphologically intact envelope fragments.

The possibility of separating these fragments from other material, and of isolating the EDTA ghost material, was next examined.

ENVELOPE ISOLATION

1. Differential centrifugation

No method was found whereby a reproducibly satisfactory yield of envelope material could be separated from other nuclear material by differential centrifugation of the EDTA ghost suspensions.

An envelope-rich fraction was, however, isolated from the sonicated nuclei by centrifugation at 30,000 X g for 30 minutes after pelleting nucleoli, unbroken nuclei and dense chromatin at 3,000 X g for 10 minutes.

2. Sucrose gradients

Both the envelope-rich material isolated from sonicated nuclei by differential centrifugation and the EDTA nuclear ghosts were pelleted through a 10-55% (w/v) sucrose gradient after 16 hrs centrifugation at 50,000 X g (average), irrespective of pH or EDTA content, unless the ionic strength was high or KCl was added to more than 0.02 M concentration. On 30-85% (w/v) sucrose gradients, however, though EDTA ghost material pelleted as with the less dense gradient, after 64 hrs at 50,000 X g (average), the envelope-rich fraction from sonicated nuclei gave a band of apparent density 1.27 g/ml except:-

(a) at pH 6.1,

(b) in 0.02 M tris-HCl buffer at pH 7.5.

In these two cases, pellets again formed. These observations, for which the explanation is not immediately obvious, will be more fully discussed in chapter 6.

Addition of KCl, 0.04 M, to the 10-55% (w/v) sucrose gradients, either unbuffered or buffered at pH 7.5, led to the formation of bands of apparent density 1.22 g/ml when the envelope-rich fraction from sonicated nuclei was applied to the gradients. After removal of the band material and exhaustive dialysis against water or 0.02 M tris-HCl-0.04 M KCl pH 7.5, no envelope material was visible in the electron microscope. Instead, the material was seen to consist of a mass of small, apparently single-membrane vesicles without pore complexes. NaCl did not mimic the effect of KCl at comparable concentrations. It was tentatively concluded that potassium ions exert a specific disrupting effect on the nuclear envelope in isolation.

Sonication or homogenisation of nuclei in media containing 0.02 M KCl releases similar single-membrane vesicles without pore

complexes; these vesicles give bands of apparent density 1.22 g/ml on sucrose gradients. Again, NaCl at similar concentrations does not have this effect; it does not appear significantly to reduce the yield of morphologically intact envelope.

The compositions of the KCl gradient band of density 1.22 g/ml, the no-KCl band (morphologically intact envelopes) of density 1.27 g/ml, the EDTA nuclear ghosts, and nuclei, are compared in table 4:1. The techniques used in the assays were those described in chapter 3.

Table 4:1 Composition of fractions

Material	µg DNA/Lowry Unit	µg Lipid/Lowry Unit
Nuclei	100	45
EDTA ghosts	76	36
1.27 g/ml band	76	210
1.22 g/ml band	61	290

The EDTA ghosts, while showing a maintenance of high DNA/protein ratio, manifest no purification of lipid. This may imply that in part at least the stability of the lipid components of the system is maintained by dications. It is also noteworthy that the RNA content of the EDTA-ghosts (4 µg/Lowry units) is much less than that of the sonicated envelopes (40-45 µg/Lowry unit), an observation consistent with the loss of ribosomes which occurs on EDTA treatment. However, the recovery of protein from the nuclei in terms of Lowry colour formation is comparable in the EDTA ghosts and the sonicated nuclei. These results constitute further criticisms of the EDTA ghost method of nuclear envelope preparation, since both ribosomes and lipids are to be found in the envelope (v. Part I). However, as the pore complexes are visible in the EDTA ghosts, this implies that the pore

complexes are not structurally dependent on either RNA or lipid.

The reduced DNA content of the membranous material recovered from the KCl gradients is consistent with the lower density of this material.

It is concluded from the foregoing that sonication in the presence of magnesium, at a pH in the range 7.2-8.0, is the most useful technique by which nuclear envelopes can be isolated. The addition of potassium ions at any stage in the procedure appears to prevent the isolation of acceptable envelopes.

OTHER ATTEMPTS TO ISOLATE THE ENVELOPE

In addition to the techniques described above, the discussion in chapter 2 also suggested that envelopesmight be isolated from nuclei by destabilising the chromatin with:-

(a) high ionic strength media, or

(b) treatment with various enzymes.

Nuclei are dispersed by suspension in media of high ionic strength, e.g. 0.5 M $MgCl_2$ (cf. Berezney, Funk and Crane[7]) and 1 M KCl. From the dispersed material, which has the form of a fairly clear viscous solution, membranous fractions can be recovered by differential centrifugation, pelleting at 30,000 or 40,000 X g in 30 minutes. These fractions appear in the electron microscope as minute vesicles lacking pore complexes.

The envelope-rich fraction prepared by differential centrifugation of sonicated nuclei (v. supra) is dispersed by 1 M KCl or by 10% (w/v) tripotassium citrate (pH = 7.5) (Kashnig and Kaspar[8]). In the membranous pellet recovered on centrifugation of the dispersed material at 30,000 X g, the morphology has again been destroyed; pore complexes are lacking, and the remaining single-membrane material is

vesicularised and aggregated. If the fraction dispersed in such a high ionic strength medium is placed directly on a 10-55% (w/v) sucrose gradient, most of the dispersed material is precipitated in the form of an intractable aggregate, unless the gradient is itself made 1 M KCl or 10% (w/v) tripotassium citrate. In this case, after 16 hours centrifugation at 50,000 X g (average), a band of apparent density 1.19 g/ml or less is formed. After dialysis to remove the salt and sucrose, electron microscopy showed this band to contain only an aggregate of morphologically unrecognisable membrane vesicles.

In the procedure described by Kashnig and Kaspar[8], membranous material was recovered from a high ionic strength sucrose gradient to which pellet from differential centrifugation had been applied. Besides the membranous band, these gradients also contain a large amount of dissolved material, and a pellet. The pellet contains nucleoli, dense chromatin, and some almost intact nuclei together with morphologically acceptable envelope material, rich in pore complexes. This pellet is, however, somewhat aggregated, and recovery of the envelope from it would be very difficult.

It is concluded that high ionic strength treatment destroys pore complexes and thus leads to morphological disruption of the envelope. Any satisfactory isolation procedure for the envelope must therefore not employ high ionic strength treatment.

RNase (British Drug Houses) added in 10 µg/mg protein concentration to a suspension of nuclei in 0.02 M sodium phosphate buffer at pH 6.1 or pH 7.5 had little visible effect after brief incubation at room temperature. Prolonged incubation at pH 7.5, however, led to the disruption of many nuclei; much loose debris of chromatin and nucleolar material was released, along with some envelope

sheets and fragments. However, it was not possible to separate the envelope sheets from other material without subsequent sonication or vigorous homogenisation, followed by differential centrifugation. The enzyme had little or no visible effect on sonicated nuclei; in some cases the outer rims of the pore annuli became a little more clearly defined after incubation for 1 hour. Nuclei pretreated at pH 4.0 are not visibly affected by RNase even after 90 minutes' incubation at room temperature.

Trypsin (British Drug Houses) in 10 μg/mg protein concentration disrupted nuclei in 0.02 M sodium phosphate buffer, pH 7.5, completely within 20 minutes. No pore complexes were visible in the membranous parts of the remaining highly fragmented material.

Nuclei in 0.02 M sodium phosphate buffer, pH 7.5, treated with 10 μg/mg protein concentration DNase I (British Drug Houses) in the presence of 10^{-4} M $MgCl_2$ were completely disrupted after 1 hour at room temperature. (These conditions were used for all the DNase digestions described in this chapter.) Again, no pore complexes were visible in the fragments remaining after this time. Brief (15-20 minute) incubation with this concentration of enzyme had no visible effect on the nuclei. Sonication after such brief treatment, however, yielded free membranous vesicles with no pore complexes. Other mechanical disruption techniques fragmented a small percentage of these partially digested nuclei. Vigorous homogenisation or passage through a hypodermic needle, for example, released amorphous membrane vesicles similar to those described above. Partially DNase digested, sonicated nuclei gave both a band and a pellet on a 10-55% (w/v) sucrose gradient centrifuged at 50,000 X g average after 16 hours, whether or not 0.04 M KCl was present in the gradient. The

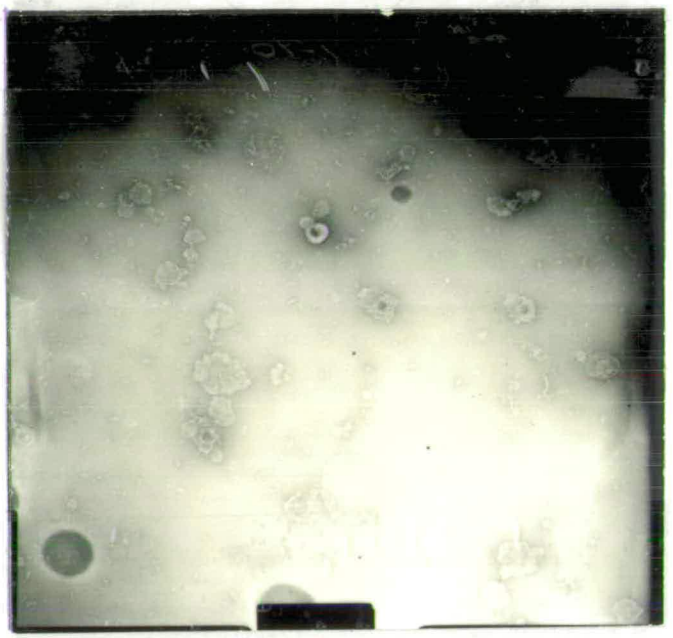
band, of apparent density 1.22 g/ml, contained only minute apparently single-membrane vesicles with no pore complexes. In the pellet, however, pore complexes were visible, but the envelopes were intractably aggregated with other nuclear material.

From these findings it is concluded that RNase, while not destroying the pore complexes, cannot usefully be employed in the isolation of the nuclear envelope. Trypsin and DNase both destroy the envelope completely.

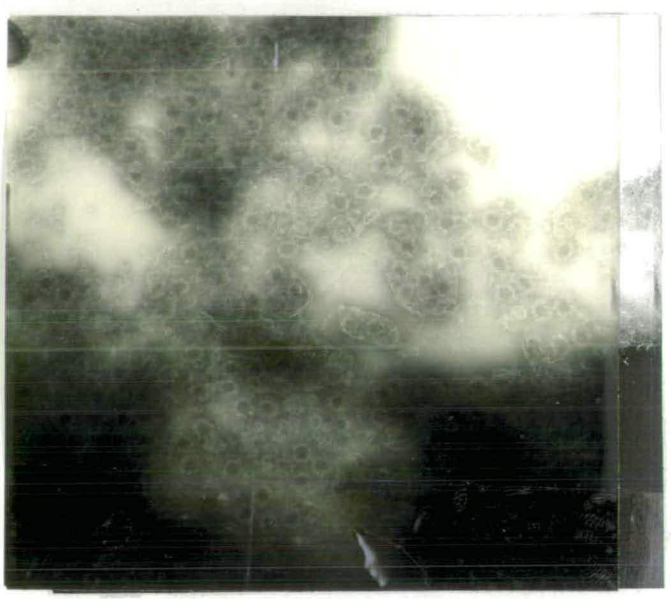
Further evidence for the destructive effect of DNase on the envelope was obtained by observing the effect of DNase I (under the conditions described above) on the envelope-rich fraction obtained from sonicated nuclei, and the effect of DNase II (Worthington Biochemical Corporation) on EDTA ghosts in 0.02 M sodium phosphate buffer-10^{-4} M EDTA pH 7.5 at a concentration of 10 µg enzyme/mg protein. In both cases the morphology of the envelope was completely disrupted after 15-20 minutes' incubation at room temperature; no pore complexes remained, and the membrane was in the form of minute vesicles.

The DNase I was shown to be protease and lipase free by the following procedure: erythrocyte ghosts were incubated with 0, 10, 20 and 40 ug/mg protein DNase I for 1 hour. At the end of this time, the suspensions were centrifuged at 40,000 X g for 30 minutes to pellet the membranes, and the E_{280} of the supernatants was measured, using a water blank. The differences in E_{280} between the four supernatants were found to be attributable entirely to the enzyme concentration. Consequently, the enzyme catalysed no measurable release of 280 nm-absorbing or scattering material from the nucleic-acid-free membranes. It was concluded that DNase I was lipase and protease free.

Plate 4:2



a



b

The conclusion is therefore that the enzymic hydrolysis of DNA associated with the nuclear envelope brings about the morphological destruction of the envelope. This is consistent with earlier results (v. table 4:1) which showed that, when the envelope morphology had been destroyed by potassium ion treatment, there was a reduction of the envelope DNA content and a concommitant reduction of density. From this comparison of the potassium ion and DNase effects on the nuclear envelope, two questions arise:-

a) Are the effects on DNase mimicked by inactivated DNase, or by the products of DNase digestion of chromatin?

b) Are the effects of DNase and KCl additive?

To answer the first question, the envelope-rich fraction prepared from sonicated nuclei by differential centrifugation was suspended in 0.02 M sodium phosphate, pH 7.5, containing 1 mM EDTA. The EDTA alone was found not to destroy the envelope morphology after incubation for 2 hours at room temperature. DNaseI (10 µg/mg protein) in this medium was inactivated by the EDTA, and had no effect on the envelope morphology after incubation at room temperature for 90 minutes. Heterochromatin prepared by the method of Frenster et al[9] was incubated at room temperature for 4 hours with 10 µg/mg protein DNase I. After centrifugation at 20,000 X g the supernatant was made 1 mM EDTA. This clear suspension had no effect on envelope morphology after incubation for 90 minutes at room temperature. It was concluded that the destructive effect of DNase on envelope morphology was mimicked neither by inactive DNase nor by the products of DNase digestion of chromatin.

To answer the second question, the envelope-rich fraction described above was submitted to the following treatments:-

a) Digestion with DNase I under the conditions described above,

followed by centrifugation on a 10-55% (w/v) sucrose gradient, ion-free, at 50,000 X g (average) for 16 hours. The band of membranous material was analysed for DNA, protein and lipid as described in chapter 3.

b) The same experiment was performed, this time using a 10-55% (w/v) sucrose gradient containing 0.04 M KCl.

c) The envelope-rich fraction was run on a similar 10-55% (w/v) sucrose gradient containing 0.04 M KCl, but without DNase digestion.

The band materials from these three types of gradient are referred to respectively as (a), (b), (c) in table 4:2 which summarises the results.

Table 4:2 Compositions of membranous fractions

Material	µg DNA/Lowry Unit	µg Lipid/Lowry Unit
Nuclei	100	45
Envelope-rich frac.	76	210
Band "a"	58	240
Band "b"	63	222
Band "c"	61	290

The effects of KCl and DNase on the composition of the nuclear envelope appear, therefore, to be comparable but not additive. It may tentatively be inferred that in molecular terms their effects are the same - that is, primarily a removal of DNA from the system.

If the removal of DNA from the system is always accompanied by morphological disruption, and vice-versa, as appears to be the case, then it must be concluded that DNA is an essential structural component of the system.

Further supporting evidence for this conclusion (which will be considered in greater detail in chapters 5 and 6, when the critical

factors in the isolation and stability of the envelope are discussed) is provided by the composition of the membranous band material found in high ionic strength (1 M KCl and 10% (w/v) tripotassium citrate) sucrose gradients. The band material in the tripotassium citrate-sucrose gradients is not, as Kashnig and Kaspar[8] claimed, entirely free of DNA. The assay procedure used here (see chapter 3) is more sensitive than that of Bartlett[10] which was used by Kashnig and Kaspar, and detects the low level of DNA present in the band. The results are summarised in table 4:3.

Table 4:3 Composition of high salt degraded envelopes

Material	µg DNA/Lowry Unit	µg Lipid/Lowry Unit
1 M KCl band	16	125
10% K_3 citrate band	14	128

It can be seen that the DNA content of the material has been drastically reduced, and the lipid content significantly reduced, by the high ionic strength treatment. The effectiveness of high electrolyte concentrations in reducing the DNA level in the envelope suggests that the association of the DNA with the envelope is essentially electrostatically mediated.

In this chapter it has been established that the most promising procedure for the isolation of the envelope is sonication in 1mM magnesium at pH 7.2-8.0. More precise definition of the appropriate conditions of isolation, and the effect of varying these conditions, will be discussed in chapter 5.

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CHAPTER FIVE

THE ISOLATION OF THE ENVELOPE: CRITICAL CONDITIONS FOR AN ACCEPTABLE PREPARATION

SUMMARY

1. A standard procedure for the isolation of nuclear envelopes, involving sonication of the nuclei in the presence of magnesium, differential centrifugation and purification on a sorbitol gradient, is established.

2. The effect of varying the conditions of this procedure (sonication conditions, concentration of nuclei, concentration and pH of buffer, cation concentration) is examined in terms of the extent of nuclear breakage, morphology of the envelope, and composition and yield of the envelope.

3. Morphological destruction of the envelope is brought about by the inclusion of potassium in the media, but not by the inclusion of sodium. This "potassium effect" is discussed.

4. The envelopes are found to contain DNA; the implications of this finding are discussed.

INTRODUCTION

In the previous chapter it was established that the most promising method for obtaining a crude nuclear envelope preparation was controlled sonication in dilute, slightly alkaline buffer in the presence of traces of magnesium, followed by differential centrifugation. In the present chapter, the precise conditions of sonication, pH, ionic strength, cation concentration and osmotic pressure necessary for the isolation of an acceptable envelope fraction (according to the criteria mentioned in part I) will be defined in the account of a standard preparation procedure. The effect of variation of these conditions, which must be studied in detail because of the logical importance of isolation (see

part I), will be examined under the following headings: extent of breakage of nuclei, as determined by phase contrast microscopy; morphology of the envelope, as determined by negative staining in the electron microscope; composition of the envelope; relative yield of envelope as the conditions are varied. The results will again be discussed in the light of possible structural interactions between the nuclear envelope and other parts of the cell, and of the observed behaviour of the envelope under various conditions. The possibility of further purifying the fraction by density gradient centrifugation will be examined.

STANDARD PREPARATION PROCEDURE

Nuclei isolated by the procedure described in chapter 3 were suspended in 0.02 M tris-HCl, pH 7.7, containing 1 mM $MgCl_2$, to a concentration of approximately 5 mg protein/ml. All subsequent operations were performed at 0°C. A 5 ml aliquot of the suspension was sonicated at 1 μm amplitude, 21 kc/sec, for 2 X 20 seconds in an MSE ultrasonicator using a 0.5 cm probe, inserted just below the meniscus. The sonicated suspension was diluted with an equal volume of ice-cold water, mixed thoroughly, and shaken gently for 10-15 minutes, then centrifuged at 3000 X g for 10 minutes. The supernatant was carefully removed and centrifuged at 30,000 X g for 30 minutes; the pellet from this was rinsed with and resuspended in distilled water, with gentle passage through a hypodermic needle. It represented the crude nuclear envelope preparation.

The suspension was layered on a continuous ion-free 0-5.0 M sorbitol gradient, or a step sorbitol gradient, 0:1.0:2.0:3.0:4.0:4.5:5.0 M, and centrifuged in a Spinco SW25 or SW39 rotor at an average of 50,000 X g for 16 hours. The band of purified nuclear envelope material, and other fractions of the gradient, were examined.

Extent of breakage of nuclei

Some 90% of the nuclei were completely fragmented; very few appeared completely intact, and in these the chromatin remained non-granular. A few aggregates, rather fibrous in appearance, were visible. There was no detectable change in light-scatter at 550 nm after the full 40 seconds' sonication.

Envelope morphology

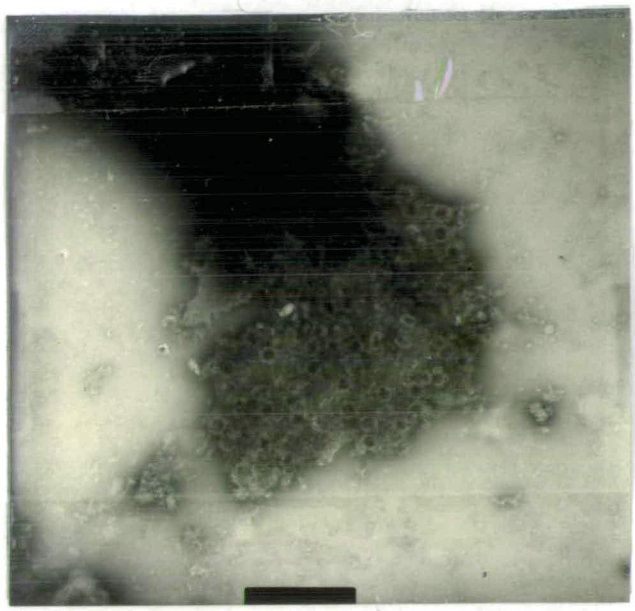
The envelope could be seen in the electron microscope to be in fragments of various sizes, containing small scattered pieces of chromatin but nothing resembling nucleolar material; only a small portion of the material was vesicularised, and the recovery of pore complexes approximated to the required 20-30% of the total envelope surface area.

Composition of envelope

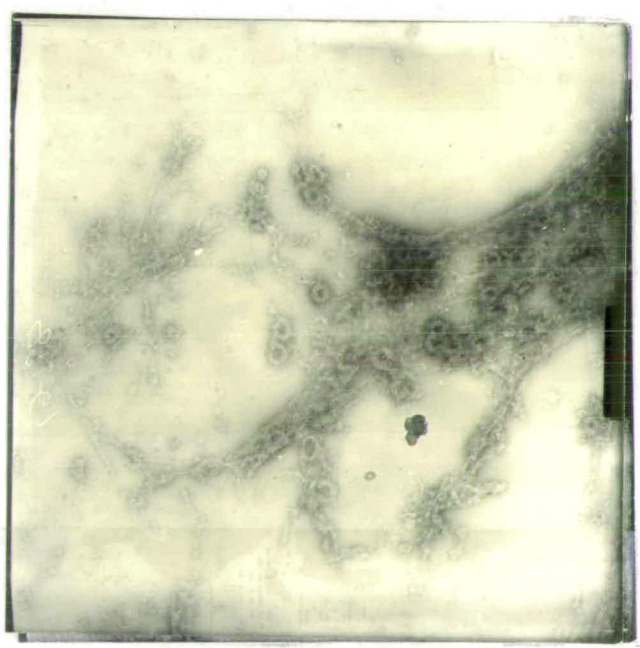
The values quoted below (table 5:1(a)) for the results of Lowry, diphenylamine, orcinol and lipid phosphate assays (see chapter 3) represent the means of three optical density readings for the colour reactions concerned; the values are subsequently converted to µg protein DNA, RNA and lipid as described below.

The position of the purified envelope band on the sorbitol gradient corresponded to a density of 1.27 g/ml after 16 hours' centrifugation. Extension of the centrifugation time to 64 hours did not alter the position of this band, which was therefore concluded to represent the equilibrium position of the envelope on the gradient. The band material was dialysed for 24 hours against a large volume of distilled water (2 changes), and assayed along with four other fractions from the gradient (see fig. 5:1). When dialysed band material was layered on a similar sorbitol gradient and re-centrifuged for 16 hours, it formed a sharp band again with density 1.27 g/ml.

Plate 5:1



a



b

Fig. 5:1 First Sorbitol Gradient

Scan at 280 nm of crude envelopes centrifuged to equilibrium on a 0.0-5.0 M sorbitol gradient (ion-free). The envelope band lies between densities 1.25 and 1.27 g/ml; the symbols I, II, III and IV refer to the fractions recovered from the gradient and assayed separately for protein, DNA and lipid phosphate (see text).

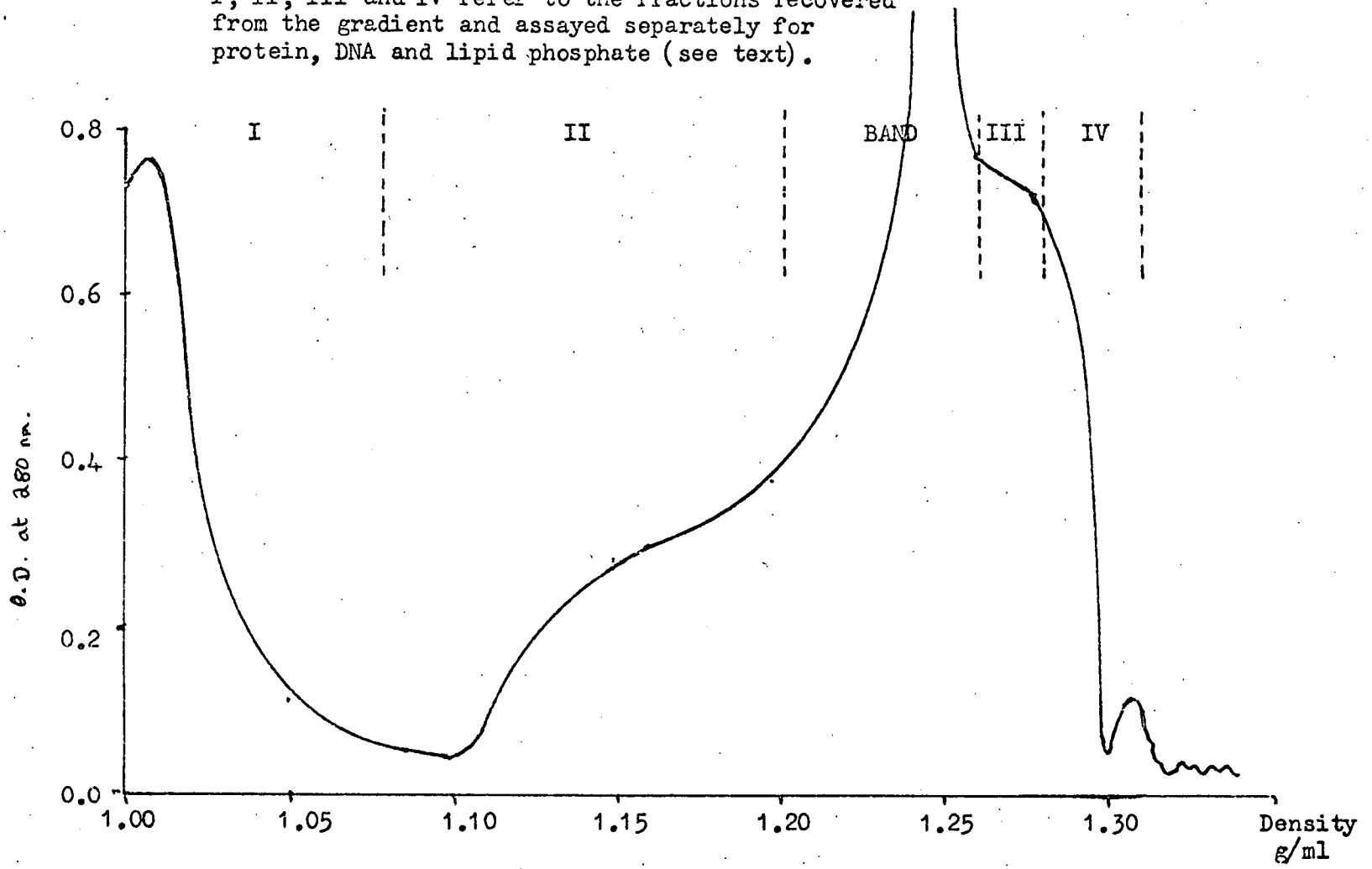


Fig. 5:2 Second Sorbitol Gradient

Scan at 280 nm of the original envelope band centrifuged to equilibrium on another 0.0-5.0 ion-free sorbitol gradient. A sharp envelope band lies at density 1.27 g/ml. Comparison with fig. 5:1 shows that, in this second gradient, material is greatly depleted in all fractions other than the envelope band, fractions II and III being more or less completelyabolished.

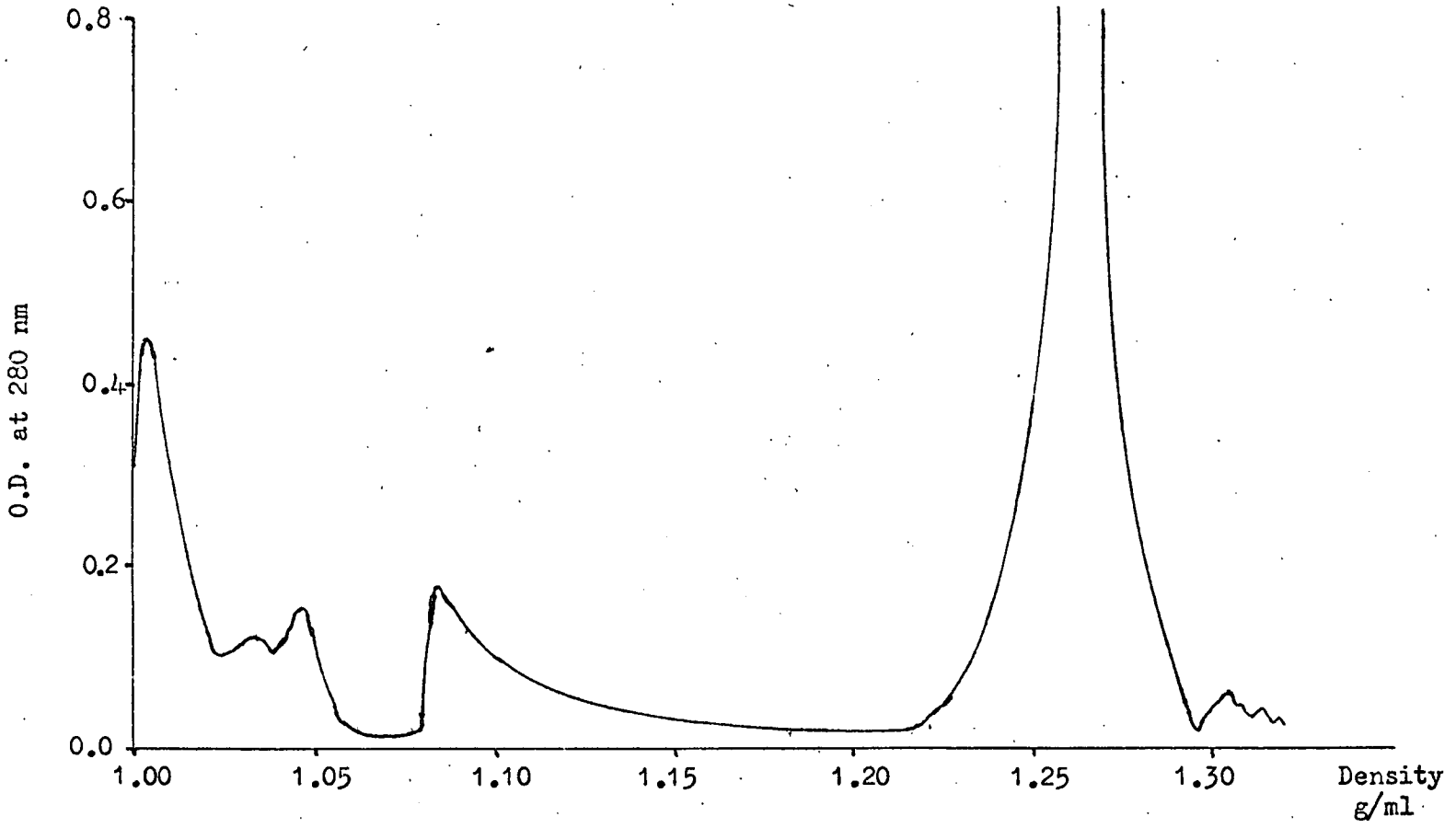


Plate 5:2

Nuclear envelopes from standard isolation procedure, fixed with 2 mM $BaCl_2$, postfixed with OsO_4, embedded in Araldite, and stained with magnesium uranyl acetate and lead citrate. Sectioned using an LKB ultratome III (diamond knife) and examined in Philips EM 300 at 80 kV using a 30 µm objective aperture.

a) Magnification X 11,500

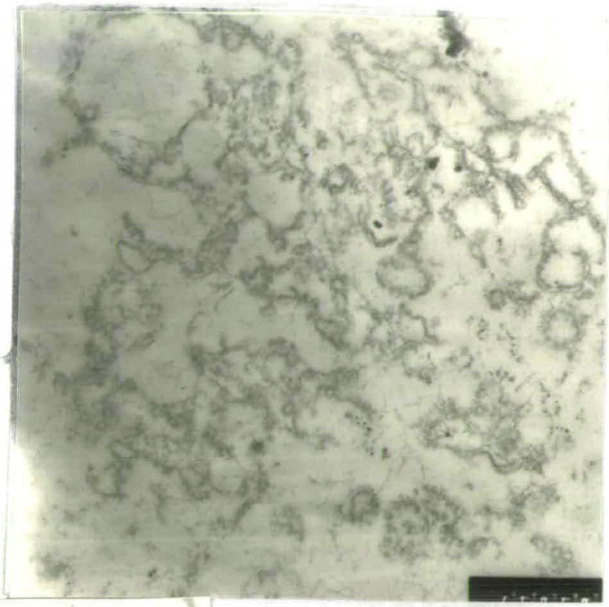
b) Magnification X 18,000

c) Magnification X 52,000

Photographs courtesy Dr. A. Everidd.

Plate 5:2

a



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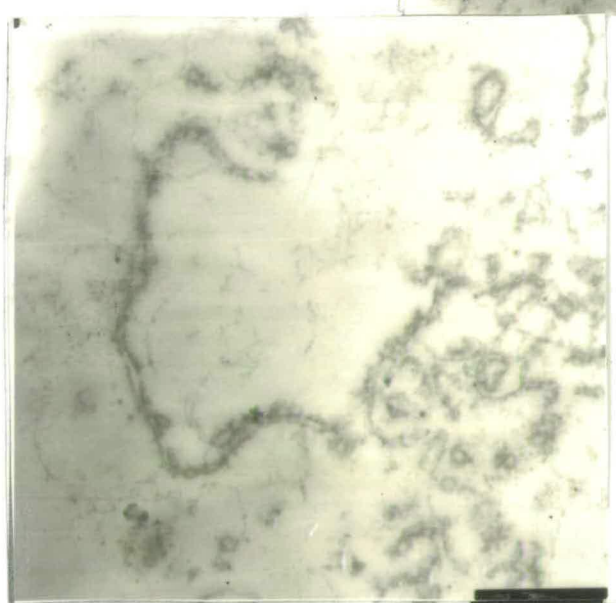


c

In this second gradient, however (fig. 5:2), significantly less material of low density was recovered. A second gradient was not therefore routinely used in the standard procedure.

Table 5:1(a)

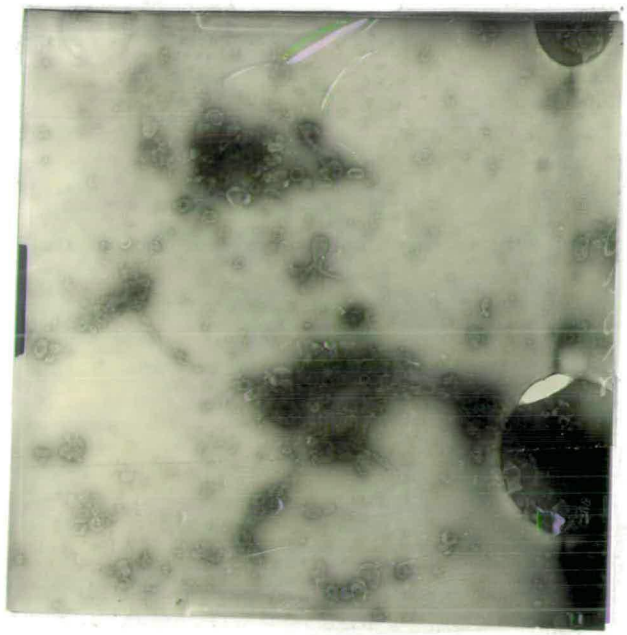
Material	Lipid P	DPN	Orcinol	Lowry
Crude envelopes	.16	.43	.40	.24
Purified envelopes	.025	.06	.08	.13
Fraction I	.075	.015	N.D.	.09
Fraction II	.02	.065	"	.12
Fraction III	Zero	.028	"	.052
Fraction IV	.005	.115	"	.16

N.B. In this table and hereinafter, "DPN" stands for "Diphenylamine".
N.D. = "Not Determined".

Aliquots of envelope suspension were sonicated at 4 μm amplitude for 60 seconds in the MSE sonicator and DNase I and RNase (10 μg enzyme/mg protein) added. After incubation for 1 hour at room temperature with gentle shaking the sonication was repeated. The whole procedure was repeated three times, and the material was then dialysed for 24 hours against 2 changes of distilled water. The dialysed suspension was freeze-dried in weighed tubes, thoroughly extracted with 2:1 chloroform-methanol, and dried again. The mass of the protein residue was compared with the mass of a dried aliquot of untreated envelopes, and the concentration of protein in the original aliquot, the Lowry coefficient (Lowry Units/mg protein/ml) and the percentage by weight of protein in the original envelopes calculated. The percentage by weight DNA, RNA and lipid were calculated from the assays as described in chapter 3. The Lowry coefficient was found to be 1.62 Lowry units/mg/ml protein (mean of 4 experiments).

In table 5:1(b), this Lowry coefficient is assumed to be valid

Plate 5:3



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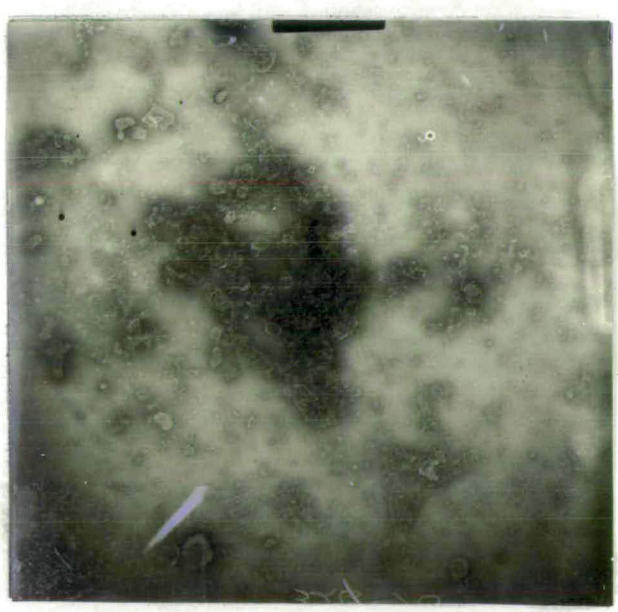


b

for the protein in all fractions from the gradient. It is further assumed (from the work of Gurr, Finean and Hawthorne[1], Keenan et al[2] and Kleinig[3]) that 70% of the lipid in all fractions is phospholipid.

Table 5:1(b)

Material	% by weight:- Lipid	DNA	RNA	Protein
Crude envelopes	20.5	8	4.5	67
Purified envelopes	23	8	5	64
Fraction I	62	2.5	N.D.	35.5
Fraction II	23	10	"	67
Fraction III	0	14	"	86
Fraction IV	5	16	"	79

N.D. = "Not Determined".

Recoveries of DNA, RNA, Lipid and Protein in envelopes

The recoveries of DNA (in terms of diphenylamine colour), RNA (in terms of orcinol colour), lipid (in terms of phosphate) and protein (in terms of Lowry) were calculated for the crude and purified envelopes, and also for the 3000 X g pellet and the 30,000 X g supernatant of the differential centrifugation. The figures presented in table 5:2 represent the means of 3 readings. The purified envelope figures were obtained by applying the same quantity of crude envelopes as contained in the 6.1 mls crude envelope suspension to the sorbitol gradient; after dialysis, the band was resuspended in a final volume of 6.0 mls distilled water.

Table 5:2(a)

Material	Vol. (mls)	Optical Densities: Lipid P	DØN	Orcinol	Lowry
3000 X g pellet	5.8	.032	.13	.08	.36
30,000 X g snat	6.3	.037	.35	.22	.575
Crude envelopes	6.1	.039	.065	.09	.21
Purified envelopes	6.0	.035	.035	.06	.12

DØN = Diphenylamine ; Lipid P = Lipid Phosphate.

Table 5:2(b)

Material	% Recovery from nuclei			
	Lipid P	DNA	Orcinol	Lowry
3000 X g pellet	24 ± 3	23 ± 1	21 ± 6	30 ± 0.5
30,000 X g amt	33 ± 3	66 ± 1	55 ± 6	52 ± 0.5
Crude envelopes	37 ± 3	11 ± 1	24 ± 6	18 ± 0.5
Purified envelopes	28 ± 3	6 ± 1	15 ± 6	10 ± 0.5

pH EFFECTS

In chapter 4 it was established that in too acidic isolation medium the envelope could not be recovered. The following experiments were designed to clarify the critical nature of the pH of the medium.

The crude envelope preparation procedure was repeated replacing the 0.02 M tris buffer with 0.01 M phosphate at pH 6.0, 6.5, 7.0, 7.5 and 8.0.

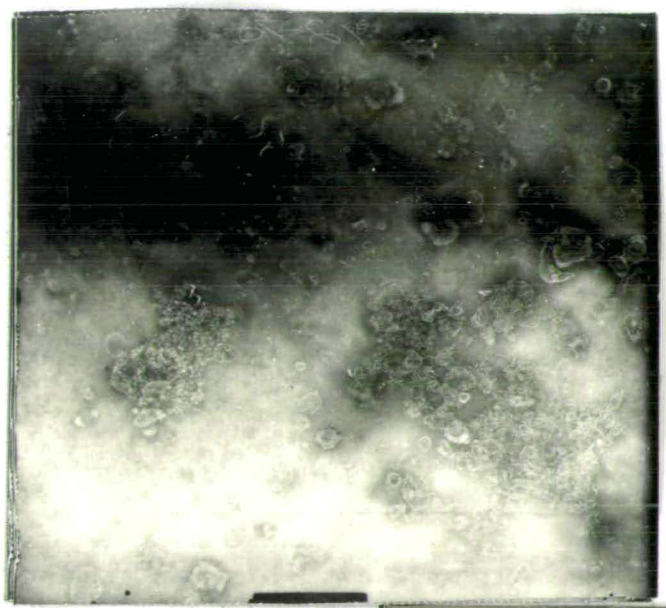
Extent and breakage of nuclei

The nuclei, spherical at higher pH values, were somewhat misshapen at pH 6.0 and 6.5. Variation of pH over this range did not significantly alter the percentage of nuclei broken. Aggregation, however, increased markedly as the pH was lowered and as the pH approached 8 the nuclei appeared more fully dispersed. Consistent with this observation was the slight (about 5%) diminution of light-scatter at the highest pH. Granularity in the nucleoplasm was not apparent.

Envelope morphology

Pores were not recovered at pH 6.0, 6.5 or 7.0, but vesicularization over this range did not seem extensive. The mean size of fragments decreased with increasing pH. At pH 7.5 and 8.0 pores were visible. Chromatin contamination was most obvious at pH 6.0, 6.5 and 8.0. The pH 7.5 preparation was indistinguishable from material from the standard procedure.

Plate 5:4



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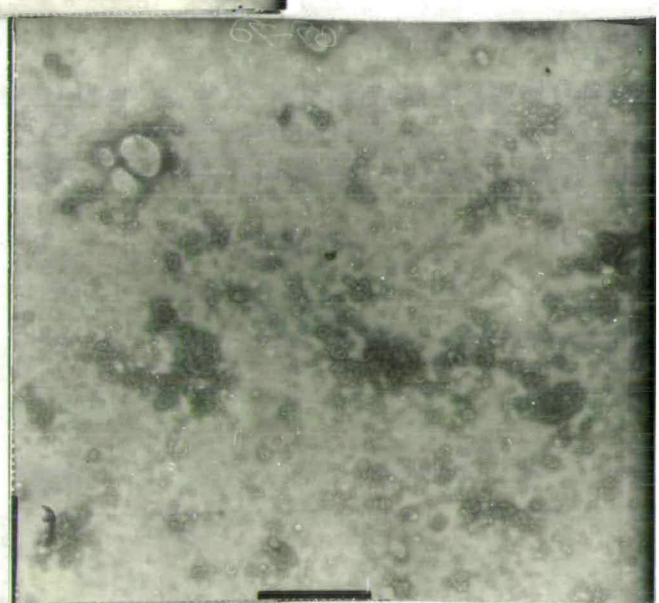

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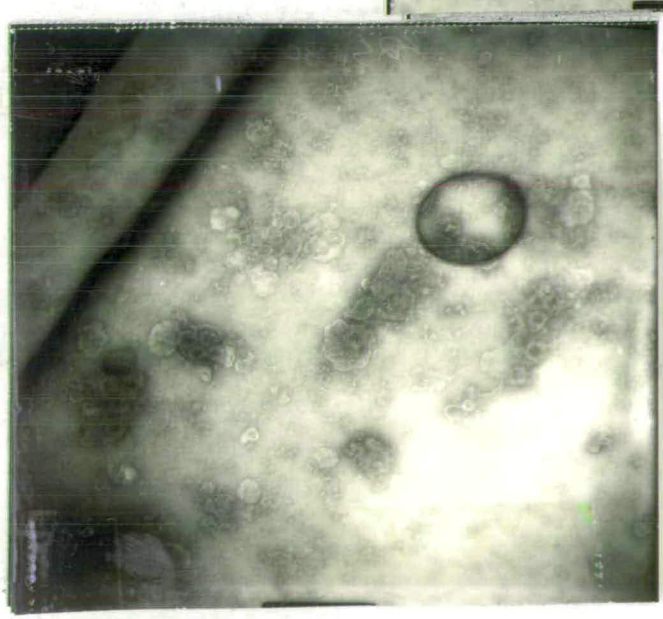


c

Composition

Table 5:3 shows the composition in terms of lipid phosphate, diphenylamine colour and Lowry colour of the 30,000 x g pellets of these components. The figures for the standard envelopes are included for comparison.

Table 5:3

Condition	Lipid P	DPN	Lowry	DPN / Lipid P	DPN / Lowry	Lipid P / Lowry
Standard	.085	.235	.50	2.8	.47	.17
pH 6.0	.08	.33	.51	4.2	.65	.16
6.5	.075	.295	.49	3.9	.60	.15
7.0	.08	.255	.47	3.2	.54	.17
7.5	.08	.215	.47	2.6	.46	.17
8.0	.075	.19	.43	2.5	.44	.17

IONIC STRENGTH EFFECTS

In chapter 4 it was shown that envelope morphology was lost (i) in the presence of potassium ions, (ii) in high ionic strength media. To clarify further these ionic effects, three sets of experiments were performed:-

a) To the tris buffer in the standard preparation, NaCl was added to (i) 0.01 M and (ii) 0.04 M concentration.

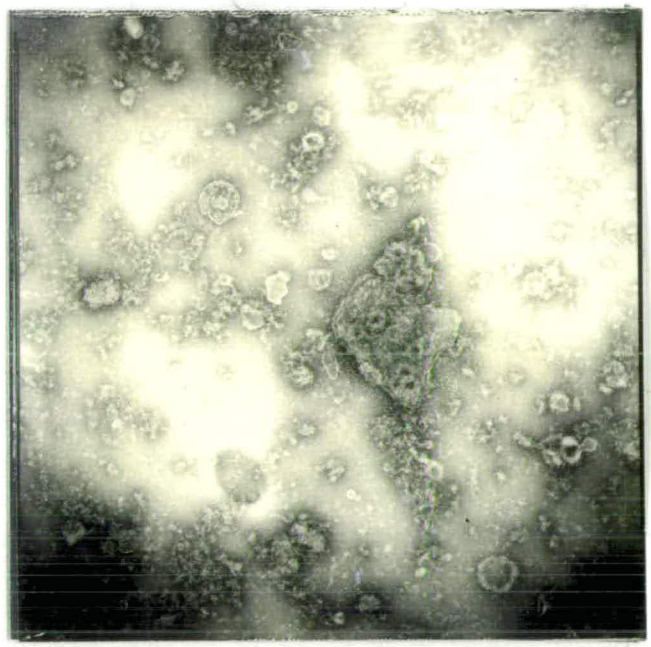
b) To the tris buffer in the standard preparation, KCl was added to (i) 0.01 M and (ii) 0.04 M concentration.

c) The 0.02 M tris buffer of the standard procedure was replaced by sodium phosphate buffer, pH 7.7, at the following concentrations: 5, 10, 20, 40 and 100 mM.

Extent of breakage of nuclei

The nucleoplasm became granular only in high concentrations (100 mM) of phosphate buffer. Potassium chloride at both concentrations

Plate 5:5



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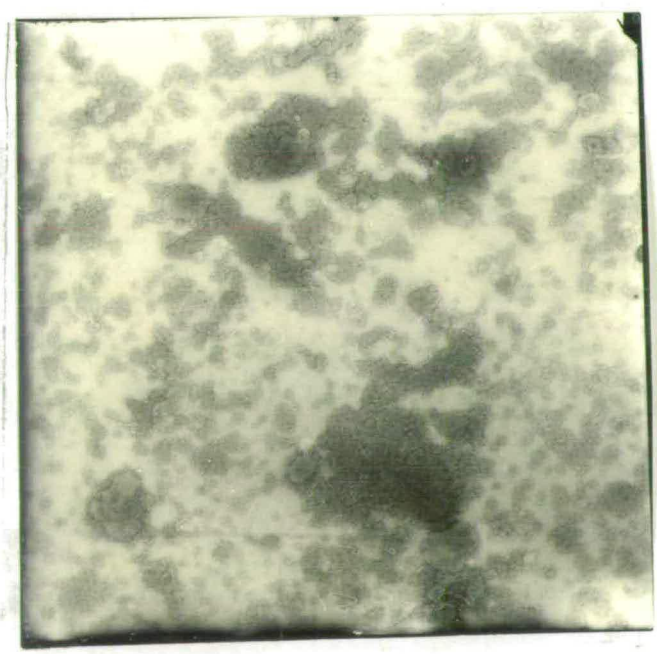

b

(unlike sodium chloride) caused complete dispersal of the nuclei after 20 seconds' sonication. In potassium chloride a 40% diminution in light-scatter occurred, while in sodium chloride the light-scatter was unchanged.

Envelope morphology

The envelopes prepared in sodium chloride were essentially of the same appearance as those prepared in the standard procedure, though the pore complexes were clearer in outline suggesting that material had been lost from the peripheries of the annuli. In potassium chloride no envelopes were obtained; instead, an aggregated mass of minute pore-free membranous vesicles was prepared, essentially free of gross chromatin or nucleolar contamination.

Composition

Table 5:4

Conditions	Ionic Strength	Lipid P	D/N	Lowry	D/N Lipid P	D/N Lowry	Lipid P Lowry
0.01 M NaCl	0.01	.085	.23	.49	2.8	.47	.17
0.04 M "	0.04	.08	.21	.45	2.6	.47	.18
0.01 M KCl	0.01	.085	.155	.435	1.8	.36	.19
0.04 M "	0.04	.085	.155	.40	1.8	.39	.21
Phosphate:-							
5 ml	0.015	-	.275	.475	-	.58	-
10 ml	0.03	.08	.21	.45	2.6	.47	.17
20 ml	0.06	.075	.195	.415	2.6	.46	.18
40 ml	0.08	-	.235	.485	-	.49	-
100 ml	0.30	-	.61	.66	-	.92	-

DICATION AND CITRIC ACID EFFECTS

The following experiments were designed to establish (i) whether the magnesium of the sonication medium could be replaced by other

dications, (ii) to what extent the magnesium concentration was critical. The effect of citric acid in the medium instead of a dication was also investigated; since the low pH removes the outer nuclear membrane, the possibility of obtaining an inner membrane preparation using citric acid was examined. The experiments performed were:-

a) The 1 mM $MgCl_2$ of the sonication medium was replaced by (i) 1 mM $CaCl_2$, (ii) 1 mM $BaCl_2$.

b) The sonication medium was made (i) 0.5 mM, (ii) 2.0 mM, (iii) 5.0 mM $MgCl_2$ instead of 1 mM $MgCl_2$, but the concentration was adjusted to 0.5 mM as in the standard procedure, prior to differential centrifugation.

c) Sonication was performed in the usual medium, containing 1 mM $MgCl_2$, but prior to differential centrifugation the suspension was diluted with (i) 4 volumes, (ii) 9 volumes, (iii) 19 volumes instead of 1 volume of water, or (iv) was left undiluted.

d) The standard procedure for the isolation of the envelope was performed on nuclei after washing 3 times in 1% citric acid, then washing twice in the sonication medium (tris-HCl + 1 mM $MgCl_2$, pH 7.7) prior to sonication. Thus, the established standard procedure was applied to nuclei from which the outer membrane had been removed.

Extent of breakage of nuclei

Slightly less nuclei were completely dispersed in the presence of calcium, and slightly more in the presence of barium, than in the presence of magnesium. The extent of breakage decreased, and the extent of aggregation of the sonicated material increased, with increasing magnesium concentration; in 5 mM magnesium, the nucleoplasm was somewhat granular. In the citric acid washed nuclei, the

nucleoplasm was very granular, and the nuclei were relatively intractable to sonication. In consequence the yield of envelope-like material was low.

Envelope morphology

In the presence of all dications, independent of concentration, acceptable envelopes with many pore complexes were recovered. At magnesium concentrations much greater or much less than 1 mM contamination was significant; when the magnesium concentration was low in sonication or differential centrifugation, marked vesiculation occurred. The material obtained from citric acid-washed nuclei consisted of a mass of aggregated and highly contaminated membranous vesicles; no envelope material was recovered.

Composition

Table 5:5(a)

$[Mg^{++}]$ in sonication	Lipid P	DNA	Lowry	DNA / Lipid P	DNA / Lowry	Lipid P / Lowry
5 mM	-	.11	.15	-	.73	-
2 mM	.09	.225	.315	2.5	.71	.29
1 mM (standard)	.075	.205	.44	2.7	.46	.17
0.5 mM	.13	.39	.84	3.0	.47	.15

Table 5:5(b)

$[Mg^{++}]$ in centrifugation	Lipid P	DNA	Lowry	DNA / Lipid P	DNA / Lowry	Lipid P / Lowry
0.5 mM	.075	.20	.425	2.7	.46	.17
0.2 mM	.12	.40	.475	3.3	.84	.25
0.1 mM	.17	.675	.90	4.0	.75	.19
0.05 mM	.11	.60	.90	5.5	.67	.12

Table 5:5(c)

$CaCl_2$, 1 mM	.05	.13	.30	2.6	.43	.17

The 30,000 X g "inner membrane" preparation was assayed as usual, and the 100,000 X g pellet of the pooled citric acid washes of the nuclei, containing "outer membrane" material (Cf. Smith et al[4]) was also examined.

Material	Lipid P	DNA	Lowry	DNA / Lipid P	DNA / Lowry	Lipid P / Lowry
30,000 X g pell.	.02	.19	.60	9.5	.31	.03
100,000 X g pell.	.007	Zero	.075	< 1	< .07	.09

OSMOTIC PRESSURE:

In chapter 2, the question of the significance of the low osmotic pressure of the isolation media of nuclei and nuclear envelopes was raised. To assess the importance of this factor, the standard sonication medium was made 2% bovine serum albumin, which is "isotonic" with the nucleoplasm (Harding and Feldhoff[5]). A macromolecular solute was required, the envelope being permeable to small molecules.

Nuclear breakage and envelope morphology

The inclusion of the serum albumin made no observable difference to the extent of nuclear breakage during sonication or to the degree of aggregation of the nuclear fragments. Envelope morphology was not significantly different from that observed in the standard procedure, but along with the envelope fragments small amorphous objects, probably aggregates of denatured protein, were observed.

Composition

The assays show differences from the standard procedure which might be explained in terms of the contamination of the envelopes with the excess protein.

Table 5:6

Condition	Lipid P	DNA	Lowry	DNA / Lipid P	DNA / Lowry	Lipid P / Lowry
Standard	.07	.19	.41	2.6	.46	.17
2% BSA	.055	.145	.37	2.7	.39	.145

CONCENTRATION OF NUCLEI

Another variable which might conceivably alter the condition of the envelopes is the concentration of the nuclear suspension sonicated. Instead of the arbitrary 5 mg/ml of the standard procedure, therefore, concentrations of 0.5 mg/ml, 1 mg/ml and 10 mg/ml protein were used.

The extent of nuclear breakage and the envelope morphology were not affected by this variation, and diphenylamine and Lowry assays also failed to show any significant change. It was concluded that the concentration of nuclei sonicated was not a critical factor in the procedure.

Table 5:7

Concentration	D/N	Lowry	D/N/Lowry
10 mg/ml	.49	1.05	.47
5 mg/ml	.19	.40	.47
1 mg/ml	.023	.05	.46
0.5 mg/ml	.010	.02	~.50

SONICATION CONDITIONS

The following factors in the sonication conditions are variable, and the choice of values used in the standard procedure requires experimental justification:-

a) The volume of suspension used — Standard = 5 ml
b) The size of the probe — " = 0.5 cm
c) The sonication time — " = 2 X 20 secs
d) The amplitude — " = 1 μm
e) The frequency of oscillations — " = 21 kc/sec

In the following experiments, the conditions were varied:-

a) Volume: 1, 2 and 10 ml aliquots of nuclear suspensions were sonicated, the other conditions being as in the standard procedure.

b) A 0.2 cm probe was used instead of a 0.5 cm.

c) The 2 X 20 secs sonication of the standard 5 ml aliquot was replaced by sonication times of 1 X 20, 2 X 15, 3 X 20 and 6 X 15 secs.

d) Sonication was performed at 2 μm amplitude and 4 μm amplitude instead of 1 μm. This necessitated some small change of frequency, but this probably did not influence the result (see below).

e) Sonication was performed at 27 kc/sec, at which frequency the amplitude is also 1 μm.

Extent of nuclear breakage

The effect of the 0.5 cm probe could be reproduced with the 0.2 cm probe, but a total of 3-4 minutes' sonication was required to effect 90% nuclear breakage. The time required for 90% disruption and dispersal was 20 secs at 2 μm and 15 secs at 4 μm amplitude. The frequency made no observable difference. Under the standard conditions the percent nuclear breakage increased more or less linearly with time (some 30-40% after 20 secs; 60-70% after 30 secs; and around 100% after 60 and 90 seconds).

Envelope morphology

In general, acceptable envelopes (as obtained under the standard sonication conditions) were obtained as long as the ratio of sonication time/volume did not exceed 8-9 seconds/ml; if it did exceed this value, then the recovery of pores decreased and the extent of vesiculariation and sonication increased; moreover, the material did not band on the sorbitol gradient but pelleted. Morphology was independent of the probe used and the frequency of the sonication energy. Increase of amplitude had the same effect on morphology as increase of the time/volume ratio.

Composition

The effect of time/volume ratio on envelope composition is shown in table 5:8.

Table 5:8

Total time	Time/volume	DNA	Lowry	DNA/Lowry
20 mins	4 secs/ml	.12	.265	.45
30 "	6 "	.215	.485	.45
40 "	8 "	.235	.50	.47
60 "	12 "	.28	.47	.59
90 "	18 "	.265	.485	.55

DIFFERENTIAL CENTRIFUGATION

The differential centrifugation of nuclei disrupted under standard sonication conditions was modified as follows:-

a) The suspension was centrifuged at 1000 X g for 10 minutes.

b) The 1000 X g supernatant was centrifuged at 3000 X g for 10 minutes.

c) The 3000 X g supernatant was centrifuged at 10000 X g for 20 minutes.

d) The 10000 X g supernatant was centrifuged at 30,000 X g for 30 minutes.

The four pellets were examined in the electron microscope by negative staining and analysed in terms of Lowry colour, DNA and lipid phosphate.

Envelope morphology

The pellets at 1000 X g and 3000 X g contained chromatin, nucleoli, intact nuclei and a quantity of amorphous material, but little recognisable envelope. At 10000 X g, material rich in pore complexes and containing apparently more chromatin than the standard

crude envelopes was recovered; practically no vesicles were found in this pellet. The 30,000 X g pellet contained more vesicles, less chromatin and somewhat fewer pore complexes. Only a very few tiny membranous vesicles were left in the 30,000 X g supernatant.

Composition

Table 5:9

Pellet	Lipid P	DØN	Lowry	DØN / Lipid P	DØN / Lowry	Lipid P / Lowry
1,000 X g	.08	.26	.69	3.3	.38	.12
3,000 X g	Zero	.07	.14	>14	.50	<.04
10,000 X g	.04	.225	.465	5.6	.48	.09
30,000 X g	.08	.155	.36	1.9	.43	.22

GENERAL DISCUSSION

Standard procedure

This procedure was shown to give a satisfactory envelope preparation according to the morphological criterion. Moreover, there was little vesicularisation, contamination by chromatin and nucleolar material was shown to be negligible, and little apparent loss occurred in the other differential centrifugation fractions. The envelope fraction contains the highest percentage of the nuclear lipid; some 6% of the nuclear DNA is finally recovered, DNA accounting for some 8% by dry weight of the envelopes (considerably more than in other published attempts at isolation of the envelope).

pH effects

In confirmation of previous results (chapter 4), the pH range over which envelopes can be prepared by the standard sonication procedure is about 7.2-8.0. Over the whole 6.0-8.0 range, DNA content varies more or less inversely with pH; low pH treatments appear to render the chromatin granular and make the nuclei resistant to sonication,

and are thus unsuitable. The degree of aggregation reflects the degree of chromatin contamination.

Ionic strength effects

The destructive effect of potassium on the envelope, which can be seen at concentrations as low as 0.01 M, is not mimicked by sodium at similar concentrations. Moreover, sodium does not cause the lowering of DNA content typical of potassium treatment. When the ionic strength of the buffer is raised, contamination appears to increase (there is an increase of DNA content and extent of aggregation); too low an ionic strength appears to have the same effect. There is, therefore, an optimal ionic strength just as there is an optimal pH, but in the case of ionic strength the actual value of the optimum depends on the ions involved (see chapter 6 for further elaboration of this point). An acceptable preparation is possible only over a limited ionic strength range.

Dication effects

Either excessive or insufficient dication in the sonication medium reduces the yield of envelope and increases its DNA content; insufficient dication also seems to promote a loss of lipid, a finding consistent with the result, quoted in chapter 4, that EDTA causes lipid depletion.

The effect of dication concentration in the differential centrifugation medium, however, requires more comment. The lipid retention is simply increased as dication concentration increases. The effect on DNA content in the envelope is, however, more complex. The results are perhaps best interpreted as follows: Consider 2 pools of DNA, one (E) bound to the envelope, the other (C) occurring in the liberated chromatin. If a certain concentration, x, of magnesium is required to maintain the integrity of the released chromatin (i.e. to

prevent DNA leaving the pool C), but any magnesium, even a small trace, in solution will bind DNA freed from pool C to pool E such that the extent of this binding is proportional to the magnesium concentration, then we have the following situation:-

Concentration of Mg^{++} in solution	Quantity of DNA in envelope
More than, or equal to, "x"	E
Between zero and "x"	$E + (\text{Const.} \times [Mg^{++}])$
Zero	E

The following experimental results would corroborate this hypothesis:-

a) If EDTA were added to the suspension after sonication and prior to differential centrifugation, the resulting envelopes would contain DNA concentrations equivalent to the pool E, i.e. about the same as in the standard procedure. This result was in fact obtained when EDTA at pH 7.7 was added to sonicated nuclei to a final concentration of 1 mM.

Material	Lipid P	DNA	Lowry	$\frac{DNA}{Lipid\ P}$	$\frac{DNA}{Lowry}$	$\frac{Lipid\ P}{Lowry}$
Standard env.	.085	.235	.50	2.8	.47	.17
EDTA treated	.04	.47	1.01	11.8	.46	.04

b) Addition of excess DNA to the suspension under the standard conditions would increase the amount of DNA bound to the envelope, since the excess would behave as DNA liberated from pool C. The validity of this prediction is investigated incidentally in chapter 7.

Replacement of magnesium by calcium makes little apparent difference to the envelope preparation, a fact which is exploited in chapter 8, when the ionic content of the envelope is examined. The use of barium, while it maintains the envelope in a reasonably intact state, is perhaps less satisfactory. The quality of the electron

micrographs of nuclear ghosts prepared in barium (see chapter 4) suggests that this cation "fixes" the protein to a considerable extent; moreover, when this ion is included, the medium strays far beyond the limits of the physiological.

The citric acid results do not confirm the prediction that acceptable inner and outer membrane preparations can be obtained by washing the nuclei in this medium and afterwards modifying suitably the envelope preparation. The pore complexes vanish after citric acid treatment, and no evidence survives to show that the preparations are in any way acceptable. Since high ionic strength methods appear from the phosphate buffer results to be inapplicable to the problem of isolation, producing contamination and morphological disruption, and since detergent methods were discussed in chapter 2, no method for obtaining separate inner and outer envelope preparations appears possible at this stage.

Sonication conditions

Another critical factor in the envelope preparation is what might be termed the "amount of sonication", which from the results may be concluded to be a function of:-

$$\frac{\text{Amplitude} \times \text{probe diameter} \times \text{sonication time}}{\text{Volume of suspension}},$$

and to be independent of the concentration of nuclei in the suspension and independent (over the range examined) of the frequency of sonication. While the amount of sonication must be sufficient to disrupt the nuclei, it must not be sufficient to destroy the pore complexes and to contaminate or vesicularise the remaining membrane. That the amount of sonication used in the standard procedure is near optimum is justified by the examination of nuclear breakage and envelope composition and morphology. It is interesting to note that it follows from the dependence of the effect on volume rather than concentration

that the critical factor is the energy applied per ml, not the energy applied per nucleus.

Osmotic pressure

Though the addition of bovine serum albumin to the medium increases protein contamination, the envelopes still satisfy the morphological criterion of integrity. Osmotic pressure cannot be concluded from the results to be an important factor in this isolation procedure.

Differential centrifugation

The variation of differential centrifugation conditions leads to the following conclusions:-

a) Material pelleted at 1000 and 3000 X g is non-membranous, and consists of nearly intact nuclei, and (as would be expected from the brief review of the isolation of submuclear fractions at the end of chapter 2) of nuclcoli and dense chromatin, probably largely heterochromatin and intermediate chromatin.

b) Material pelleted at 10,000 X g is largely envelope and is very rich in pore complexes; some chromatin is visible, an observation corroborated by the assay results.

c) The 30,000 X g material after centrifugation at 10,000 X g contains rather fewer pore complexes than standard envelopes and is proportionately more vesicular. Since little material of a membranous appearance remains in the supernatant, it is concluded that the nuclear envelope, even when vesicularised, is considerably denser than microsomal membrane. Density is increased, along with chromatin "contamination", when the pore complexes are more in evidence.

The value of the nucleoli preparat

In the standard preparation, crude envelopes from differential

centrifugation are "purified" by zonal gradient centrifugation (zonal is used in preference to sucrose because of its lower viscosity at high density - see chapter 6). However, the composition of the purified envelopes is closely similar to that of the pure envelopes, though the relative yields of material and analysis of the remainder of the gradient indicate that a loss of all components occurs on the gradient. This loss may be regarded either as the removal of contaminants or as the product of envelope degradation.

The first viewpoint is more tenable than the second since a considerable amount of envelope persists on the gradient, since a second gradient removes very much less material than the first, and since on the second gradient the density remains at around 1.27 g/ml. It follows that after zonal gradient centrifugation the envelopes are to be regarded as less contaminated than the crude envelopes from differential centrifugation.

This conclusion illuminates the difficulty of discussing concepts such as "contamination", "purity", etc. with reference to a macromolecular system of biological origin of which the definition is largely or entirely operational. Superficially, it might be expected that the DNA/lipid ratio would be a useful indicator of contamination in the case of the nuclear envelope, but it can be seen that crude and purified envelopes have more or less the same DNA/lipid ratio; this ratio is not, therefore, an adequate marker of contamination. Alternatively, one might say that the nuclear envelope consists of only that set of materials which is both necessary and sufficient to generate a complex which has a morphology identical with that stated in the criterion of definition of the system. However, it has been shown that DNA treatment removes lipids without necessarily destroying the envelope morphology. The implied conclusion that lipids are unnecessary

in the system, and hence contaminants, is unsatisfactory: it gives to the lipids a highly ambiguous structural role in the nucleus.

The problem of contamination and purity will be discussed thoroughly in chapter 11. For the present time, the only possible definition of the nuclear envelope is in the last analysis operational. The isolated nuclear envelope is the material found in the 1.27 g/ml sorbitol band at the end of the standard preparation described above.

DNA CONTENT

In the absence of an acceptable definition of contamination, the question "is the DNA a contaminant?" is not meaningful. Positive evidence to show that DNA is an essential component of the envelope is as follows:-

a) When the DNA content of the material falls below a certain level, the experimental findings indicate that morphological integrity is always lost, the pore complexes being in each case destroyed.

b) This observation, which appears to be generally true, may be explained in one of two ways:-

(i) Certain sets of conditions disrupt the envelope, thereby releasing the DNA attached to it;

(ii) Certain sets of conditions remove DNA from the system, and as a consequence of this morphological disruption occurs.

c) In chapter 4, a case was described which belonged unequivocally to the second of these possible classes. This was the effect of treatment of the envelope with protease, lipase and RNase free DNase. This must necessarily disrupt the envelope as a consequence of the removal of DNA.

Moreover, when the envelope is intact in vivo it is always intimately associated with DNA; condensation of chromosomes in mitosis is more or less coincident with envelope breakdown; and it is

probable that close attachment of the DNA with the rest of the envelope can be seen on the molecular level, as the envelope appears to be the site of initiation of DNA synthesis. Moreover, all attempts to isolate the nuclear envelope which have so far been published are content to isolate from nuclei membranous material with low DNA content. When DNA content is low, the morphological criterion is never satisfied. Finally, in the (essentially operational) definition of the envelope adopted in this thesis, DNA is reproducibly found to be a component of the isolated system. It is concluded that DNA is an essential component of the nuclear envelope.

The range of experiments in this chapter has shown that the standard procedure for envelope isolation produces material in which the DNA content is as low as possible while maintaining morphological integrity. Large-scale "contamination" (obvious inclusion of organised chromatin in the preparation) is present only when the pH is too low, the ionic strength or the dication content too low or too high, or the amount of sonication excessive or insufficient. So far as can be determined, the standard procedure, in terms of integrity and of DNA and lipid content, and in terms of yield, optimises all the variables involved in the procedure. Variation of any of those conditions makes the preparation in some way less satisfactory.

THE POTASSIUM EFFECT

A special finding of this chapter, consistent with results in chapter 4, concerns the potassium effect. Sodium (or caesium - which also fails at 40 mM concentration to disrupt the envelope in preparation) do not mimic this effect. It is clear that potassium of all monocations, must be omitted from the isolation medium.

However, all workers with the exception of Zbarsky et al[6] have used potassium, with or without high ionic strength, in attempts to

isolate the nuclear envelope. The effect constitutes one reason for the failure of these attempts (see chapter 2). However, to interpret the effect in molecular terms is not straightforward, and requires:-

a) The investigation of the effect of potassium on the isolated envelope rather than on nuclei during disruption. Intact nuclei are not destroyed by low potassium concentrations;

b) The investigation of the effects of other ions, pH conditions, etc. over a range of concentrations on the isolated envelope.

Chapter 6 is devoted largely to such investigations; however, it should be noted in advance that a satisfactory interpretation of this curious ionic effect may be possible only in terms of the structural interactions within the nucleus, rather than in terms of the envelope structure itself.

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CHAPTER SIX

THE STABILITY OF THE ISOLATED ENVELOPE

SUMMARY

1. The behaviour of the isolated nuclear envelope on sucrose gradients differed from that on sorbitol gradients containing the same ions and having the same pH.

2. Sucrose and sorbitol were themselves found to affect the composition of the system.

3. The effect of sodium and potassium ions on the stability and composition of the envelope depended on the concentration of ion in the medium.

4. Anion effects on composition and stability differed from anion to anion; no two ions appeared to affect the envelope in exactly the same way at the same concentrations.

5. The effects of chelating agents, changing diction concentrations and changing pH on the envelope were also investigated.

6. The results are discussed with a view to their possible physiological significance, taking account of the observed differences in behaviour between the isolated envelope and the envelope in the nucleus.

7. It is argued that no satisfactory explanation for the results at the molecular level can be offered.

INTRODUCTION

It was established in chapter 5 that the crude nuclear envelope pellet prepared by differential centrifugation of sonicated nuclei could be freed of "contaminants" by the use of sorbitol gradients. Two questions which arise from this finding are:-

a) Can the sorbitol be replaced by other nonelectrolytes, e.g. sucrose?

b) What is the effect on the behaviour of the envelope on the

gradient of pretreatment with EDTA or of inclusion of ions, e.g. K^+ ions?

The finding described in chapters 4 and 5 that potassium ions decrease the stability of the envelope, leading to a reduction in RNA content and loss of morphological integrity, suggests that further investigation of the effects of various ions on the system might give useful information about the factors tending to increase or decrease the stability of the envelope in isolation.

The aim of the work described in this chapter is to establish information about the stability of the envelope in isolation, and compare this to its stability in the intact nucleus, which information may have some physiological significance. Stability is monitored as morphological integrity as determined by electron microscopy; it is correlated with the composition and gradient behaviour of the envelope under the conditions used.

COMPARISON OF SUCROSE AND SORBITOL GRADIENTS

These experiments demonstrate differences between the effects on the envelope of sorbitol and sucrose.

Methods

Crude nuclear envelopes prepared by the standard procedure (see chapter 5) were suspended in water to a concentration of about 10 mg protein/ml. 0.25 ml of this suspension were layered on a 5 ml 0.0-5.0 M ion-free sorbitol gradient as described in chapter 5, or on a 0.0-2.5 M (ion-free) sucrose gradient. Sorbitol gradients were centrifuged for 16 hours at 50,000 X g (average) in a Spinco SW39 rotor at 5°C; sucrose gradients were centrifuged for 4 hours under the same conditions.

The envelope band recovered from such a sorbitol gradient was dialysed thoroughly (2 X 12 hours against a large volume of water)

and re-centrifuged on a sucrose gradient; similarly, the envelope band recovered from sucrose gradient centrifugation of the crude envelopes was re-centrifuged on a sorbitol gradient.

These experiments were repeated using crude envelopes resuspended in 0.02 M KCl rather than water.

Results

Though no band was visible in the highly viscous sucrose gradient after 16 hours, this being too short a time for the band to sharpen, an envelope band appeared with apparent density 1.27 g/ml after 64 hours centrifugation and more prolonged centrifugation did not alter its position. The material of this band had the morphology and composition of the standard envelopes.

However, when the crude envelopes were suspended in 0.04 M KCl rather than water, the equilibrium density of the band appearing in the sucrose gradient was reduced to 1.22 g/ml. On the sorbitol gradient, the crude envelope suspended in KCl gave no band but a pellet.

The band recovered from sorbitol gradient centrifugation of crude envelopes resuspended in water gave a band of the same density on sucrose. Similarly, the band of density 1.27 g/ml recovered from the sucrose gradient gave an apparently identical band on sorbitol. However, the light band of material observed in the sucrose gradient after the envelopes had been suspended in KCl solution gave, after dialysis, a pellet on sorbitol. The pellet formed on sorbitol gradient centrifugation of the KCl-envelope suspension gave a band of density 1.26 g/ml subsequent centrifugation on sucrose.

These results were independent of the concentration of envelope suspension applied to the gradient in most cases. However, a volume of material greater than 10% the volume of the gradient led to distortion of the gradient and irreproducible results.

Bands and pellets obtained from envelope material which had been treated with KCl solution did not constitute morphologically acceptable envelope, but consisted of aggregates of minute apparently single-membrane vesicles lacking pore complexes. Potassium ions appear to disrupt the isolated envelope much as they disrupt the envelope during the isolation process (see chapter 5); exposure to 0.01 M KCl at room temperature for 1 minute (this being the minimum time required to prepare specimens for electron microscopy) brought about more or less complete disruption of the isolated envelope.

In an attempt to explain the difference between sucrose and sorbitol gradients, the effects of sucrose and sorbitol on envelope composition during isolation were investigated.

EFFECTS OF SUCROSE AND SORBITOL ON ENVELOPE COMPOSITION

Methods

The standard procedure for the isolation of crude envelopes was modified by the addition of sorbitol or sucrose at (a) the sonication stage, (b) the differential centrifugation stage, no carbohydrate being present in the medium at earlier stages. The composition of the resulting envelopes was established by the procedures for lipid, protein and RNA assays described in chapter 3.

Results

Table 6:1 shows the results of this experiment; the composition of crude envelopes prepared by the standard procedure are included for comparison.

Table 6:1

Condition of envelope isolation	P/L Lowry	RNA/ Lipid P	Lipid P/ Lowry
Sonication in 2 M sorbitol	0.43	2.6	0.17
Sonication in 1 M sucrose	0.56	2.9	0.20
Diff. centrifugation in 2 M sorbitol	0.42	2.1	0.20
Diff. centrifugation in 1 M sucrose	0.43	2.8	0.15
Standard procedure	0.46	2.7	0.17

N.B. In this table and hereinafter, the figures quoted refer to the means of three optical density readings, no attempt being made to convert these values to µg of protein, DNA or lipid. "DPA" stands for "diphenylamine colour" and "Lipid P" for "lipid phosphate colour" (Cf. chapter 3).

Discussion

Comparison of the effects of inclusion of 2 M sorbitol or 1 M sucrose with the standard procedure leads in itself to no obvious interpretation of the results. One possible interpretation could be as follows:-

a) Water depletes the envelope of all components, particularly lipid.

b) Sorbitol protects the envelope against lipid depletion by water. Lipid phosphate values therefore tend to increase relative to values for other components if sorbitol is present.

c) Sucrose protects the envelopes against both depletion of lipid and depletion of protein by water. DNA assay values therefore appear to be lowered relative to values for other components.

From the foregoing results there is no reason for concluding that this is the best possible interpretation. However, results of experiments on the effects of sonications on envelopes in sorbitol and sucrose gradients (v. infra) are readily explicable only if this interpretation is accepted.

Conductivity measurements showed that no significant contamination by electrolytes occurred in either the sucrose or the sorbitol. Both the sucrose and the sorbitol solutions had approximately the same pH values as distilled water. It appears, therefore, that the effects on the separation must be understood in terms of the carbohydrate solutes themselves.

EFFECTS OF ENVELOPE DISRUPTION ON GRADIENT BEHAVIOUR

The results described above (*v. supra*) suggest that potassium chloride may have a disruptive effect on the nuclear envelope in isolation, as it appears to have during the process of recovery from the nucleus. It may also be predicted that DNase treatment at room temperature, or brief sonication of the envelope suspension, will similarly disrupt the system and lead to comparable behaviour on gradients. The results of such experiments may be regarded as typical of the behaviour of disrupted nuclear envelopes on gradients, and may provide useful comparisons with the results of more methodical investigations of ionic effects on gradients.

Methods

a) Crude envelopes were resuspended in 0.04 M KCl to a concentration of about 10 mg protein/ml, then dialysed for 12 hours against a large volume of distilled water. The resulting suspension was layered on a 0.0-5.0 M sorbitol gradient, ion-free, as described earlier in this chapter, and centrifuged for 16 hours at 5°C.

b) Crude envelopes were resuspended in a small volume of 0.01 M sodium phosphate buffer, pH 7.5, containing 10^{-4} M $MgCl_2$, and incubated for 20 mins at room temperature with 10 µg/mg protein pancreatic DNase. The digested envelopes were then subjected to sorbitol gradient centrifugation as described above.

c) Crude envelopes were resuspended in 5 mls water and sonicated at 27 kc/sec, 1 µm amplitude, for 5 seconds using a 0.5 cm probe which just penetrated the meniscus. During this process the suspension was cooled in ice. The suspension was then centrifuged on a sorbitol gradient as described above.

Results and discussion

Table 6:2 summarises the appearance of the three above gradients

after 16 hours' centrifugation, scanning at 280 mμ. Material at the meniscus of the gradient ("top"), material distributed between the meniscus and the normal location of the envelope band at 1.27 g/ml ("solubilised"), the band, and the pellet, are roughly compared as -, +, ++, etc. The term "solubilised" was used for material in the upper part of the gradient since this material did not scatter measurably at visible wavelengths.

Table 6:2

Description of pretreatment	Band density (g/ml)	Pellet	Top	Solubilised
None (Cf. Chapter 5)	1.27	-	+	+
5 secs sonication	-	++	++	++
EDTA, 20 minutes, room temp.	Diffuse	(+)	+++	+++
0.04 M KCl; dialysed	Diffuse	+	+++	++

From these results it appears that, on disruption of the envelope, the following changes in the gradient behaviour may be expected:-

a) The envelope band disappears or becomes diffuse.

b) A pellet generally becomes evident.

c) There is a marked increase in low-density, generally non-scattering material in the upper part of the gradient, and in material collected at the meniscus.

The meniscus material released from the disrupted envelope, having a density of 1.0 g/ml or less, is probably lipid (Cf. gradient results described in chapter 5); the pellet material appears to be released nucleic acid or nucleoprotein. The disappearance of the band at density 1.27 g/ml demonstrates that more or less all the envelope is destroyed, rather than a specific fraction of it, by the conditions employed.

EFFECTS OF pH ON THE ENVELOPE

The stability of the isolated envelopes over a range of pH

values was investigated by treatment with various sodium phosphate and tris-HCl buffers and examining the composition of the washed envelope and its behaviour on sorbitol gradients.

Method

a) Crude envelopes were resuspended to a concentration of about 10 mg protein/ml in 0.01 M sodium phosphate buffer (pH 6.0-9.0 in steps of 0.5) or 0.02 M tris-HCl pH 7.5, 8.5 or 9.0, and were centrifuged for 16 hours on sorbitol gradients (v. supra) containing the same buffer at the same pH. At the end of this time the gradients were scanned at 260 nm and the band density and quantities of material in the pellet, meniscus and upper part of the gradient recorded as -, +, ++, etc. as above. More precise estimation of the distribution of material in the gradient was not necessary since assays were performed on envelopes washed in buffers at the same pH values (v. infra).

b) Crude envelopes were suspended (5 mg protein/ml, 5 ml volumes) in the same range of phosphate and tris-HCl buffers over the same pH range and, after incubation for 20-30 minutes at room temperature, were centrifuged at 30,000 X g for 30 minutes. The pellets were assayed for DNA, protein and lipid as described in chapter 3, and the ratios of diphenylamine colour/Lowry colour (DPN/Lowry), diphenylamine colour to lipid phosphate colour (DPN/Lip. P) and lipid phosphate to Lowry colour (Lip. P/Lowry) recorded.

c) The supernatants from these washes were recovered and the optical densities at 260, 280 and 500 nm determined using a Unicam SP 500 spectrophotometer. The value at 500 nm was taken as an estimate of light-scatter, and the values at 260 and 280 nm roughly corrected for light-scatter by the following procedure:-

From the Debye equation, if R_1 = light-scatter at wavelength λ_1 and R_2 = light-scatter at wavelength λ_2, then, for a solution of constant concentration, particle size, path-length, etc.:-

$$\frac{R_1}{R_2} = \frac{\lambda_2^4}{\lambda_1^4}$$

Hence,

$$\frac{R_{260}}{R_{500}} = \frac{(500)^4}{(260)^4} = \underline{14.7}$$

Therefore, if E_{260} is the observed optical density at 260 mμ and E_{260}° is the same optical density corrected for light-scatter,

$$E_{260}^{\circ} = E_{260} - (E_{500} \times 14.7)$$

Similarly, if E_{280}° represents the E_{280} corrected for light-scatter,

$$E_{280}^{\circ} = E_{280} - (E_{500} \times 10.1)$$

The figures E_{260}° and E_{280}° provide some information on the quantities of nucleic acid and protein dissolved in the supernatant after the washing. If the supernatant contains DNA but no protein, then E_{260}° would be expected to be approximately twice E_{280}°.

Insofar as the spectrophotometer is an inefficient device for determining scatter, apparently zero scatter at 500 mμ does not imply that scatter is necessarily zero at 260 or 280 mμ. The E_{260}° and E_{280}° constitute, therefore, upper limits for the absorbance values.

Results

Tables 6:3, 6:4 and 6:5 summarize, respectively, the distribution of envelope materials in the gradients at different pH values, the compositions of the washed 30,000 X g pellets, and the optical densities of the washing supernatants.

Table 6:3 Effect of pH on gradient behaviour

Buffer	pH	Band density (g/ml)	Pellet	Top	Solubilised
Phosphate	6.0	-	◇◇	-	(◇)
	6.5	-	◇◇	-	(◇)
	7.0	-	◇	(◇)	◇
	7.5	1.27	-	(◇)	◇
	8.0	1.27	(◇)	◇◇	◇◇
	8.5	Diffuse	-	◇◇◇	◇◇◇
	9.0	Diffuse	-	◇◇◇	◇◇◇
Tris-HCl	7.5	-	◇◇	◇◇	◇
	8.5	Diffuse	(◇)	◇◇◇	◇◇◇
	9.0	Diffuse	(◇)	◇◇◇	◇◇◇

The salient features of this set of results appear to be:-

a) As the pH rises over this range, so more material appears to be removed from the envelope until the envelope becomes diffuse.

b) Phosphate and tris-HCl appear to have somewhat different effects, particularly at pH 7.5; this is an example of the differences between the effects of different ions on the envelope.

c) The envelope band at pH 7.5 can be obtained only over a very limited range of pH conditions. This is consistent with the results described in chapter 5, which showed that the envelope could only be isolated from the nucleus over a very limited pH range.

Table 6:4 Effect of pH on envelope composition

Buffer	pH	DNA/Lowry	DNA/Lipid P	Lipid P/Lowry
Phosphate	6.0	0.47	2.1	0.23
	6.5	0.41	2.0	0.21
	7.0	0.42	1.9	0.22
	7.5	0.46	2.2	0.21
	8.0	0.42	2.2	0.20
	8.5	0.26	0.6	0.43
Tris-HCl	7.5	0.40	3.3	0.13

The values for tris-HCl at pH 8.5 and both buffers at pH 9.0 were not determined.

Here, again, a difference between phosphate and tris-HCl buffer at the same pH can be detected. It is apparent from the results that increasing the pH to a value greater than 8.0 brings about extensive breakdown of the envelope; this again is consistent with results discussed in chapter 5. Composition does not otherwise appear to vary dramatically with pH, though the results could be interpreted as showing a maximum percentage lipid at pH 7.5.

Table 6:5 Effect of pH on removal of protein and nucleic acid

Buffer	pH	E_{260}	E_{280}	E_{500}	E_{260}°	E_{280}°
Phosphate	6.0	.08	.135	0	.08	.135
	6.5	.12	.125	0	.12	.125
	7.0	.19	.12	0	.19	.12
	7.5	.26	.105	0	.26	.105
	8.0	.28	.145	0	.28	.145
	8.5	.32	.175	.002	.29	.155
Tris-HCl	7.5	.46	.12	.005	.37	.07

Here, differences between the two buffering systems are even more striking, and pH 7.5 emerges as a more obviously significant value in the range, representing the point at which the ratio $E_{260}^{\circ}/E_{280}^{\circ}$ is maximal. This ratio may be regarded as the ratio of DNA removed to protein removed; however, comparison with table 6:4 shows that the interpretation cannot be so simple (the DPN/Lowry ratio being greater at this point than at neighbouring values).

Discussion

Electron microscopic studies on the material resulting from envelopes treated by the above-described range of buffers indicate that morphology is destroyed by pH values less than 7.5 and greater than 8.0. No pore complexes are found; the membrane is predominantly

vesiculariscd; and the vesicles appear to be by and large single-membrane. Perhaps the best interpretation of results would be as follows. At pH 7.0 or below, DNA is removed from the envelope as an insoluble nucleoprotein complex which pellets in the sorbitol gradients. The removal of DNA tends to destabilise the envelope and lead to loss of morphological integrity. At pH 7.5, the DNA is not removed to the same extent and morphological integrity is retained. However, the E_{260}° of the washing supernatant appears to contradict this statement. The contradiction may be overcome by noting that crude envelopes were used in these experiments; "contaminating" DNA may have been removed in soluble form at the higher pH, leaving the essential "structural" DNA in position. It is notable that water-washing of the envelopes, which also removes a large quantity of DNA in the supernatant, nevertheless leaves morphologically intact envelopes with a DØN/Lowry ratio of 0.46-0.47.

The differences between tris and phosphate buffering may be related to the cation-binding properties of unionised tris. It may therefore be predicted (Cf. chapter 4) that tris will remove cations from the system and hence cause lipid depletion. The assay results suggest that DNA depletion has also occurred to some extent.

The most important general conclusion from these experiments is that small pH changes can incur marked modifications of morphological integrity and envelope composition. This may suggest that electrostatic interactions are significant in stabilising the envelope; it also suggests that the critical nature of the pH of the isolation medium is a function of the properties of the envelope rather than the properties of the whole nucleus.

EFFECTS OF MONOCATIONS ON THE ISOLATED ENVELOPE

In chapters 4 and 5 it was established that sodium and potassium

ions differed in their effects on the nuclear envelope during the process of isolation. Earlier in this chapter (v. supra) it was shown that potassium also exerted a destructive effect on the isolated envelope. The experiments now described constitute a more thorough investigation of the similarities and differences between the effects of alkali metal ions on the nuclear envelope.

Method

The experiments conducted were analogous to those performed in the investigation of pH effects on the envelope (v. supra):-

a) Crude envelopes were centrifuged on sorbitol gradients containing (i) 5-150 mM KCl, (ii) 25-600 mM NaCl. In addition, equilibrium centrifugation on sucrose gradients (0-2.5 M) over the same range of ion concentrations was performed. Results of these experiments were interpreted in terms of the difference between sucrose and sorbitol effects as well as the difference between sodium and potassium effects on the nuclear envelope.

b) Crude envelopes were resuspended in solutions containing (i) 5-250 mM KCl, (ii) 10-500 mM NaCl, and the pellets formed on centrifugation at 30,000 X g for 30 minutes assayed as described above.

c) The supernatants from these washings were recovered and the $P_{260}°$ and $E_{260}°$ determined as described in the pH experiments.

Results

Table 6:6 Effects of ions on sorbitol gradients

Ion concentration	Band density (g/ml)	Pellet	Top	Solubilised
KCl 5 mM	1.24	(◇)	◇	◇
7.5 mM	1.27	◇	◇	◇
10 mM	1.27	◇	◇◇	◇◇
15 mM	1.22	◇	◇◇◇	◇◇
20 mM	-	◇◇	◇◇◇	◇
40 mM	-	◇◇	◇◇◇	◇◇

/(cont/d)

Table 6:6 (cont'd)

Ion concentration	Band density (g/ml)	Pellet	Top	Solubilised
NaCl 25 mM	1.27	-	◇◇	◇◇
60 mM	1.24	(◇)	◇◇	◇◇
120 mM	1.27	◇	◇◇◇	◇◇◇
300 mM	-	◇◇	◇◇◇	◇◇◇
600 mM	-	◇◇	◇◇	◇◇◇

As before, the symbol "-" under "band density" implies that no band was formed in the gradient at equilibrium.

At sufficiently high concentrations, both sodium and potassium destroy the envelope morphology. At this point, the envelope density decreases; the point is reached at different concentrations of the two cations. Further increase in ionic strength leads to increase of density of the membranous remnant of the envelope, and pelleting.

Table 6:7 Effects of ions on sucrose gradients

Ion concentrations	Band density (g/ml)	Pellet	Top	Solubilised
KCl 10 mM	1.27	-	◇	◇◇
20 mM	1.27	-	◇◇	◇◇
40 mM	1.22	(◇)	◇◇	◇◇
80 mM	-	◇◇	◇◇	◇◇◇
150 mM	-	◇◇	◇◇◇	◇◇◇
NaCl 130 mM	1.27	-	◇	◇
250 mM	1.27	-	◇◇	◇◇
600 mM	-	◇◇	◇◇◇	◇◇

It is apparent from these data that the concentration of either ion required to cause some particular change in the envelope is two or three times higher on a sucrose gradient than on a sorbitol gradient. This is consistent with the observation (v. supra) that 0.04 M KCl causes pelleting on sorbitol but banding on sucrose gradients. The value of the hypothesis that sucrose protects the envelope against

depletion of both lipid and protein, while sorbitol protects it only against lipid depletion (v. supra) may become apparent in interpreting these observations.

Table 6:8 Effects of concentrations on envelope composition

Ion concentration		DØN/Lowry	DØN/Lipid P	Lipid P/Lowry
KCl	5 cm	0.39	2.5	0.16
	10 cm	0.42	2.2	0.19
	15 cm	0.46	2.6	0.18
	20 cm	0.52	3.1	0.17
	40 cm	0.47	2.9	0.16
	250 cm	0.67	1.7	0.04
NaCl	10 cm	0.40	2.2	0.18
	20 cm	0.32	1.9	0.17
	30 cm	0.49	3.2	0.16
	60 cm	0.52	3.3	0.16
	120 cm	0.68	4.9	0.14
	300 cm	0.70	5.0	0.14
	500 cm	< 0.10	< 0.5	0.20
10 cm KCl + 60 cm NaCl		0.42	2.8	0.15
10 cm KCl + 10 cm Na phosphate pH 7.5		0.24	1.3	0.19

Sodium and potassium chloride solutions of the same concentration were found to have the same pH (in the same range as the pH of distilled water); it was concluded that the effects observed are due to differences in action of the ions themselves. With increasing concentration, potassium may be regarded as removing first RNA (reducing the density), then protein, and finally lipid. The effects occur at lower concentrations in water than they do in the presence of sorbitol or sucrose; sodium may be regarded as having roughly the same effect as potassium, but at higher ionic strengths. This is at best a tentative explanation at present, but serves to indicate the general trends of the results in a simple and reasonably accurate manner.

It will be noted that this hypothesis does not explain the values obtained at high salt concentrations, when complete breakdown of the envelope is apparent.

Table 6:9 Effects of cations on removal of DNA and protein

Ion concentration		E_{260}°	E_{280}°
KCl	5 ml	1.70	1.04
	10 ml	1.86	1.13
	15 ml	2.00	1.21
	20 ml	1.85	1.09
	40 ml	1.56	0.85
	250 ml	1.23	0.69
NaCl	10 ml	Not determined	
	20 ml	Not determined	
	30 ml	1.62	0.95
	60 ml	1.30	0.74
	100 ml	0.79	0.47
	120 ml	0.65	0.42
	300 ml	1.61	0.95
	500 ml	∞	>2

On comparison of the results summarised in table 6:9 with those in tables 6:6 and 6:7, it may be concluded that the effects of a given ionic strength are comparable on washing and on gradient centrifugation; however, table 6:9 suggests that rather large amounts of protein and nucleic acid are removed by washing in the low ionic strength media (5 ml KCl; NaCl up to 60 ml). It is possible that there is incomplete pelleting in these media at 30,000 X g after 30 minutes, perhaps because envelope fragments repel one another electrostatically and the ionic strength is too low to counteract such repulsion. Incomplete pelleting is also apparent in the case of the 500 ml NaCl wash; here, however, the ionic strength must be sufficient to suppress repulsion, and the failure of the particles to pellet at

30,000 X g may be explained in terms of the very small mean particle size. 500 mM NaCl brings about extreme DNA depletion but not so much lipid depletion (table 6:8); the particles of membrane in this suspension will therefore probably be of low density. If this is the case, presumably sucrose and sorbitol potentiate the reassociation of the removed DNA with the fragments, since pelleting occurs in gradients at this salt concentration.

Discussion

The very marked effects of ionic strength on envelope composition, morphology and gradient behaviour, and the different effects exerted by different cations, support the hypothesis (v. supra) that electrostatic interactions are of major importance in determining the structure of the nuclear envelope. (It may be remarked that electron microscopy shows pore complexes to be stable in 20% aqueous ethanol at 0°C for at least 90 minutes. This may suggest that hydrophobic interactions are not of primary importance in determining the morphology of these structures, such interactions being weakened by the lowered dielectric constant; however, the same conditions would also strengthen the already significant electrostatic bonds.)

It is notable that at concentrations below those required to degrade the envelope morphologically, e.g. 0.041 M NaCl, the pore complexes become sharply defined as if amorphous material has been lost from the annulus perimeter (Cf. Abelson and Smith[1]). This may in fact represent a first stage in degradation; a very small amount of material is lost, insufficient significantly to alter the density of the envelope but sufficient measurably to enrich the upper parts of a gradient - e.g. 25 mM NaCl. The results with 60 mM sodium and 10 mM potassium, in which medium the envelope morphology is destroyed, suggest that the sodium and potassium effects are additive at these concentrations;

this mixture of salts has much the same effect on the envelope as 15 or 20 mM potassium. Probably, therefore, the sodium and potassium effects are similar in mechanism at these low concentrations.

In summary of these results, it appears that, at low concentrations, sodium and potassium ions lead to the loss of components from the envelope and a consequent lowering of density, and a fragmentation of the cytos which, increasing the mean charge density around the particles probably increases electrostatic repulsion at the pH values used in the experiments. At a certain concentration, a critical amount of "structural" DNA has been removed and the envelope morphology collapses; the concentration of potassium required to bring about this effect is in the order of 15 mM; the sodium concentration is considerably higher (around 60-100 mM). At concentrations of salts above those at which the morphology is destroyed - i.e. at which the density of the cytos is minimised - lipid and protein are removed from the cytos, the density rapidly increases again, and the membranous but largely nucleoprotein remnant of the cytos pellets on gradients.

During this density-increase, however, the effects of the two cations are once again rather dissimilar. Lipid is removed and dispersed more completely by the potassium; a viscous suspension of low light scatter results when the potassium concentration is in the range 250-300 mM. Sodium over this concentration range removes less lipid, and hydrophobic interactions (presumably) between DNA-depleted fragments lead to aggregation and increase in light-scatter.

At very high salt concentrations, in the order of 1.5 M (detailed investigations of such conditions were not carried out since destruction of the envelope is more or less total), aggregation is less in evidence. Probably the minute, nucleic-acid depleted membrane

vesicles which remain at such ionic strengths adsorb ions and electrostatic repulsion is again significant. This hypothesis is supported by the rapid and irreversible aggregation which occurs when the salt is removed by dialysis against distilled water or phosphate buffer pH 7.5.

Under conditions of high ionic strength, in general, material rich in lipid is separated from material rich in NNA; on gradients, the former tends to float and the latter to pellet. As the ionic strength is further increased, the DNA-rich material is further degraded; DNA is dissolved and vesicles are released which band on gradients at a density of around 1.20 g/ml (Cf. Kuehnig and Kasper[2]; Franke et al[3]).

OTHER IONIC EFFECTS ON THE ISOLATED ENVELOPE

The foregoing results suggest that, while electrostatic effects are important in controlling the composition and hence the morphology and gradient behaviour of nuclear envelopes, not all ions have the same effects. Differences between monocations have been demonstrated; differences between anions remain to be investigated. Furthermore, additional evidence is required for the hypothesis that lipid is removed from the envelope by chelating agents, and that a consequence of removing lipid is to increase the density of the system. The comparison between the effects of EDTA and magnesium will therefore be investigated.

Methods

As before, zonal gradient centrifugation and the contents of supernatant and pellet before and after washing and centrifuging at 30,000 X g for 30 minutes were examined. In these experiments, the following ionic conditions were investigated:-

a) 60, 120 and 300 mM $NaNO_3$ and NaI (to compare with similar concentrations of NaCl);

b) 0.1, 0.5 and 3.0 mM $MgCl_2$;

c) 10 mM and 100 mM EDTA.

The disodium salt of EDTA was adjusted to pH 7.5 with 1 M NaOH prior to use; the $MgCl_2$ was found to have a pH of around 5.0, but adjustment of the pH to 7.5 with NaOH made no detectable difference to the results.

Results and discussion

Table 6:10 summarises the effects of these various conditions on sorbitol gradients.

Table 6:10 Miscellaneous effects on gradients

Ionic conditions	Band density (g/ml)	Pellet	Top	Solubilised
$NaNO_3$ 60 mM	-	◇◇	◇◇	(◇)
120 mM	-	◇◇	◇◇◇	◇
300 mM	-	◇◇	◇◇◇	◇◇
NaI 60 mM	1.27	-	(◇)	(◇)
120 mM	1.27	-	◇	(◇)
300 mM	1.27	-	◇	(◇)
$MgCl_2$ 0.5 mM	-	◇◇◇	(◇)	-
EDTA 10 mM	1.27	(◇)	◇◇	◇◇
100 mM	-	◇◇	◇◇◇	◇◇

Here, again, the dash (-) signifies "nothing detected".

The salient features of these results appear to be, first, that different anions, like different cations, do indeed have dramatically different effects on the nuclear envelope; second, that magnesium ions cause pelleting of the envelope, possibly because of the removal of bound water (v. infra) and the consequent increase in buoyant density; third, that EDTA does, even at low concentration, enrich the membrane of the gradient and therefore probably removes lipid,

but may also remove other components from the envelope. 100 mM EDTA causes morphological destruction of the envelope, but this may be a simple ionic strength effect rather than a specific chelating action. The sodium ion concentration of 100 mM EDTA is fairly high at pH 7.5.

Table 6:11 Miscellaneous effects on envelope composition

Ionic conditions	DNA/Lowry	DNA/lipid P	Lipid P/Lowry
$NaNO_3$ 120 mM	0.54	2.6	0.21
NaI 120 mM	0.47	3.6	0.13
$MgCl_2$ 0.1 mM	0.51	3.6	0.14
0.5 mM	0.48	4.8	0.12
3.0 mM	0.49	2.5	0.19
EDTA 10 mM	0.47	3.1	0.15
100 mM	0.82	4.1	0.20

The sodium nitrate and iodide were compared at only one concentration since their effects at all concentrations used were closely comparable on gradients. In the above results, the 10 mM EDTA envelope composition differed from that of the standard preparation conditions only in having a higher DNA/lipid and a lower lipid/protein ratio, i.e. showing specific lipid depletion. At 100 mM concentration, however, EDTA appears to result in extensive protein depletion as well as lipid depletion. The consequent apparent DNA enrichment may well account for pelleting on the gradient.

Sodium iodide, too, appears to cause some limited lipid depletion, but this is presumably not sufficient to increase the envelope density. Nitrate ions appear to cause specifically a depletion of protein.

Explanation of the results with magnesium ions is not so straightforward. If water is regarded as depleting the envelope of all three major components to more or less comparable extents, then it may be suggested that, as the magnesium concentration is increased,

it protects against the removal of, first, DNA, second, protein, and finally, lipid. Further increase of magnesium concentration does not bring about any significant change in composition, though the envelope material continues to pellet on gradients, until it reaches about 100 Å. The envelope is then dispersed, morphological integrity is destroyed, and the membranous remnant tends to vesiculation. The viscous suspension resulting is of low light-scatter, and DNA-depleted membrane fragments can be recovered by differential centrifugation (Cf. Doreswy, Funk and Crane[4]).

Table 6:12 Miscellaneous effects on removal of DNA and protein

Ionic condition	E_{260}°	E_{280}°
$NaNO_3$ 120 Å	1.80	1.42
NaI 120 Å	∞	0.80
$MgCl_2$ 0.1 Å	1.70	1.28
0.5 Å	0.89	0.54
3.0 Å	0.205	0.16
EDTA 10 Å	0.37	0.07
100 Å	0.59	0.175

The relatively low $E_{260}^{\circ}/E_{260}^{\circ}$ ratio of the sodium nitrate washing supernatant supports the explanation for the assay results (v. supra) that this anion potentiates the removal of protein. Similarly, the decrease in this ratio as the EDTA concentration is raised supports the hypothesis that the chelating agent also tends to remove protein at the higher concentration. The suggestion that increase of magnesium concentration decreases the tendency for water to remove all components, DNA being protected somewhat before protein, is also roughly supported by the figures quoted in table 6:12.

GENERAL DISCUSSION

Since the effects on the nuclear envelope of a wide range of

ionic conditions have now been investigated, the significance of these effects for the understanding of the system as a whole will be discussed under three headings: the molecular mechanism of the effects, comparison of the stability of the envelope in the nucleus and in isolation, and the physiological significance of the findings.

1. The molecular mechanism of the ionic effects

An explanation of the ionic effects at the molecular level must have recourse to the theories of solution chemistry, and might be corroborated by empirical accounts of ionic effects on pure proteins in solution.

In aqueous solution, the effective size of the ion (the hydrated ion size) is a function of the unhydrated ion size and the hydration number. The former of these can be obtained with considerable precision from crystallographic studies, but the latter (the average number of water molecules bound per ion), which can be calculated from the transport number, diffusion properties, ion-exchange properties, etc. of the ion, varies enormously according to the method of determination. Consequently, the hydrated ion size shows more dependence on the method of calculation than on the ion concerned; it is not unusual, in discussion of problems in physical chemistry, to select the values which most satisfactorily explain the phenomena under observation without consideration of theoretical appropriateness. Furthermore, several interpretations of ionic effects on macromolecular complexes such as the nuclear envelope are possible. For example, each ion may be considered in terms of (a) the probability of its entry into a particular structural site (a function of hydrated ion size and of charge distribution and steric hindrance around the site) and (b) the probability of its causing disruption after entry. Using this approach, it might be argued that (1) potassium has a higher probability of

entering the DNA-protein linkage at the pore complex than has sodium, (ii) after a certain number of ions have entered the linkage, the probabilities of disruption are equal for sodium and potassium. Such an interpretation requires that the hydrated sodium ion has a greater radius than the hydrated potassium ion. Alternatively, the envelope structure might be considered dependent on bound water, and the effect of any ion is therefore determined by its effect on this water-structure. This presumably means that potassium has a greater effect than sodium on the water structure over the temperature range considered; hence its tendency to bind water must be greater; hence potassium must have a greater hydrated ion size than sodium.

Further complications are introduced when consideration is given to disturbances induced in the system by ions which have already entered; that is, the n^{th} ion to enter is interacting with a physically different system from the $(n-1)^{th}$, and itself changes the system still further, no matter whether the effect be described in terms of probability of entry or of water-structure. A reasonably consistent explanation might be reached if the effect of, say, all the alkali metal halides were thoroughly investigated. However, the value of attaining such a solution would have to be weighed against the lack of physiological relevance of the greater part of such a study, and against the quantity of information about the factors determining stability of the nuclear envelope in general which the study would yield.

The difficulties inherent in molecular interpretations of the observed effects are further illuminated by accounts of ionic effects on pure proteins in solution. Such effects are usually considered under two headings: moles of water bound per mole of protein, and moles of salt bound per mole of protein, at a given salt and protein

concentration in solution. Bull and Breese[5], in a paper dealing with the effects of a range of salts on aqueous solutions of egg albumin, show no drastic differences between sodium and potassium chlorides; in the presence of the latter, the protein binds relatively more of both water and ions, but the difference is not great. In sodium iodide and election halides, very little water but a great deal of salt is bound; on the nuclear envelope, however, the effects of these salts were seen to be very different. Both guanidine hydrochloride and urea were found to bind extensively to egg albumin (up to 32 or 33 moles per mole of protein) and water binding was very greatly reduced. (Urea, even at 1 M concentration, was found to disrupt the nuclear envelope structure; this may suggest the significance of water binding, or a greater significance than hitherto suspected of hydrophobic interactions, in the maintenance of envelope structure). The impossibility of extrapolating from the findings of Bull and Breese to the situation regarding the nuclear envelope is clear, and further emphasises the enormous difficulty of attempting at this stage an explanation at the molecular level of the observations.

2. <u>The stability of the envelope in the nucleus and in isolation</u>

The factors tending to disrupt the envelope in isolation and to destabilise it <u>in situ</u> in the nucleus are largely comparable. Beyond the superficial resemblances, however, several points of difference can be noted, and these points of difference seem to depend on the presence or absence of an asymmetry in the distribution of organised bulk macromolecules on the two sides of the envelope:-

1) The conditions disrupting the isolated envelope do not actually disrupt the envelope in the nucleus; the envelope in the nucleus can still be recognised after prolonged treatment with DNase, potassium, or a pH of around 6.

2) If an attempt is made to isolate the envelope at a pH of 7 or below, the material obtained is very rich in DNA; treatment of the isolated envelope at such a pH does not give DNA-rich material.

3) Treatment of the isolated envelope with EDTA readily destroys its morphology; again, contiguous chromatin appears to protect the envelope in this case (see chapter 4).

It may be inferred from these considerations that if chromatin be attached to some part of the envelope, then, independent of the isolation procedure used, the envelope will be morphologically intact in those parts. In the electron micrographs published by Kashmig and Kasper[2] and by Franke et al[3], such intact regions are visible; repetition of these preparations in this laboratory show that such regions indeed account for an unpredictably, always very small, but always present part of the total membranous material obtained.

Conversely, it may be argued that the standard procedure described in chapter 5 yields only a specialised chromatin fraction - a nucleoprotein complex of defined morphology. However, when the definitions of the various chromatin fractions are considered, this argument breaks down. The morphological, or cytological, definitions of the recognised chromatin fractions rely on their overall appearance under defined conditions of electron microscopy, and the appearance of the nuclear envelope does not coincide with that of any of these recognised fractions. The alternative, operational definition of chromatin fractions as macromolecular complexes derived from nuclei by procedures such as those outlined at the end of chapter 2 does not include any procedures comparable with the standard procedure for envelope isolation. Thus, the nuclear envelope qualifies as a chromatin fraction neither morphologicallynor operationally.

Nevertheless, the envelope is morphologically dependent on the

presence of DNA, if not chromatin, and undoubtedly participates in peripheral chromatin organization (see chapter 1). The two points are presumably not unrelated. DNA and protein, with perhaps other components, make up the pore complexes which appear to organize the rest of the envelope (certainly the envelope collapses and vesiculates when the pore complexes are destroyed). The DNA attached to the envelope (presumably the inner membrane) may be regarded as a focus around which other peripheral intranuclear material is organized; such material presumably includes the pore-complex DNA. Thus, total disruption of the peripheral chromatin necessitates disruption of the envelope, and vice-versa. This conclusion is consistent with the observations of peripheral chromatin organization and of the disappearance of the envelope when the chromatin is reorganized in mitosis (see chapter 1).

This account suggests that most of the lipid is not centrally involved in the structural organization of the envelope-chromatin complex, and this in turn explains - given the direction dependence of lipid binding - the apparent stability of the system in chelating agents. However, to explain the difference of observed effect at pH 6 in the isolated and non-isolated envelope, the titration of surface groups and the consequent random adsorption of any chromatin present must be invoked. The structural stability of the total nucleus to EDTA and potassium may depend either on steric hinderance of the envelope sites labile in isolated preparations, or on competition for the destructive agent by the bulk intranuclear chromatin. Alternatively, the DNA-pore complex linkages may be broken but the peripheral chromatin may remain sufficiently intact to keep the envelope in an apparently stable state. If this last explanation is the most usefully applicable, it may also explain why isolation of the

envelope after potassium or DNase treatment of the nuclei is impossible; the envelope, after the treatment, is entirely stabilised by the peripheral chromatin; thus, breakage of this chromatin (which is inevitable in the isolation attempt) destroys the envelope.

3. Physiological relevance of the findings

Mitosis, as discussed above, may be regarded as involving envelope destabilisation generalised over the whole nuclear surface. Blebbing activity (see chapter 1) may be regarded as a more or less localised destabilisation. The onset of chromatin condensation in mitosis is associated with increased cation flux into the nucleus, and also with maximum sodium and potassium content of whole cells (Jung and Rothstein[6]). The chromosomes are thought to condense on the pore complexes (see chapter 1), and the extreme changes in charge distribution which presumably accompany this process may distort the predominantly electrostatic binding forces which stabilise the envelope tangentially, thus triggering the collapse and disposal of the envelope. It is interesting that this collapse - degeneration into minute single-membrane vesicles - is morphologically comparable to the degeneration caused by potassium and DNase in the isolated system. It is, given the above information, conceivable that a sharp increase of potassium influx into the nucleus is responsible for the removal of the perinuclear dense chromatin which precedes chromosome condensation; the condensation may itself be due in part to the cation influx, though this cannot be the whole explanation since simple increase in the ambient cation concentrations does not induce changes similar to those involved in prophase (see discussion in chapter 1). Further to this hypothesis, it is interesting to note (Jung and Rothstein[6]) that a rapid efflux of ions from the cell occurs after the onset of division. If the fluxes in the nucleus

roughly parallel the fluxes in the whole cell, then ionic conditions in telophase must be suitable for the regeneration of the envelope and of the perinuclear chromatin zone. Moreover, Nicolas[7] has reported that sea-urchin oocytes deprived of potassium do not divide; division recommences when potassium is added to the medium.

Taking into account the suggestion (see chapters 1 and 2) that DNA replication is initiated in DNA attached to the envelope, it may be inferred that a stable chromatin-envelope complex is necessary during the S phase. It is interesting, therefore, to note that Jung and Rothstein[6] report minimum cell potassium content at the onset of this stage in the cell cycle.

It is reasonable to suppose, therefore, that concentrations (and particularly potassium) are important in bringing about the various structural and functional changes in the nuclear envelope which have been observed in a number of cells. Bivalions are, presumably, always present in sufficient concentrations to stabilize lipid, and changes in magnesium and calcium levels become important only in chromatin condensation in prophase. Changes in local hydrogen ion activity, if they occur, may not be of great significance in mitosis and DNA synthesis; in blebbing, however, it is conceivable that such changes around "pH 7.2" are important in varying the ionic binding energies between inner and outer membrane, or inner membrane and chromatin, and between the membranes and pore complexes. A large, "pH"-induced decrease of strength of chromatin and pore complex binding to one or both membranes may induce an outpocketing in that region. Clearly, however, other explanations of blebbing are possible.

The observed protecting effects of sucrose and sorbitol may be due to changes in bound water structure around the envelope which may be regarded as a slight lowering of dielectric constant, either

increasing the energy required to disrupt electrostatic bonds or destabilising hydrophobic interactions. The physiological relevance of this finding may be extended beyond the effects that these and other carbohydrates may have, to factors which in general tend to lower "dielectric constant"; the general tendency of intracellular water molecules to be less free to move randomly than is the case in bulk extracellular water may be involved here. The generally rather low intracellular "dielectric constant" may make electrostatic bonds more stable *in vivo* than they appear to be in isolated systems.

For this and for other reasons caution must be observed in attempting to extrapolate from observations made on the isolated envelope to the situation *in vivo*. The major problem here concerns the definition of parameters like pH, dielectric constant, ionic strength, etc. and will be discussed at greater length in part IV. Nevertheless, the findings of this and the two previous chapters give rise to several interesting suggestions about the mechanisms involved in observed changes during mitosis and blebbing, and about the general maintenance of envelope stability during interphase.

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PART III

COMPONENTS OF THE NUCLEAR ENVELOPE

CHAPTER SEVEN

THE DNA COMPONENT OF THE NUCLEAR ENVELOPE

SUMMARY

1. The standard procedure for the isolation of the nuclear envelope is applied to mouse liver and, with minor modifications, to L-cells in tissue culture.

2. The DNA of the envelope is found, in the case of liver, to contain no detectable satellite. In envelopes from L-cells, however, the DNA consists of both main-band and satellite.

3. Free DNA added to a suspension of sonicated or unsonicated nuclei adheres to the surface and is recovered in the envelope fraction. The implications of this finding are discussed.

INTRODUCTION

The existence of DNA in the intact nuclear envelope and its apparent status as an essential structural component of the system generate the following problems:-

(a) Is the envelope DNA a random fraction of the total nuclear DNA, or is it a specific fraction, relatively homogeneous?

(b) Is the envelope DNA exchangeable with any free DNA added to the nuclear suspension? It may be either that the DNA recovered in the envelope is present in the envelope in the intact nucleus, and is not contaminated during isolation with other nuclear DNA; or that the envelope in the nucleus is essentially free of and structurally independent of DNA, and that the high DNA content observed and the structural dependence on this DNA are artifacts of preparation. By adding labelled DNA to sonicated nuclei and determining the fraction of the label recovered in the envelope fraction, it would appear possible to distinguish between these possibilities.

Heterogeneity in the DNA can be determined by centrifugation

through caesium chloride of the total DNA extracted from the system. In the case of rat, the heterogeneity is not clearly seen. Mouse DNA is, however, clearly differentiated into main band (some 90% of the total) and a single satellite band (10% of the total)[1]. For the experiments described in this chapter, therefore, the procedure for the isolation of the nuclear envelope established in chapter 5 was adapted to mouse material. The entire procedure proved to be directly applicable to mouse liver; the results were identical to those for rat liver in the conditions used and in the morphology, composition and gradient behaviour of the resulting envelope. In the case of a mouse L-cell strain in tissue culture, however, though the method for isolating the envelope from the nuclei was identical with that described for liver, the method for isolating the nuclei differed from that used in the case of liver. L-cell nuclear envelopes have the same overall composition as liver envelopes (see chapter 5).

THE ISOLATION OF L-CELL NUCLEI

L-cells recovered from the culture medium by centrifugation at 800 X g for 10 mins were ruptured by osmotic shock and mild mechanical disruption (v. infra — bottom of page) and the nuclei were recovered by differential centrifugation. The cells were suspended in 10-20 vols 0.01 M sodium phosphate buffer, pH 6.5, containing 1 mM $MgCl_2$; this concentration of magnesium was sufficient to stabilise the nuclei. After incubation at 0°C for 5-10 mins, the cytoplasm was swollen but the nuclei had retained their original diameter so far as could be judged under the phase contrast microscope.

Three ~~gentle~~ passages through a hypodermic needle, insufficiently vigorous to produce frothing, was in most cases sufficient to break the cells in this condition; 1-2 more passages were used if more than 20% of the cells remained intact as

determined by phase contrast microscopy. The nuclei were pelleted by centrifugation of the suspension at 800 X *g* for 10 minutes, and after 1 wash in the same buffer appeared to be free of cytoplasmic contamination.

THE ISOLATION OF DNA

DNA was isolated from total nuclei and from envelopes by a modification of the procedures of Marmur[2] and Walker and McLaren[3]. The suspension was dispersed in 1% sodium dodecyl sulphate and shaken vigorously with chloroform:octanol (24:1 v/v). After centrifugation to separate the phases, the upper aqueous phase and interface were recombined and made 0.3 M sodium trichloracetate. Extraction of the suspension with an equal volume of chloroform:octanol was repeated a further six times, the lower phase and interface being discarded after centrifugation following each extraction. Nucleic acid was precipitated from the remaining aqueous solution by the addition of 2 vols ethanol and storage overnight at 5°C. Much of the nucleic acid precipitates almost immediately on addition of the ethanol, but some material is precipitated rather more slowly. Phenol was avoided in this sequence of extractions, since it was found to produce a rubbery and intractable aggregate with envelope protein; it is possible that DNA could have been lost in this aggregate.

The nucleic acid was redissolved in 1 ml 0.1% sodium dodecyl sulphate - 0.01 M sodium citrate, pH 7.3, and RNase added to 0.1 mg/ml concentration. After incubation at room temperature for 90 minutes, the solution was submitted to a further 3 extractions with chloroform: octanol. The DNA was finally precipitated by the addition of NaCl to 0.5 M concentration and 2.5 vols absolute ethanol. All the insoluble material was precipitated in 2 hours at -20°C.

Lowry and orcinol assays of the final material in solution at a concentration of 1 mg/ml showed no detectable colour formation. It was concluded that the DNA preparation contained less than 0.8% protein and less than 2.5% RNA.

EXAMINATION OF THE DNA IN THE ANALYTICAL ULTRACENTRIFUGE

Analytical ultracentrifugation of DNA isolated from mouse liver nuclear envelopes was carried out to establish whether or not the DNA represented a random or a specific fraction of the total nuclear DNA.

Methods

DNA prepared as described above was dissolved in 10 mM tris-HCl, pH 8.0 and the solution mixed with CsCl (British Drug Houses), AnalaR grade, to a final DNA concentration of 3-5 µg/2.5 ml and a density of 1.715-1.720 g/ml. DNA from Micrococcus lysodeikticus, having a density of 1.731 g/ml (Schildkraut et al[4]) was added in 1-3 µg concentration as a marker. The solution was then centrifuged at 44,700 rev/min in an M.S.E. Analytical Ultracentrifuge at 25°C for 24 hours, and the gradient photographed in the ultraviolet (260 nm), using bromine or chlorine gas filters and Kodak film (Hennig and Walker[5]). The photographs were scanned with a MK IIIC double-beam recording microdensitometer (Joyce-Loebl and Co.). This experiment was performed on envelopes andtotal nuclear material from both mouse liver and mouse L-cells.

Results

Fig. 7:1 shows the results for liver. The main band and satellite fractions are clearly distinguishable in fig. 7:1(a), representing DNA obtained fromwhole nuclei. In the envelope DNA, however, the satellite is not visible (fig. 7:1(b)). The liver envelope DNA appears to contain only main band material.

Fig. 7:1

Microdensitometer scans of CsCl gradients of mouse liver and liver envelope DNA. (See text for experimental details.)

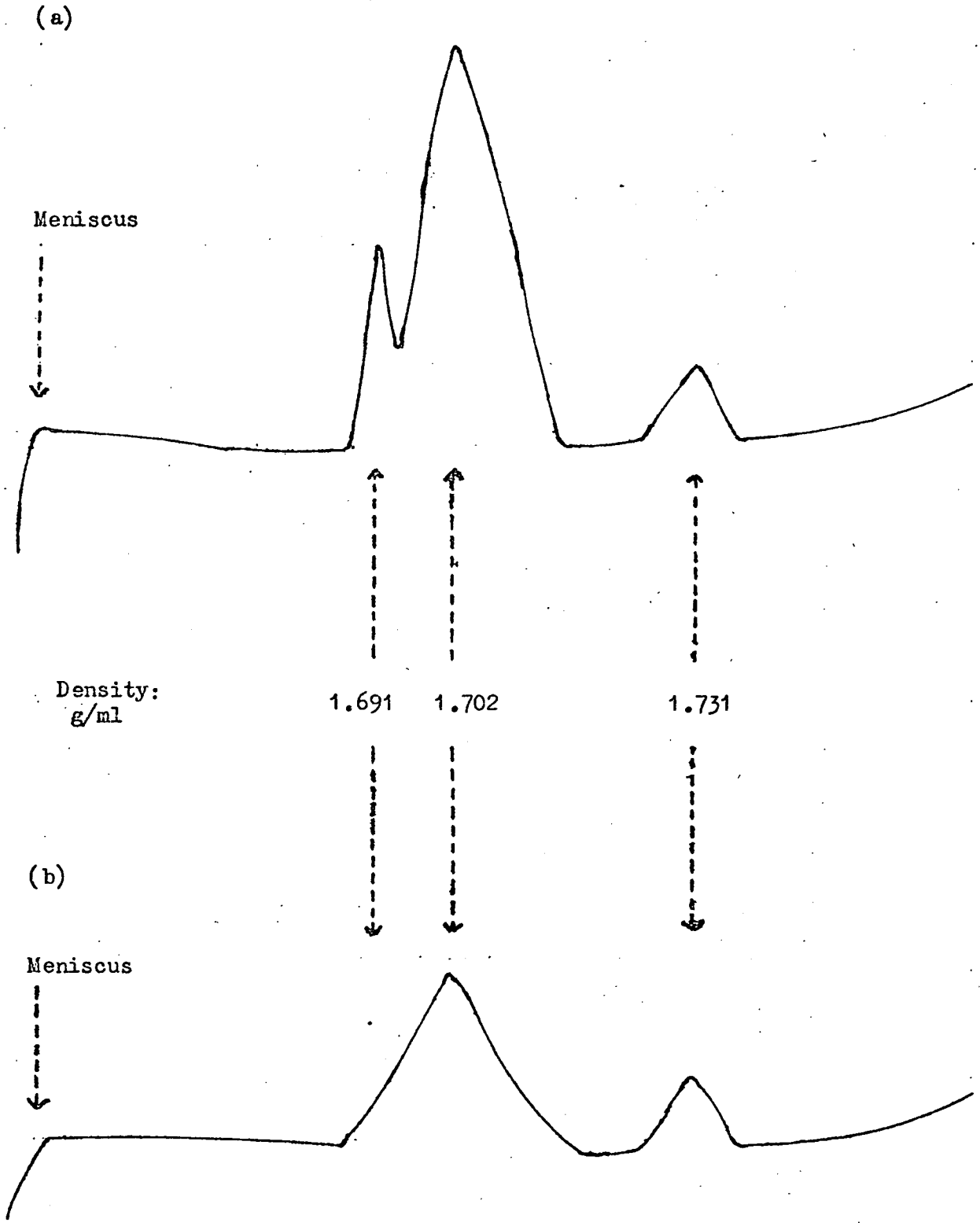
(a) Total mouse liver DNA

The satellite (density = 1.691 g/ml) and main band (density = 1.702 g/ml) fractions are clearly visible and clearly distinct. The *Micrococcus* marker DNA is represented by the peak of density = 1.731 g/ml.

(b) Mouse liver envelope DNA

The main band peak (density = 1.702 g/ml) is visible as before, but no satellite DNA is apparent. The relative diffuseness of the main band peak may be attributable either to fragmentation of the DNA during the sonication procedure, or to marked heterogeneity in base composition, and hence in density, of this small fraction of the total liver DNA. The *Micrococcus* marker (density = 1.731 g/ml) appears as before.

Fig. 7:1



The results obtained from L-cell nuclei are shown in fig. 7:2. Both nuclei and envelopes contain both main band and satellite fractions.

In fig. 7:3, the results for the DNA isolated from envelopes prepared from liver nuclei in the presence of 0.02 M KCl are presented. These envelopes are disrupted. However, it is once again apparent that the satellite fraction is absent. The gross DNA composition of the envelope appears therefore to be independent of the integrity of the envelope.

Fig. 7:4 shows the presence of both satellite and main-band material in DNA isolated by the procedure described earlier in this chapter from the 3,000 X g pellet of the sonicated liver nuclei. This pellet contains dense chromatin (largely heterochromatin and some intermediate chromatin, Cf. Frenster et al[6]) along with nucleoli.

Discussion

The results obtained with mouse liver indicate that the DNA of the envelope is not a random fraction of the total nuclear DNA. The satellite fraction, which accounts for some 10% of the DNA in the nuclei (Bond et al[1]) is absent. This finding is consistent with reports which suggest the satellite is associated with either the nucleoli[7] or the heterochromatin[8].

In L-cells, however, there is satellite associated with the envelope. This difference between the liver and L-cell material indicates no more than a difference in the mean affinity of satellite for the envelope in the two cell-types, such that the satellite is readily removed by the isolation procedure in the case of liver but not in the case of L-cell envelopes; the affinity in the case of liver may or may not be so low as to be functionally insignificant in vivo. One possible explanation for the observed difference lies

Fig. 7:2 L-cell Envelope DNA

Microdensitometer scan of CsCl gradient of mouse L-cell nuclear DNA. In contrast to the liver envelope DNA, it can be seen that both satellite (density = 1.691 g/ml) and main band (density = 1.702 g/ml) DNA fractions are present, in more or less the same proportions as in total nuclei. The Micrococcus marker appears at density = 1.731 g/ml.

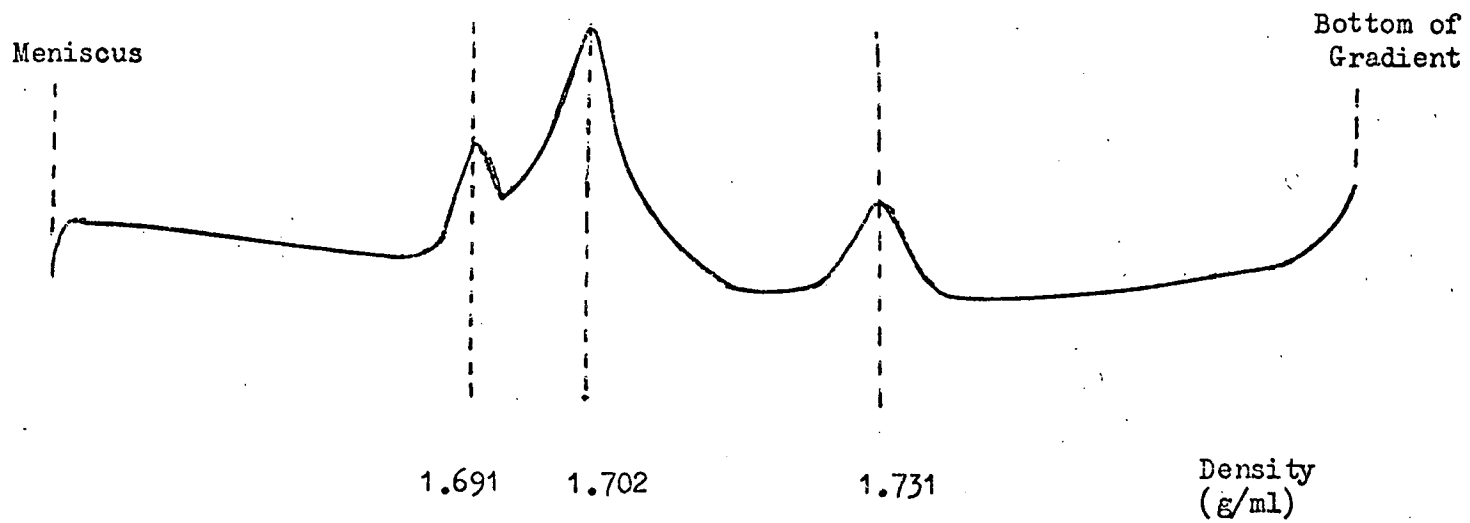


Fig. 7:3

CsCl gradient of DNA from mouse liver envelopes isolated in the presence of 0.02 M KCl. The morphological disruption of the envelope does not significantly alter its DNA composition; the satellite is still apparently absent.
(Cf. fig. 7:1(b))

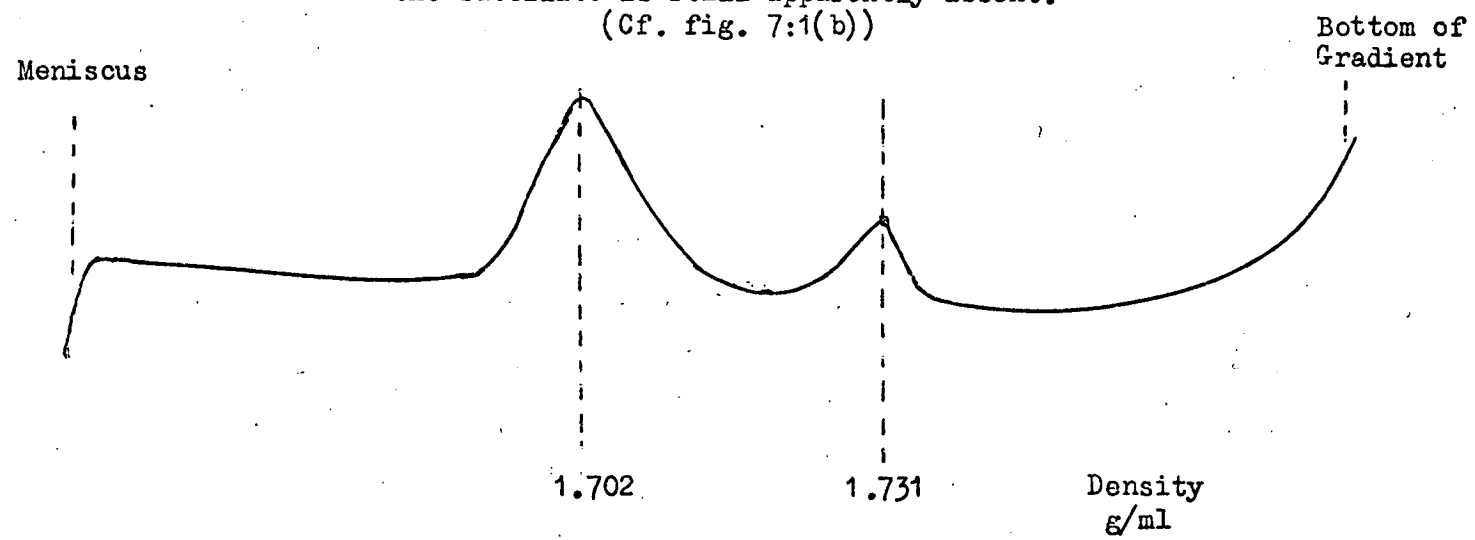
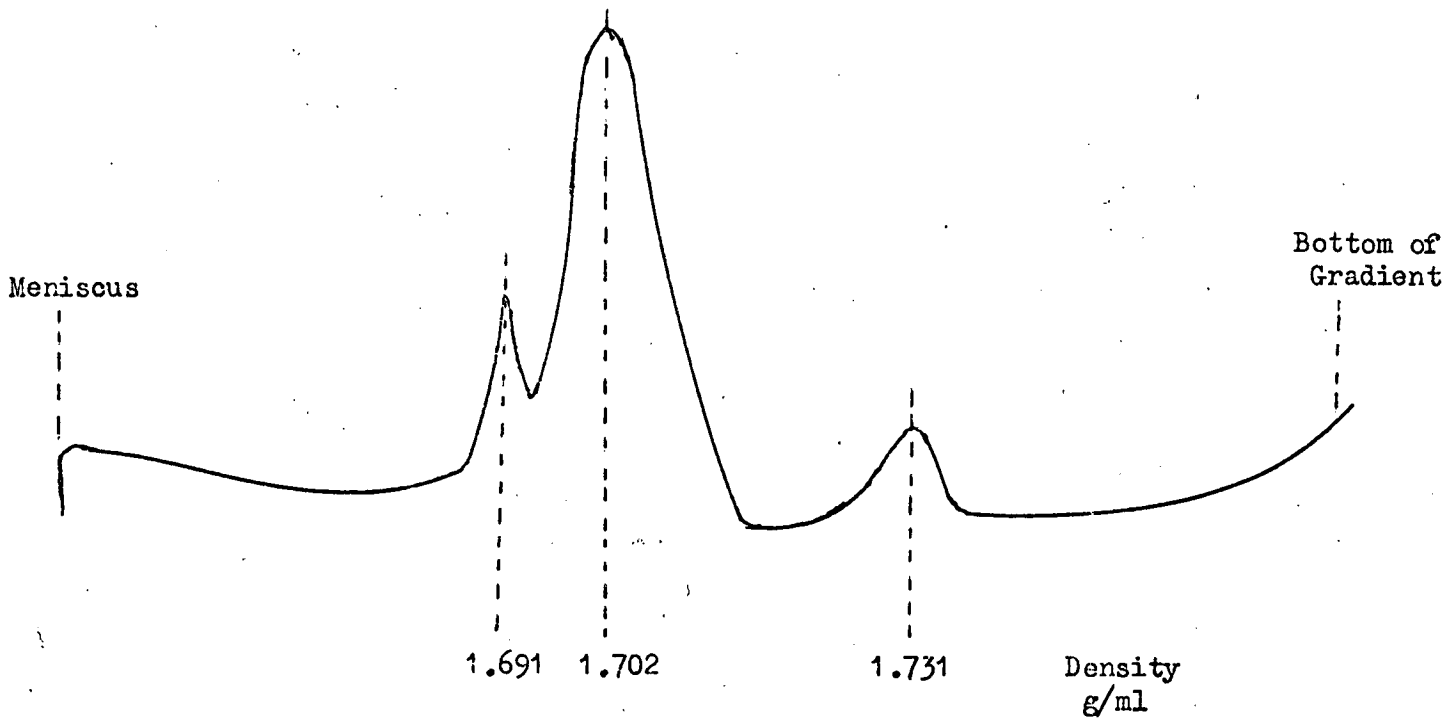


Fig. 7:4

CsCl gradient of DNA from 3,000 X g pellet of sonicated mouse liver nuclei. Both main band and satellite fractions are visible.



in the rates at which the cells are dividing. The rates for the two types are such that, in a random sample of n cells, the probability of finding at least 1 cell in S-phase is significantly greater in the case of the L-cells than in the case of liver. If DNA replication is associated with the envelope (see chapter 1), then the probability of isolating envelopes with replicating DNA is much greater in the case of L-cells than in the case of liver. If satellite is associated with the envelope only while it is being replicated, then envelope-associated satellite might be expected to appear in an asynchronous L-cell culture, but not in comparable amounts in liver.

The first conditional hypothesis, that DNA replication occurs on or is initiated on the envelope, is supported by considerable experimental evidence as reviewed in chapter 1. For the second hypothesis, however, no independent empirical support can be found. It is possible that transcription might be to some extent associated with the envelope, and that only transcribable DNA will therefore be associated with the envelope during interphase. If the satellite fraction is not transcribed *in vivo*, cf. Flamm *et al*[9], then this is consistent with the observations and foregoing arguments.

The extent of association of RNA synthesis with the envelope is, however, questionable. RNA polymerases appear to be soluble in most cases[10]; if this is so, then they cannot have a high affinity for a membrane fraction. Nevertheless, synthesis of RNA in the region of the envelope, perhaps within the pore complex, might in part overcome the energetic problem of passage of these macromolecules from nucleus to cytoplasm.

THE EXCHANGEABILITY OF THE ENVELOPE DNA FRACTION

An attempt was made to determine whether the envelope DNA was exchangeable, by isolating envelopes from unlabelled nuclei in the

presence of excess ^{32}P-labelled DNA. The extent of label in the isolated envelopes might be regarded as a measure of the exchangeability of the envelope DNA, and hence as a measure of potential DNA contamination during isolation.

However, it was found that the excess DNA bound to intact nuclei as well as to the envelope fraction of sonicated nuclei. This made interpretation of binding to the envelope difficult. Further experiments to circumvent the difficulties of interpretation were therefore undertaken on the basis of the following argument, though simple conclusions are not possible:-

Since DNA binds to intact nuclei, presumably to their surfaces, to an extent (v. infra) which depends on the ratio between the concentration of excess DNA and the concentration of the nuclei, the DNA may be regarded as occupying binding-sites (not necessarily specific, or homogeneous, or even discrete structural entities) on the nuclear surface. If some of these binding-sites are occupied during sonication by DNA released from the interior of the nucleus, this may be regarded as contamination. Such a process will leave less sites available for binding by excess (labelled) DNA. Therefore, the comparison of the amount of label bound to intact nuclei with the amount bound to the envelope fraction of sonicated nuclei (and therefore recovered in the isolated envelopes) will give a measure of the contamination of the envelope by intranuclear DNA during sonication and isolation. It was found (v. infra) that extraneous labelled DNA bound in similar amounts to both intact nuclei and the envelope fraction of sonicated nuclei. From this it was tentatively concluded that the contamination of the envelope by DNA during sonication is negligible.

Methods

^{32}P-labelled main band mouse DNA was obtained from L-cells in culture by the modification by Flamm et al[9] of the method of Walker and McLaren[3]. After recovery from the CsCl gradient, the DNA was dialysed for 2 X 12 hours against a large volume of distilled water.

The following experiments were performed:-

1) A series of concentrations of labelled DNA was added to aliquots of nuclei; the nuclei were then recovered and counted.

2) A similar series of concentrations was added to aliquots of sonicated nuclei; the envelopes were then isolated and counted.

These experiments gave a measure of the binding of label to nuclei and to envelopes, and hence were expected to provide an indication of the exchangeability of the DNA. Results were expressed as $\frac{\text{counts added}}{\text{counts recovered}}$ X 100%. Since the surface area of nuclei = area of envelopes was proportional to the concentration of nuclei (sonicated or unsonicated), and since the quantity of nuclear DNA or protein was similarly proportional to the concentrations of nuclei, the results from these two experiments were comparable with one another.

3) The effect of more or less prolonged incubation of the nuclei with the label on the recovery of label in nuclei and envelopes was investigated. Incubations were carried out at 5°C for 0, 2 and 16 hours.

4) The binding of labelled main band DNA was compared with the binding of labelled satellite.

In all these experiments, the nuclei (sonicated or unsonicated) were obtained from mouse liver and suspended at a concentration of 5 mg protein /ml in 0.02 M tris-HCl buffer, pH 7.7, containing 1 ml $MgCl_2$. The labelled DNA solution (x mls) and distilled water (y mls) were in each case added such that x + y = 0.5 mls.

Results and discussion

In tables 7:1 and 7:2, the figures quoted are means of three readings and refer to the total counts/min added, not to the counts/min in any given volume.

Table 7:1 Recovery of counts in intact nuclei

Counts/min added	Counts/min recovered	% counts recovered
219	201	93
1,097	1,033	94
2,194	2,077	94
5,484	2,019	37
10,968	2,133	21
22,030	603	2.7

The results are consistent with the model of a finite number of binding-sites for DNA on the nuclear surface, with very strong binding at each site. The last value, however, suggests a completely different effect. The specific activity of the DNA here was 4,860 counts/min/O.D. unit at 260 mμ, implying that 22,030 counts/min = 4.7 O.D. units. The implication is that this concentration of DNA (0.5 mls added to 2.5 mls nuclei) is sufficient to disrupt nuclei equivalent to 2.5 X 5 = 12.5 mg protein in such a way as to destroy the binding of the excess DNA.

Table 7:2 Recovery of counts in envelopes

Counts/min:-

Added	Recovered	% Recovery	Recovery in envelopes / Recovery in nuclei
219	171	80	0.86
1,097	987	91	0.97
2,194	1,739	79	0.84
5,484	1,884	35	0.94
10,968	1,826	18	0.86
22,030	518	2.3	0.85

The results in table 7:2 show a pattern similar to those of table 7:1, implying that a similar model can be used in the explanation of the findings. If the binding sites on the nuclei are assumed to be located entirely on the surface, then the last column of table 5:2 suggests that a roughly constant percentage of the sites (11% ± 5.6%) is removed when the nuclei are sonicated. This removal may be interpreted as disruption by the sonication (e.g. the site may be made unavailable by membrane vesicularisation) or as occupation by the intranuclear material.

Since both nuclei and envelopes take up free DNA the interpretation of this experiment is more complex than planned; clearly, simple exchange of labelled with unlabelled DNA cannot be assumed. However, if the removal of binding-sites on the nuclear surface is taken to represent occupation by intranuclear material, maximum contamination is being assumed and the results make it possible to give an upper limit for the extent of contamination of the envelope by intranuclear material during the isolation.

From table 7:1 it can be concluded that nuclei equivalent to 12.5 mg nuclear protein are saturated by some 2,075 counts/min = 0.46 O.D. units DNA/ml at 260 nm = 0.011 mg DNA[12]. Loss of 11% of the binding sites thus implies occupation of the surfaces of the nuclei by 1.2×10^{-3} mg DNA. The 12.5 mg nuclear protein is equivalent to about 1.25 mg envelope protein. Thus the extent of DNA contamination during isolation as determined by this procedure is in the order of 1 µg/mg envelope protein.

This suggestion that contamination of the envelopes by intranuclear material during isolation is negligible is, however, dependent for its validity on the validity of the foregoing assumptions. Possible binding-sites for the excess labelled DNA may already be

occupied by intranuclear material. Since, however, the binding of labelled DNA is very strong, this would imply that extremely strong forces must bind the intranuclear material to the envelope (since the excess DNA cannot, from the assumption of this argument, replace such material). If the forces are extremely strong, however, then they must presumably operate *in vivo*; and if this is so, it is of doubtful value to consider the bound material as a contaminant.

It is concluded from these results, therefore, that the envelope DNA is to only a small extent (<1%) contamination; the remainder is to be regarded as a real component. However, a result that remains to be explained is that which suggests breakdown of the binding sites on addition of excess DNA (approx. 2.2×10^4 counts/min = 0.1 mg/12.5 mg nuclear protein; i.e. 10 µg/mg total nuclear protein). Further to this result, envelopes isolated after addition of 3×10^3 counts/min labelled DNA to the sonicated nuclei, and therefore containing about 10 ug label/mg protein, are completely disrupted on treatment with a further 25 µg labelled DNA/mg protein; centrifugation of the dispersed envelope at 30,000 X g for 30 minutes led to the recovery of only 200-300 counts/min in the pellet, and the bulk of the protein remained in the supernatant. This is consistent with the finding by Bach and Johnson[11] that DNA removes a lipid-rich, protein-containing fraction from nuclei if added in excess.

In conclusion it may be suggested, therefore, that free DNA in low concentrations binds very strongly to nuclear envelopes. In higher concentrations, it disrupts the system. According to Bach and Johnson[11], other polyanions might be expected to have comparable detergent-like actions on the envelope.

Two further questions arise as a consequence of these findings:-

a) Does satellite DNA show the same binding properties as main-band DNA?

b) Does further binding, or disruption of the envelope, occur on incubation of the envelope with a more than saturating amount of DNA?

Table 7:3 presents the results obtained when intact nuclei were incubated with ^{32}P-labelled satellite DNA, in the absence and in the presence of main-band material. It can be seen that the binding is very closely similar in the two types of DNA, and separate binding sites cannot usefully be assumed. (1.7×10^5 counts/min/ml = 1 mg/ml.)

Table 7:3 Binding of satellite DNA to nuclei

(a) Main band absent

Counts/min added	Counts/min recovered	% Recovery
180	141	78
942	895	95
1,856	1,610	87
4,740	1,574	33
9,318	1,639	18
18,502	697	3.7

(b) In presence of 2,194 counts/min main band

Counts/min satellite added	Counts/min recovered	% Recovery°
180	2,091	88
942	2,003	64
1,856	2,180	54
4,740	2,067	30
9,318	1,958	17
18,502	487	2.4

° Recovery here is calculated as $\frac{\text{Counts recovered}}{\text{Counts main band + counts satellite}}$

Binding of the two types of DNA only occurs at concentrations less than those required fully to saturate the available sites on the nuclear surfaces.

Table 7:4 compares the recoveries of labelled main band DNA in the envelopes, 3,000 X g pellet and 30,000 X g supernatant fractions of sonicated nucleoi (Cf. chapter 5) after incubation at 5°C for 0, 2 and 16 hours. The values express the counts recovered in each fraction as percentage of counts added (10,968/min incubated with 12.5 µg nuclear protein in all cases).

Table 7:4 Effect of incubation on distribution of label

Fraction	Immediate isolation	Incubation 2 hrs.	Incubation 16 hrs.
3,000 X g pell.	6.3	5.9	5.0
30,000 X g snat.	76.5	80.0	88.6
Envelopes	17.2	14.1	6.4

It can be seen that a steady decrease of the amount of label in both insoluble fractions (one consisting mainly of heterochromatin, nucleoli, etc., the other of envelopes) occurs over the period of incubation, with consequent enrichment of the supernatant fraction. This result may be taken to indicate that there is a time-dependent element in the dispersal of the envelope by DNA, and that DNA also disperses insoluble bodies other than the envelope.

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CHAPTER EIGHT

IONIC COMPONENTS OF THE NUCLEAR ENVELOPE

SUMMARY

1. The sodium, potassium, calcium and magnesium contents of intact and disrupted envelopes were determined. The dication contents were found to be fairly high (10-100 pM/Lowry Unit) and to depend in a complex way on the integrity of the system.

2. When the envelope preparation was repeated using 1 mM $CaCl_2$ instead of 1 mM $MgCl_2$, it was found that the magnesium content of the system was increased and the calcium content decreased.

3. The results are discussed in the light of published data on ion-protein and ion-DNA interactions.

INTRODUCTION

In part II of this thesis it was established that electrostatic interactions play a major role in the determination and maintenance of the envelope structure. The inorganic ion content of the system may therefore be important in controlling its integrity both *in vitro* and *in vivo*. In chapter 6, a complex range of ionic effects on the composition and morphology of the envelope was described. Furthermore, the ionic content of the nucleoplasm is high, and the ions (particularly dications, Cf. Mirsky and Osawa[1]) appear to be of considerable significance in the organisation of chromatin. For these reasons, an attempt was made to determine the ion content of the isolated envelope.

Of the ions investigated, magnesium and calcium were estimated because of their significant levels in various chromatin fractions, and because of their probable significance in organising the envelope lipid (see chapter 4). Sodium and potassium are present in high concentrations in the nucleoplasm, and have widely different effects on envelope morphology. Their levels in the isolated system are therefore of some interest.

Among the factors expected to determine the results of this investigation was the presence or absence of a particular ion in the isolation media. Magnesium content, for example, might be expected to be higher than calcium content because the former, but not the latter, ion is present in the sonication medium. To control for such an artifact, the preparation was repeated using calcium instead of magnesium in the isolation medium, and the dication contents of the two preparations were compared.

MATERIALS AND METHODS

Isolation of the envelope

Envelopes referred to in this chapter as "Mg-envelopes" were isolated according to the standard procedure described in chapter 5. The preparation described in this chapter as "Ca-envelopes" was made by a procedure identical to the standard procedure, except that the 1 mM $MgCl_2$ of the sonication medium was replaced by $CaCl_2$.

Intact envelopes isolated by such procedures were of the same DNA/protein ratio (see chapter 5). They were disrupted by resuspension in 20 mM KCl and centrifugation at 30,000 X g for 30 minutes.

After isolation and disruption, the envelopes were resuspended in glass distilled water and dialysed against a large volume of glass distilled water for 2 X 12 hours.

Liberation of ions for analysis

A volume of the dialysed envelope suspension having a determined protein content was dried in a test-tube and digested for 5 hours at 100°C with 0.5 mls AnalaR nitric acid (British Drug Houses). At the end of this time the organic matter had been destroyed and a clear colourless solution resulted. This solution was diluted with 4.5 vols glass distilled water and its ionic content determined. For the

determination of Mg^{++} and Ca^{++} an aliquot of the diluted acid digest was neutralised with 1 M NaOH and disodium EDTA added to a final concentration of 5 mM.

Care was taken to ensure that all glassware was thoroughly washed with glass distilled water before use. Contamination was assessed by the use of an envelope-free HNO_3 digest (blank).

Determination of ion content

The diluted digests were analysed for ionic components using a Unicam SP 90A Atomic Absorption Spectrophotometer. The emission spectra of sodium (589 nm) and potassium (766 nm) and the absorption spectra of calcium (423 nm) and magnesium (285 nm) were used in the determinations. The instrument was calibrated using standard solutions of the ion under investigation. All determinations were performed in triplicate. The results were expressed as mM ion/Lowry Unit in the original envelope.

RESULTS

Table 8:1 Envelopes prepared by standard procedure ($HgCl_2$)

Ion	mM/Lowry Unit (intact envelopes) Experiment 1	Experiment 2
Sodium	105	38
Potassium	0.6	0.4
Calcium	50	54
Magnesium	30	27
	mM/Lowry Unit (disrupted envelopes)	
Sodium	26	23
Potassium	0.8	1.1
Calcium	25	31
Magnesium	28	27

It appears (neglecting the high, probably anomalous, 105 mM/Lowry Unit for sodium in the first experiment on intact envelopes) that

disruption of the system with 20 mM KCl reduced the quantity of calcium and sodium bound to the system. The bound magnesium remained at approximately the same level, while the potassium level increased.

In table 8:2, these findings are compared with the results obtained with envelopes prepared in calcium chloride.

Table 8:2 Envelopes prepared in $CaCl_2$

Ion	pM/Lowry Unit (intact envelopes) Experiment 1	Experiment 2
Sodium	28	29
Potassium	0.3	0.3
Calcium	18	26
Magnesium	51	47
	pM/Lowry Unit (disrupted envelopes)	
Sodium	33	25
Potassium	0.7	0.5
Calcium	21	19
Magnesium	24	26

Here, sodium and calcium levels are not markedly affected by disruption; the potassium level is again increased, but the magnesium level is markedly lowered.

DISCUSSION

The apparent similarity of envelopes prepared in calcium and magnesium (chapter 5) does not extent to their ionic compositions. This presumably implies some qualitative difference in the electrostatic interactions in the envelope, and hence a difference of structure at the molecular level, between calcium and magnesium preparations. The results are difficult to explain given the information available, and the following discussion is necessarily speculative.

Before an explanation of these differences is attempted, it is important to note that these results are consistent with the values given by Mirsky and Osawa[1] for the quantities of cation associated with chromatin. Assuming

1 Lowry Unit = 1.62 mg envelope protein/ml

then the levels of magnesium, for example, are in the range

$(3.2\text{-}8.1) \times 10^{-11}$ gm ions/mg protein.

Assuming further that the lipid-free dry mass of nuclei consists of protein, RNA and DNA, then

100 g lipid-free dry nuclei contain 76 g protein

(Cf. Busch[2]).

Thus, if the magnesium concentration in the envelope were equal to that over the nucleus as a whole, the concentration in the nuclei would be

$(2.5\text{-}6.2) \times 10^{-6}$ gm ions/100 g lipid-free dry mass

= 0.059-0.146 mg Mg^{++}/100 g lipid-free dry mass.

The value quoted by Mirsky and Osawa for total nuclei is 0.115 mg/100 g lipid-free dry mass.

Moreover, this level of magnesium is equivalent to 1 ion/50 nm^2, a mean spacing between the ions (assuming random distribution) of some 7 nm. This value is calculated from the assumption that the envelope contains 10% by weight of the total nuclear protein, i.e. 4.4 pg protein/envelope (Cf. Busch[2]), and that the surface area of the envelope is about 100 μm^2 ($= 10^8 nm^2$), assuming a radius of some 3 μm for the nuclei.

Taking as axiomatic the presence of magnesium and calcium in both chromatin and the envelopes, the results may be explained by the following two hypotheses. Other explanations are possible; the one presented here is chosen because of the relatively small number of

untested assumptions it involves, but it remains very tentative at present:-

a) In the presence of magnesium ions, the envelope takes up a configuration such that it binds calcium strongly but magnesium very weakly.

b) The maintenance of this configuration depends on those structural factors which also determine the morphological integrity of the system.

In a nuclear suspension containing some 5 mg protein/ml, then according to the magnesium levels in the nucleus quoted by Mirsky and Osawa (*v. supra*), viz. 0.45 mg/100 g lipid-free dry mass, each 1 ml of suspension contains some 10^{-10} gm-ions Mg^{++} associated with protein. Each 1 ml of the buffer in which the nuclei are suspended contains 10^{-6} gm-ions Mg^{++} (1 ml solution). This large excess of magnesium will lead to magnesium binding with the chromatin, and probably to a consequent displacement of calcium. However, according to postulate (a), the liberated calcium will combine with the envelope and the excess of magnesium will not. In the isolated envelope, therefore, which has been washed free of the excess magnesium, the calcium level but not the magnesium level has been increased. On treatment of the envelopes with KCl, the structural factors which determine morphological integrity and the set of molecular configurations of the system are modified. The consequent weakening of calcium binding causes loss of much of the excess calcium.

Similarly, two hypotheses may be evoked to explain the results obtained using envelopes prepared in $CaCl_2$. These are:-

a) In the presence of calcium ions, the envelope takes up a configuration such that it binds magnesium strongly but calcium very weakly.

b) The maintenance of this configuration depends on those structural factors which also determine the morphological integrity of the system.

In this case, the excess calcium liberates magnesium from the chromatin, and the magnesium, but not the excess of calcium, binds strongly with the envelope. However, the resulting excess of magnesium in the envelope fraction is observed only so long as the envelope is morphologically intact.

The monovalent binding is remarkable for the difference observed between sodium and potassium levels. This difference may have been exaggerated by the fact that the nuclei were isolated in the presence of sodium and the absence of potassium; however, a number of steps in the isolation - sonication, differential centrifugation, washing, gradient centrifugation and dialysis to remove sorbitol - are all carried out in the absence of sodium. Any loosely-bound sodium would therefore be removed from the system.

Generally, potassium treatment appears to increase the amount of potassium bound and to decrease the amount of sodium bound. This may be interpreted as a direct displacement effect. The level of bound sodium in the intact envelope is very high - comparable with the magnesium level - and may represent the counterions of the nucleic acids at pH 7-8. Replacement of sodium with potassium as the counterion may be the initial change leading to destabilisation of the system by potassium.

It is possible that such a process may be physiologically significant. At the end of mitosis, the envelope resynthesis seems to involve conversion of the envelope from vesicular to sheet form, i.e. destabilising the vesicular form relative to the sheet form. This conversion may involved, as a first step, the replacement of potassium

with sodium as the counterions of the nucleic acid groups associated with the envelope. Such a replacement might be initiated by, in part, the extrusion of potassium from the cell (Jung and Rothstein[3]).

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CHAPTER NINE

ENZYMIC ACTIVITIES ASSOCIATED WITH THE NUCLEAR ENVELOPE

SUMMARY

1. The investigation in the nuclear envelope of six enzymes is discussed; the enzymes are NADH-cytochrome c oxidoreductase (E.C. 1.6.99.3), ATPase (E.C.3.6.1.3), glucose-6-phosphatase (E.C.3.1.3.9), succinoxidase (E.C.1.3.99.1), alkaline phosphatase (E.C.3.1.3.1.) and proteases.

2. Of these enzymes, glucose-6-phosphatase, succinoxidase and protease were found to be absent. The implications of this are discussed.

3. Alkaline phosphatase activity was found to be inhibited by both calcium and magnesium, but high in the absence of diction.

4. ATPase activity was found to be diction dependent but mono-cation independent. No support was found for the hypothesis that the activity is associated with the pore complex. NADH and cytochrome c in combination reduced the net ATP lysis in nuclei and intact envelopes, suggesting that oxidative phosphorylation is coupled to the electron transport chain of the system.

INTRODUCTION

In chapter 1, the activities of enzymes associated with the nucleus and possibly associated with the envelope was discussed. In this chapter, four of these enzymes - NADH cytochrome c oxidoreductase (E.C.1.6.99.3), ATPase (E.C.3.6.1.3), alkaline phosphatase (E.C. 3.1.3.1) and protease - are examined in the nuclear envelope, and the implications of the findings for work on the structure and function of the system are discussed. In addition, the microsomal and mitochondrial marker enzymes used in the investigation of the contamination of the nuclei (see chapter 3), namely glucose-6-

phosphatase and succinamidase, are investigated here in the isolated envelope. It is possible that these enzymes are recovered in the envelope fraction and are detectable in it, while they are not detectable in the nuclei, where there is some ten times as much protein and hence only 1/10 of the specific activity.

The investigation of each enzyme is presented and discussed in turn in the light of relevant data already in the literature.

NADH-CYTOCHROME C OXIDOREDUCTASE (E.C.1.6.99.3)

Evidence for the occurrence of this enzyme in nuclei found by Rees and Rowland[1], Pennisall et al[2] and Botel and Klouwen[3], and in membranous preparations from nuclei by Kuzmina et al[4], and Berezney, Funk and Crane[5], was discussed in chapter 1. It remains to demonstrate the occurrence of the enzyme in the nuclear envelope and to estimate its recovery in this fraction. In view of the findings discussed in chapter 1 by the above-mentioned workers, it is also necessary:-

a) to investigate the rotenone sensitivity of the enzyme in the envelope;

b) to establish whether or not the enzyme is coupled to a mechanism of ATP synthesis. This may be determined by observing the effect of ATP on the enzyme activity;

c) to estimate the effect on the specific activity of the enzyme of disrupting the envelope morphologically, for example by pretreatment of the envelope with 0.02 M KCl.

Method

The assay procedure was derived from that of Slater[19] and used ferricyanide as an electron acceptor.

The assay mixture consisted of:-

1.5 mls 0.1 M phosphate buffer, pH 7.5: final concentration 0.05 M

0.1 mls potassium cyanide, 9 mM: final concentration 0.3 mM

0.1 mls 0.03 M potassium ferricyanide: " " 1 mM

0.1 mls 0.015 M N.NADH: " " 0.5 mM

1 ml water

0.2 mls sample containing some 6 Lowry units/ml (aqueous suspension of nuclei, or envelopes)

The potassium ferricyanide and N.NADH solutions were freshly prepared, and all solutions were preincubated at 37°C for 30 minutes before assay. The assay was performed in silica cuvettes in a Unicam SP800 spectrophotometer, the change in absorbance at 420 nm being read over an interval of 2-5 minutes against a blank containing no NADH, a scale expansion of X5 being employed.

The following variations of the basic assay procedure were performed:-

a) The 1 ml water replaced by 0.1 mls 1 mM rotenone in ethanol + 0.9 mls water (final rotenone concentration 0.03 mM).

b) 1 ml water replaced by 0.2 mls 0.05 M $MgCl_2$ + 0.8 mls water (final magnesium concentration = 3.3 mM).

c) 1 ml water replaced by 0.2 mls freshly-prepared sodium ATP, 0.05 M, pH 7.5, + 0.8 mls water (final ATP concentration = 3.3 mM).

d) 1 ml water replaced by 0.2 mls 0.05 M $MgCl_2$ + 0.2 mls 0.05 M ATP, pH 7.5, + 0.6 mls water.

In all cases, glass distilled water was used. N.NADH and ATP were obtained from Sigma Biochemicals, rotenone from British Drug Houses.

Results

After the assay procedures, Lowry assays were performed on the samples used and the enzyme activity expressed in E_{420} units/Lowry Unit/minute.

Table 9:1(a) Recovery in envelope

Material	Enzyme activity Experiment 1	Experiment 2	Experiment 3
Nuclei	.035	.038	.070
Intact envelopes	.25	.19	.26
Disrupted envelopes	.21	.23	.25
Purification:-			
Intact envelopes	7.1	5.0	3.7
Disrupted envelopes	6.0	6.1	3.6

From these results it appears that NADH-cytochrome c oxidoreductase is concentrated in the nuclear envelope independent of its state of integrity. The close similarity in the purifications of the enzyme in both intact and potassium-disrupted envelopes suggests once again that, while potassium treatment disorganises the system it does not solubilise any large quantity of protein (Cf.chapter 11).

Table 9:1(b) Rotenone sensitivity

By comparing the activity of the enzyme in the absence of rotenone with that in the presence of rotenone, the percentage rotenone sensitivity is calculated, assuming more or less complete inhibition of the sensitive component.

Treatment	Experiment 1	Experiment 2
No rotenone	.285	.27
Rotenone	.255	.255
% Total activity:-		
Rotenone-sensitive	10.5%	5%
Rotenone-insensitive	89.5%	95%

The enzyme thus appears to be predominantly rotenone-insensitive; the diminution in activity brought about by rotenone, if it is significant, may represent trace mitochondrial contamination.

Table 9:1(c) ATP effect

The activity of the enzyme was measured in the presence of ATP and magnesium, and also, for controls, in the presence of ATP alone and magnesium alone. The results shown refer to intact envelopes.

Conditions	Experiment 1	Experiment 2
Control	.25	.21
Mg	.23	.26
ATP	Zero	Zero
ATP + Mg	Zero	Zero

Similar results were obtained with nuclei and disrupted envelopes. While magnesium has no inhibitory effect on the enzyme, activity is completely abolished by ATP whether or not magnesium is present. Two explanations for this observation are possible:-

(i) ATP has some direct inhibitory effect on the enzyme;

(ii) ATP lysis ("reductive dephosphorylation") drives the electron transport chain backwards so that no net NADH oxidation is observed; presumably this would require sufficient intrinsic magnesium in the envelope to activate the ATPase (Cf. chapter 8). It should be noted that 0.05 M ATP represents a considerable excess, NADH and ferricyanide being present in much lower concentration.

ATPase (E.C.3.6.1.3)

ATPase activity in nuclei (Rees and Rowland[1]; Botel and Klouwen[3]) and in membranous material derived from nuclei (Zbarsky et al[6]; Dolektorskaya and Porovoshchikova[7]; Franke et al[8]) was discussed in chapter 2, where the questions of monocation dependence were raised. In the following experiments, both monocation and dication effects are investigated, and the possibility that the ATPase is associated with the pore complex (Yasuzumi and Tsubo[9]; Yasuzumi et al[10]) is examined. In addition, since the possibility remains from the

NADH-cytochrome c oxidoreductase experiments that oxidative phosphorylation occurs in the envelope, the effect on net ATPase activity of NADH + cytochrome c is investigated.

Methods

The assay procedure was based on that of Lowry and Lopez[20] and the basic assay mixture was as follows:-

1.5 ml 0.15 M Histidine-imidazole, pH 7.3; final concentration = 0.11 M

0.1 ml 0.05 M Sodium ATP, pH 7.3; " " = 2.5 mM

0.1 ml 0.05 M $MgCl_2$; " " = 2.5 mM

0.3 ml Sample (approx. 1 Lowry unit/ml)

All solutions were freshly prepared and preincubated for 30 minutes at 37°C before assay. Incubation of the assay mixture was performed at 37°C for 30 minutes and 0.15 ml 70% perchloric acid were then added. The suspension was centrifuged briefly and the supernatant assayed for phosphate as described in chapter 3.

The following variations of this basic procedure were performed:-

a) To determine the pH sensitivity of the enzyme, the histidine-imidazole buffer was made (i) pH 6.8, (ii) pH 8.0.

b) To determine the dication dependence of the enzyme, the $MgCl_2$ was replaced by (i) $CaCl_2$, (ii) Sodium EDTA, pH 7.3, both 0.05 M.

c) To determine the monocation dependence of the enzyme, the buffer was made 0.01 M NaCl and 0.01 M KCl.

d) To determine the effect of electron transport on net ATP lysis, 1 mg NADH and 50 µg cytochrome c were dissolved in the buffer. The large excess of NADH was used to ensure the occurrence of electron transport for a significant portion of the incubation time; if oxidative phosphorylation was coupled, this would insur a significant reduction in the final concentration of inorganic phosphate.

All these experiments were carried out on standard envelopes, and the oxidative phosphorylation and monovalent cation activation experiments were also performed using envelopes disrupted by washing in 0.02 M KCl. Blanks were assayed to determine (a) the amount of phosphate released from the samples (envelopes and nuclei) in the assay mixture in the absence of ATP, (b) the extent of spontaneous lysis of ATP. The results of the assays, corrected according to these blanks, were expressed as μg phosphate released/10 mins/Lowry unit.

Results

Table 9:2(a) Basic ATPase assay

Material	μg P_i/10 mins/Lowry unit	
	Experiment 1	Experiment 2
Nuclei	4.7	2.5
Intact envelopes	16.0	17.9
K^+-disrupted envelopes	16.3	15.8
Purification in envelope:-		
Intact envelopes	3.4	7.1
Disrupted envelopes	3.5	6.3

These results indicate that the nuclear ATPase is concentrated in the envelope, but no significant difference between intact and disrupted envelopes can be seen. This may imply either that (as previously suspected) potassium treatment disorganises rather than solubilises the pore complex protein, or that contrary to the findings of Yasumumi[9,10] ATPase is not primarily associated with the pore complex; a more or less random distribution of the activity over both the pore complex and the rest of the envelope would have to be assumed if the pore complex protein were removed on potassium treatment.

Table 9:2(b) Dication dependence

Assay conditions	µg P_i/10 mins/Lowry unit	
	Experiment 1	Experiment 2
$MgCl_2$	16.0	17.9
$CaCl_2$	8.0	7.7
EDTA	1.1	0.3

These results show that when magnesium is replaced by calcium the ATPase activity is approximately halved, while removal of all dication reduces the activity to 2-7% of that in the presence of magnesium.

Table 9:2(c) pH effects

pH	µg P_i/10 minutes/Lowry	
	Experiment 1	Experiment 2
6.8	1.9	2.7
7.3	16.0	17.9
8.0	8.3	9.1

The reduction of activity is very marked as the pH falls below 7.3, but somewhat less marked as the conditions become more alkaline. This implies either an asymmetry of the curve of enzyme activity/pH or a pH optimum slightly above 7.3.

Table 9:2(d) Monocation activation

Conditions	µg P_i/10 minutes/Lowry	
	Experiment 1	Experiment 2
Intact envelopes:-		
Without monocations	16.0	17.9
With monocations	15.5	19.1
Disrupted envelopes:-		
Without monocations	16.3	15.8
With monocations	13.2	16.8

No significant effect is observed; this is consistent with the findings of Zbarsky et al[6], Franke et al[8], and Dolokterskaya and Perevoshchikova[7] on the concentration independence of nuclear ATPase activity.

Table 9:2(e) Effect of NADH

Conditions	μg P_i/10 minutes/Lowry Experiment 1	Experiment 2
Intact envelopes:-		
Without NADH + Cyt. c	16.0	17.9
With NADH + Cyt. c	1.8	3.3
Disrupted envelopes:-		
Without NADH + Cyt. c	16.3	15.8
With NADH + Cyt. c	10.8	13.1

In both cases a reduction of the apparent ATPase activity has occurred, but the reduction is significantly greater in the case of the morphologically intact envelopes. Two explanations are possible:-

(i) NADH or cytochrome c, or both, directly inhibit the ATPase of the envelope. If this is so, then the results suggest that morphological disruption of the envelope with potassium partially prevents the inhibition, presumably by some rearrangement of the inhibitor binding sites, or blocking of such sites, which leaves the substrate binding sites functionally unaffected;

(ii) Oxidative phosphorylation can occur in intact but not in disrupted envelopes. The net ATP lysis is therefore less, provided the envelope is intact, in the presence of NADH than in its absence.

In view of the observed effect of ATP on NADH-cytochrome c oxidoreductase activity, the explanation invoking oxidative phosphorylation becomes preferable. It involves the use of only one hypothesis,

viz. that NADH or cytochrome c directly inhibits ATPase, and that ATP directly inhibits the NADH-cytochrome c oxidoreductase.

GLUCOSE-6-PHOSPHATASE (E.C.3.1.3.9)

In the preparations of membranous material from the nucleus described by Boreaney, Funk and Crane[5] and by Franke et al[8] glucose-6-phosphatase was present in only trace quantities. The preparation of Kashnig and Kaspar[11], however, showed glucose-6-phosphatase with a specific activity 50% of that in the microsomal fraction. The investigation of this enzyme in isolated intact nuclear envelopes was undertaken to explain the contradiction in these results. At the outset, two explanations appear possible:-

(i) Since glucose-6-phosphatase is an unstable enzyme (Beaufay and de Duve[12]) it may have been destroyed in those preparations in which its activity was detected only in trace amounts;

(ii) Since it is difficult to remove all traces of microsomal contamination from nuclei, it is possible that the nuclei prepared by Kashnig and Kaspar[11] were more highly contaminated than those used in other preparations. Extensive microsomal contamination of the nuclei may have resulted in similar contamination of the final material.

If the former explanation, implying a high glucose-6-phosphatase activity in the envelope, is preferred after further investigation, then this will have considerable significance in discussions of the structural and functional comparability of endoplasmic reticulum and nuclear envelopes.

Method

The assay procedure used was that of Swanson[13], described in chapter 3 of this thesis. Intact and potassium-disrupted envelopes were used. Envelopes were prepared from both "pure" nuclei and "crude"

nuclei, the latter being made by the following procedure: the liver homogenate prepared as described in chapter 3 was centrifuged at 800 X g for 10 minutes. The pellet, after careful decantation of the supernatant, was resuspended in 10 volumes ice-cold homogenising buffer and again centrifuged at 800 X g for 10 minutes. This washing was repeated twice, and the final pellet was taken to represent a crude nuclear preparation.

Results

Table 9:3 Glucose-6-phosphatase

Material	µg P_i/30 mins/Lowry	
	Experiment 1	Experiment 2
Pure nuclei:-		
Intact envelopes	Zero	Zero
Disrupted envelopes	Zero	Zero
Crude nuclei:-		
Intact envelopes	5.0	6.5
Disrupted envelopes	4.5	4.5

Here, "Zero" inorganic phosphate release implies less than 2 µg/30 minutes/Lowry unit.

The results show that when nuclei are extensively contaminated with microsomal material, envelopes prepared from them contain glucose-6-phosphatase activity. The integrity of the envelopes is not relevant. Since envelopes from pure nuclei have no detectable activity, the activity would appear to represent contamination. However, in the material prepared from the crude nuclei, the activity of the enzyme is low (compare results for total liver homogenate, chapter 3). This suggests that the isolation procedure may partially inactivate glucose-6-phosphatase. Nevertheless, the procedure described by Keshnig and Kaspar[11] involved, like the procedure

described in chapter 5 of this thesis, sonication and gradient centrifugation; it may therefore be predicted that in the material prepared by those workers much of the glucose-6-phosphatase was inactivated.

It is concluded that nuclear envelopes contain no detectable glucose-6-phosphatase. A nuclear envelope preparation containing this enzyme is contaminated with microsomes.

SUCCINOXIDASE (E.C.1.3.99.1)

Nuclei prepared as described in chapter 3 contain a trace of succinoxidase activity (specific activity = 3% that in total homogenate). Though this may represent quantitatively negligible mitochondrial contamination, if all the activity were recovered in the envelopes its specific activity would now be some 30% of that in the total homogenate. This is not negligible, and further investigation of the extent of mitochondrial contamination would be required. For this reason, the succinoxidase activity of the envelope was investigated.

Method

The assay method derived from that of King[14] was used as described in chapter 3. The activities of intact and potassium-disrupted nuclear envelopes were compared with those of nuclei and of total liver homogenate.

Results

The results were calculated in terms of change of optical density at 400 nm/minute/Lowry unit.

Table 9:4 Succinoxidase

Material	Change in E_{400}/minute/Lowry unit	
	Experiment 1	Experiment 2
Total homogenate	0.42	0.37
Nuclei	0.014	0.010
Intact envelopes	0.019	0.012
Disrupted envelopes	0.007	0.012
	Specific activity:-	
Nuclei	3.3%	2.7%
Intact envelopes	4.5%	3.2%
Disrupted envelopes	1.7%	3.2%

From these results it is concluded that:-

a) The recovery of succinoxidase activity in both intact and disrupted envelopes is such that no purification of the enzyme from the nuclei is discernable. Presumably the enzyme is distributed more or less randomly over the sub-nuclear fractions obtained during the isolation procedure, its specific activity being similar to that of the nuclei in all fractions.

b) The activity appears to be less in the disrupted than in the intact envelopes, but it is doubtful whether this difference is significant.

c) Nuclear envelopes are essentially free of succinoxidase activity. The small trace of activity which is present may best be regarded as mitochondrial contamination, though electron microscopic results show that no distinguishable mitochondrial fragments are present in the envelopes. However, the extent of succinoxidase contamination is roughly equal to the extent of apparent contamination by the rotenone-sensitive component of the NADH-cytochrome c oxidoreductase.

ALKALINE PHOSPHATASE (E.C.3.1.3.1)

Magnesium-independent alkaline phosphatase activity in liver is concentrated in the nuclear fraction (Emery and Deamer[15]). In other systems, alkaline phosphatase is frequently associated with membranes (Cf. Fennell[16]); it is, therefore, possible that it has high activity in the nuclear envelope.

Methods

The assay procedure was a modification of the method of Morton[17]. The assay mixture consisted of:-

1 ml 0.1 M Ethanolamine-HCl, pH 9.5

1 ml 0.05 M EDTA, adjusted to pH 9.5 with NaOH

1 ml 0.1 M glucose-6-phosphate, adjusted to pH 9.5 with NaOH

0.5 mls water

0.25 mls suspension of envelopes in water

All solutions were preincubated at 37°C for 30 minutes; the incubation was carried out at 37°C for 30 minutes. At the end of this time, 0.3 mls 60% perchloric acid were added to the mixtures and the precipitated protein removed by centrifugation. The supernatants were then estimated for inorganic phosphate as described in chapter 3.

To determine the extent of diction dependence of the alkaline phosphatase activity, the experiment was repeated using 0.05 M $MgCl_2$ or 0.05 M $CaCl_2$ instead of EDTA. In all cases, the enzyme activity was expressed as μg phosphate released/Lowry unit/15 mins. Both intact and potassium-disrupted envelopes were used, and their activities compared to that of unsonicated nuclei.

Results

Table 9:5 Alkaline phosphatase in presence of EDTA

Material	µg P_i/Lowry unit/15 min	
	Experiment 1	Experiment 2
Nuclei	1.1	1.8
Intact envelopes	2.3	3.1
Disrupted envelopes	3.1	2.4

When the EDTA was replaced with calcium or magnesium, no activity was detected, i.e. phosphate released was less than 0.5 µg/Lowry unit/15 minutes.

From these results it was concluded that:-

a) The alkaline phosphatase activity undergoes a 2- or 3-fold purification in the isolation of the envelope; the activity may therefore be said to be largely associated with the envelope.

b) There is no significant difference between the activities of intact and disrupted envelopes.

c) Cations at a final concentration of 13 mM inhibit the enzyme both in the nuclei and in the isolated envelopes.

PROTEASES

In nuclei isolated using a range of procedures, protease activities with local maxima at pH values of 3.0, 3.5, 4.5, 7.0 and 9.0 and a minimum (zero activity) at pH 5.8 have been reported (Dounce and Uruña[18]). None of these isolation procedures, however, involved the use of centrifugation through dense sucrose.

Since the activities are increased when disruption of extranuclear organelles is marked, it is possible that the proteases adhere to the surfaces of the nuclei and are consequently purified in the isolated envelope. In the following experiments, the protease

activities of "pure" and "crude" nuclei were compared with the activities in envelopes prepared from them.

Methods

Nuclei were isolated as described in chapter 3 ("pure" nuclei) and "crude" nuclei were prepared by the method described in this chapter under "glucose-6-phosphatase"(v. supra). Envelopes were isolated from the nuclei by the standard procedure described in chapter 5; potassium-disrupted envelopes were not used.

The assay procedure was that described by Dounce and Umaña[18]. The assay mixture consisted of:-

1 ml suspension of nuclei or envelopes in water

1 ml 2.5% haemoglobin in 8 M urea

1 ml 0.1 M buffer. Acetate buffer was used at pH 3.0-4.5, phosphate buffer at pH 5.8 and 7.0, and tris-glycine at pH 9.0.

All solutions were preincubated at 37°C for 30 minutes and the incubations were carried out at 37°C for 2 hours. At the end of this time 2 mls 5% trichloracetic acid were added, the precipitated protein and nucleic acid removed by centrifugation, and the optical density of the supernatant at 280 mμ determined, using a Bausch-Lomb spectrophotometer. Results were expressed in terms of O.D. units/Lowry unit nuclear or envelope protein.

Results

Table 9:6(a) Proteases in crude nuclei

pH	O.D. units/Lowry unit nuclei	
	Experiment 1	Experiment 2
3.0	.23	.195
3.5	.185	.205
4.5	.125	.110
5.8	.010	Zero
7.0	.110	.145
9.0	.085	.090

Those values are in close agreement with the relationship between pH and protease activity in nuclei prepared in 0.25 M sucrose-3 mM $CaCl_2$ shown by Bounce and Umaña[18]. The figures quoted by these authors did not, however, refer to specific activity, but to the relative activities at the range of pH values used.

Table 9:6(b) Proteases in envelopes from crude nuclei

pH	O.D. units/Lowry unit envelopes	
	Experiment 1	Experiment 2
3.0	.88	.93
3.5	.71	.84
4.5	.43	.38
5.8	.003	.010
7.0	.34	.435
9.0	.25	.18

Comparison between the values in this and the previous table shows a 3-4-fold purification of protease activity in the envelope fraction from the nuclei. This enhancement of activity appears to be more marked at the lower pH values, though the significance of this observation is doubtful.

In pure nuclei (the preparation of which involved centrifugation through 2.3 M sucrose), and in the standard envelopes prepared from pure nuclei, no protease activity was detectable. It was concluded from this observation that the protease activity of the nucleus, and hence of the envelope, represents some form of extranuclear contamination. The alternative explanation - viz. that protease activity is readily inhibited in pure nuclei, while the presence of contaminants protects the enzyme - was dismissed on the grounds that disruption of the nuclei by sonication, and consequent removal of the protection - the initial step in the isolation of the envelopes - fails to decrease the apparent activity.

The presence of proteases active over a wide pH range in other sub-cellular fractions (mitochondrial, microsomal etc.) is generally regarded as representing lysosomal contamination. It is therefore suggested that the apparent nuclear protease activity depends on contamination of the nuclear preparation with lysosomes. However, until other sub-cellular fractions are removed from the nuclei by centrifugation through the dense sucrose medium, the nuclei will be in contact with such protease-rich bodies. This justifies the use in the procedure for isolation of nuclei of a pH close to that at which the protease activity is minimal (see chapter 3).

GENERAL DISCUSSION

Two classes of enzymes have been considered in this chapter:-

a) Those which seem to be best regarded as contaminants of nuclei, and hence of nuclear envelopes, in isolation.

b) Those which appear to be native to the envelope. Of these, ATPase, alkaline phosphatase and NADH-cytochrome c oxidoreductase have been considered here.

Evidence has been presented for a high rate of breakdown, and perhaps a measure of synthesis, of ATP in the system. This suggests a high local energy requirement, which may possibly be related to synthetic processes, e.g. DNA replication, or to energy-requiring transport processes operating between nucleoplasm and cytoplasm (see chapter 1). It is uncertain which transport processes are likely to require energy; possibly the passage of messenger RNA into the cytoplasm, or the assembly of ribosomes (if final stages of this process occur near the surface of the nucleus), may be endothermic. Information on this possibility could be gained by comparing the ATPase activities of nuclear envelopes from cells differing in their rates of protein synthesis.

The phosphatase activity at high pH values appears to be fairly non-specific; inorganic phosphate was released from both ATP and d-AMP at pH 9.5, as well as from glucose-6-phosphate. The biological significance of the alkaline phosphatase is mysterious. Its presence in the envelope may, however, account for the apparent asymmetry in the plot of ATPase activity against pH.

The absence of glucose-6-phosphatase, succinoxidase and protease activities from the envelope provides, first, a method whereby the purity of a nuclear envelope preparation can be estimated, and second, an indication that the envelope is not functionally comparable with microsomal, mitochondrial or lysosomal membranes. Any speculation on the degree of identity between the nuclear envelope and one or other of these systems must take this conclusion into account.

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PART IV

GENERAL DISCUSSION

CHAPTER TEN

THE PROBLEMS OF ISOLATION AND IDENTIFICATION

The significance of the careful study of the isolation procedure developed for a membrane system was fully discussed in part I of this thesis, where a number of specific problems associated with the isolation of the nuclear envelope were raised. It was seen that the first logical requirements were:

a) Definition of the "nuclear envelope";

b) Definition of the term "isolation".

The required procedure then became one which, in accordance with these definitions, gave a reproducibly satisfactory envelope preparation. A number of contingent problems then arose. These were:

a) Arising from the definition of "isolation", the problems of "purity", "contamination" and "completeness";

b) Arising from the study of structural interactions of the system with other systems - notably the appended chromatin and endoplasmic reticulum - the problem of the dependence of the envelope, as defined, on the presence of these other systems.

While all the previous problems may be illuminated by empirical study, only the last-mentioned is capable of direct empirical solution. A major part of the experimental work described in part II of the thesis was, therefore, directed towards an investigation of the significance of the structural interactions. This was shown to be particularly important in view of the work done in other laboratories, in which "envelope" preparations failing to meet the criteria of definition were described. It was necessary to ask of such work (v. chapter 2) why morphological breakdown of the envelope had occurred:

a) From the point of view of the envelope itself; could some relationship between the loss of integrity and the loss of one of the principal components of the system (protein, lipid, DNA, RNA) be established?

b) From the point of view of the experimental procedure; what techniques or parameters defining the composition of the medium were necessary in maintaining integrity, and, conversely, were responsible for the loss of integrity?

This chapter will be devoted to a full discussion of all these problems and questions in the light of the experimental results.

1. THE DEFINITION OF THE ENVELOPE

In chapter 1 it was established that the only acceptable definition of the nuclear envelope was morphological; a functional definition based on enzymic or permeability properties is not possible in the light of current knowledge. According to this definition, a nuclear envelope preparation has the following properties:

a) It is obtained from nuclei uncontaminated by other cellular membrane systems and containing no internal membranes, such as intranuclear annulate lamellae.

b) It is a double membrane system, at least one of the component membranes bearing ribosomes.

c) Its surface area is occupied, in the case of rat liver, to the extent of 20-30% by pore complexes, consisting essentially of annuli of eight subunits and in some cases central tubules or "granules".

While this definition is in itself acceptable and while it provides a sound basis for experiment, as described in this thesis, it contains certain inherent problems when the object of research is to consider the composition, structure and function of the system. These problems are as follows:

a) The definition of the envelope is independent of its enzymatic activity, its permeability properties, etc. It is possible that two or more procedures whereby acceptable envelopes can be isolated would give material which differed in these respects.

b) Provided the above morphological criteria are satisfied, other morphological factors - e.g. the gross integrity of the system, whether it be in the form of nuclear ghosts or of small fragments - are irrelevant.

c) Moreover, the definition of the material is <u>independent of its composition</u>. This point is very important. It is possible that the composition of the system varies according to the isolation procedure chosen; hence, since presumably composition determines enzymatic and permeability functions, the enzymology of the system will also depend (in principle) on the isolation procedure. In addition, the details of the organisation of the system in space, being dependent on the composition to a greater or lesser extent, will not be precisely constrained by the definition.

2. THE ISOLATION PROCEDURE

Fundamental to the problems stated above is the fact that no single set of isolation conditions is required by definition. Provided the isolation procedure is in accordance with the criteria stated, i.e. provided that it results in morphologically acceptable material, it is not otherwise constrained. An indefinite number of procedures is from this point of view possible; and it is not unreasonable to suppose that each procedure will give material unique in composition, organisation, enzymology, gross morphology, permeability etc.

Further consideration of the problems and the definition, however, complicate this viewpoint. The morphology of the system, as required in the definition, is a product of the ~~special~~ spatial organisation of the system; while it remains impossible to argue that the specific morphology requires a unique macromolecular organisation, it is probable that only a limited range of such modes of organisation can account for it. Macromolecular organisation, in turn, depends on two factors:

i) The composition of the system,

ii) The physico-chemical parameters defining its immediate environment.

Again, it is probable that several different sets of components in combination with a number of sets of physico-chemical parameters can account for a single observed mode of organisation, but notwithstanding this redundancy on two levels, these conclusions follow:

a) While several different compositions are possible in a system of which the definition is morphological, the composition is not completely unrestricted in principle;

b) The physico-chemical parameters defining the isolation procedure are, because they affect organisation and hence morphology, also of significance in the definition of the system itself. This means that in any meaningful definition of the isolated nuclear envelope, the conditions of isolation and storage must themselves be specified.

It now follows that the isolation procedure attempted may fail for one of two reasons (distinguishable logically but not empirically):

a) Under the conditions used the separation of a morphologically intact nuclear envelope from the surrounding structures cannot be achieved;

b) While the nuclear envelope can be separated from surrounding structures, its composition under the conditions used is such that under those same physico-chemical conditions it fails to maintain morphological integrity.

The adoption of a particular single preparation procedure for the nuclear envelope, no matter how arbitrary the choice, is therefore essential if the system is to be defined consistently. In terms of this choice, the problems of contamination, purity and completeness can be considered.

3. PURITY, CONTAMINATION AND COMPLETENESS

In defining these three terms, the basic problem lies in the inapplicability of the notion of "chemical purity" to a macromolecular system of biological origin. In the past, a similar argument has been applied to the debatable chemical purity of isolated proteins (Pirie[1]). Recent advances in the understanding of protein structure, and the theories of enzymology which are dependent on the concept of a protein species of which the activity can be considered in isolation from other species, have greatly diminished the relevance of such arguments. It is possible that, if in the future an understanding of membrane structure reaches the level of current understanding of protein structure, and theories of membrane function become as clearly established as theories of enzymology, the problem of purity will lose relevance here, too: the situation will then be one in which membranes can be clearly and simply defined in terms of composition, structure and function. At present, however, these terms are the object of research rather than definable concepts in view of which further research can take place.

In the absence of an applicable notion of chemical purity, the definition of the nuclear envelope, like that of other membrane

system, is operational. Hence purity, contamination and completeness are defined in relatively arbitrary terms. These are as follows:

a) As shown above, the isolation procedure is of primary importance in the definition of the system. Material reproducible in both morphology and composition is to be expected of a single isolation procedure, and deviations from the standard composition under these stated conditions may be regarded as contamination (e.g. if DNA is present in excess) or as incompleteness (e.g. if only a low quantity of lipid phosphate has been recovered).

b) The history of experiments on biological membranes has shown that, as an empirical generalisation, membrane systems contain in most tissues most of the cellular lipid, while they contain little or no DNA. In the investigation of the nuclear envelope, it is therefore reasonable to expect to isolate membranous material richer in lipid but poorer in DNA than total nuclei. As discussed in chapter 2, many workers, while recognising this empirical constraint, have failed to recognise the primacy of the morphological criterion in all such work. As a result, they have isolated material rich in lipid and almost free of DNA but impossible to describe as nuclear envelope; in this thesis, it has been shown that any acceptable nuclear envelope preparation must contain a certain minimum level of DNA. It becomes possible to speak of contamination when, for example, the DNA level significantly exceeds this minimum value.

The "pure nuclear envelope", in current operational terms, must be defined as the material found in the sorbitol band in the standard preparation procedure described in chapter 5. This definition is necessarily tentative, but forms the basis for definitions of "contamination" and "completeness" which are in accordance with the foregoing restrictions on the meaning of the terms.

4. THE IMPORTANCE OF STRUCTURAL INTERACTIONS

Closely related to the problem of contamination and completeness and bearing directly on the experimental investigation of the problem of isolation of the nuclear envelope, is the question of its dependence on the systems with which it manifests structural interactions *in vivo*. In terms of the morphological criterion of definition of the envelope, it was shown in part II of this thesis:

a) That lipid does not appear to be of primary importance in the definition of the system - i.e. removal of lipid does not necessarily cause morphological breakdown;

b) That, similarly, RNA does not appear to be important;

c) That the protein content is the factor most highly variable with variations in the conditions of preparation, and is probably of considerable importance in determining the acceptability of morphology;

d) That DNA is essential to the integrity of the system.

To these four points may be added a fifth:

e) That the endoplasmic reticulum, which was shown in chapter 1 to be very probably associated with the outer nuclear membrane, cannot be wholly removed from the nucleus without serious damage to, and probably removal of, the outer membrane. (The principal evidence for this statement comes from scanning electron microscopy[2].) Nevertheless, either the endoplasmic reticulum concerned is very small in quantity or it is atypical, since no glucose-6-phosphatase activity is detectable in the envelopes to which it presumably remains attached (Cf. ref. 3).

The essential DNA is associated with peripheral chromatin, either the perinuclear zone discussed in chapter 1 or some distinct fraction directly associated with the pore complex and possibly concerned in DNA replication. In principle, it is possible under a definable set of experimental conditions to minimise the content of this DNA (and

the content of endoplasmic reticulum appended to the envelope), the limiting requirement of these appended systems being dependent on the conditions.

Given the arbitrary restricting criteria established in the discussion of purity, contamination and completeness, it now appears that the best possible isolation procedure is the one in which the morphological criterion is satisfied and in which the content of DNA and endoplasmic reticulum can reach the lowest minimum.

5. THE ABSOLUTENESS OF THE REQUIREMENTS FOR STRUCTURAL INTERACTION

It has been argued that the requirement for structural interactions with chromatin, and perhaps with endoplasmic reticulum, in the maintenance of envelope integrity, is dependent upon the isolation conditions used. Thus, maintenance of the asymmetry of distribution of macromolecules on the two sides of the envelope was seen in part II of this thesis to protect the envelope against disruption by potassium ions, by high concentrations of EDTA, and even for a time by DNase. It is possible that in the isolation medium there are mildly disrupting factors, and that the quantity of DNA in envelopes of the standard preparation could with impunity be lowered if more suitable conditions were found. However, it is argued that such lowering is not in practice possible; the evidence for this claim is:

a) The range of experiments described in chapters 5 and 6 of this thesis, where the variations in the conditions of isolation and the ionic and other effects on the envelope were rigorously explored;

b) The fact that <u>in vivo</u> the envelope is throughout the cell cycle in contact with DNA and other macromolecules, distributed asymmetrically across it, except perhaps during the formation of certain blebs (single-membrane types, which include no pores and hence

are not complete or recognisable envelope), and during mitosis when the entire envelope is disrupted.

Hence, although the composition, and therefore the apparent dependence on structural interaction with RNA, of the envelope would appear prima facie to vary with the isolation conditions, it may be argued that the requirement, at least for chromatin and possibly for a portion of attached endoplasmic reticulum, in maintenance of envelope integrity is absolute. The alternative argument (in many ways preferable, and implicitly used in part II of this thesis) extends the definition of the envelope to include the required contiguous structures. According to this argument the envelope is defined as a double membrane system, derived from intact but essentially uncontaminated nuclei, bearing the required distribution of pore complexes and also the minimum amounts of endoplasmic reticulum and chromatin to maintain its morphological integrity. This definition has important implications for the composition, and hence the structure and inherent function, of the envelope.

6. THE DEFINITION OF THE PARAMETERS OF THE ISOLATION MEDIUM

It was shown in part II of the thesis that, under the conditions for standard envelope isolation, the inclusion of potassium, or the adjustment of the medium to too high an ionic strength or too high or too low a pH, brought about disruption. It now remains to determine whether these conditions are absolute; that is, whether potassium in more than 15 mM concentration, or pH below 7.2, etc., will always disrupt the morphology of the nuclear envelope.

Again, the effect of such conditions must be related to the composition of the system. The susceptibility of the envelope to these conditions, indeed, may be regarded as illuminating further the protection of the envelope by contiguous macromolecules *in vivo*.

(The mean intracellular potassium concentration, for example, is rather more than 15 mM, and it is improbable that the mean intracellular pH never falls below 7.2.) However, this leads to the interesting problem of the definability of such terms as concentration, pH, ionic strength etc. in intracellular compartments. The question of the physiological relevance of the ionic effects described in detail in chapter 6 is important because of their possible involvement in the modification of the state of the envelope and the peripheral chromatin during the cell cycle; to make a clear statement of these implications requires the transference of concepts from the *in vitro* to the *in vivo* situation.

The problem of definability has three major facets, which are as follows:

a) The definitions of concentration, ionic strength and pH (understood in its "practical" sense, as the negative logarithm of concentration) depend on the applicability of statistical considerations to a very large number of molecules or ions of the species under consideration. When the number of ions or molecules is not large, as in a subcellular compartment, such considerations are inapplicable. For example, in a bulk solution of pH = 7, it is meaningful to speak of a concentration of some 6×10^{16} hydrogen ions/litre, but "a concentration of about 60 hydrogen ions per cubic micron" has no meaning. 1 μm^3 would be a reasonably large volume to consider as a homogeneous subcellular component at any given instant. It follows that (while by definition an ion or molecule must have an activity, a chemical potential, etc.) in general the commonly used chemical parameters have no meaning *in vivo*.

b) Even if such concepts could be applied *in vivo*, the problem of the distribution of "fixed" charges on membranes, large proteins,

etc., the problem of "bound" and "freely moving" water molecules, and the problem of the percentage of any given volume occupied by water as against the percentage occupied by non-aqueous material, make it clear that the activity coefficient of any ion or molecule will in general be very far from unity. Again, activity is meaningful in this context, but cannot be determined experimentally. The determination of the factors controlling activity coefficient in a single compartment, even at one instant in time, is a technically impossible task.

c) The matter is further complicated by the dynamic (as defined in chapter 1) nature of the cell. At each instant in time all properties of all compartments require redefinition. A time-averaged pH in a small volume has no significance in the description of the integration of intracellular activity; an average pH over the whole cell has no meaning in terms of the functional interactions between compartments, even at a single instant in time. Certainly, the "intracellular pH" of discussions of experimental work, which is usually both time-averaged and space-averaged, has no meaning at all in terms of cellular activity.

Returning to the case of the nuclear envelope, the factors to be considered include sodium and potassium activities in subcompartments of the peripheral chromatin and the cytoplasm contiguous with the envelope, and the pH (negative log of hydrogen ion activity) of the same subcompartments. The subcompartments must be considered to be small and numerous, in accordance with the demonstrable heterogeneity of the peripheral chromatin (Cf. Davies[4]), the tangential heterogeneity of the envelope itself (e.g. the distribution of pore complexes and, at least on the outer surface, ribosomes), and the distribution of other membrane systems in the contiguous cytoplasm. For each

compartment, with an understanding of the local envelope composition and the quantity of DNA attached, etc., it may be possible to state a critical potassium concentration, pH, etc., above which the envelope is disrupted and below which it is intact; but the method by which this can be achieved from *in vitro* experimentation is not obvious.

In summary, while it is unnecessary and even misleading to think of the various disruptive ionic effects in absolute terms, and while these effects clearly depend on envelope composition, there is at present no way of extrapolating from *in vitro* studies of the subject to the *in vivo* situation and of establishing the physiological significance of the effects in other than speculative terms.

The problem discussed here is not specific to the nuclear envelope, but appears in all attempts in cell biology to provide a physico-chemical interpretation of intracellular phenomena. In combination with difficulties such as those (described for the case of the nuclear envelope in chapter 6) associated with the molecular interpretation of *in vitro* modifications of macromolecular systems, it presents at this time a conceptual impasse in cell biology, to overcome which new techniques, and possibly a wholly new approach to the subject, may be required.

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CHAPTER ELEVEN

PROSPECTS FOR FURTHER RESEARCH

Many of the findings described in this thesis suggest that further series of experiments designed with similar aims in mind might illuminate further important properties of the nuclear envelope, and perhaps have implications for the study of membrane systems in more general terms. Future investigations of this type might fall into one of the following classes:

1. ENZYMOLOGY

a) ATPase. The basic properties of a very active ATPase in the nuclear envelope were described in chapter 9. The high activity of this enzyme tempts speculation as to its function. First, some part of the activity may be associated with an oligomentary electron transport chain. Although direct demonstration of oxidative phosphorylation by determination of P/O ratios is not possible when the ATP is being rapidly consumed, the reduction of net ATPase activity in the presence of NADH and cytochrome c arguably provides an indirect demonstration. In the absence of NADH, therefore, the phosphorylation system may act as an ATPase.

However, there must remain some ATPase not explained by this argument. It is conceivable that this may function *in vivo* to supply energy for certain stages in the process of DNA replication, if indeed replication is initiated at the nuclear surface. Again, it may provide the energy for transport of m-RNA and ribosomal precursors into the cytoplasm. Little is known about the details of such transport processes; they would appear *a priori* to be energy-requiring.

b) Adenyl Cyclase. On the basis of the same high ATPase activity, and the weight of evidence suggesting that certain steroid hormones,

the actions of which are probably mediated through 3', 5'-cyclic AMP, act at the nuclear level, it is suggested that the nuclear envelope might show adenyl cyclase activity. This enzyme in general appears to be associated with the plasma membrane; it may therefore be necessarily a membrane enzyme. If this is so and if it occurs in the nucleus at all, it must be associated with the envelope.

c) Cytochrome oxidase. The existence of NADH-cytochrome c oxidoreductase in the envelope suggests that other electron transport chain components might be present. Cytochrome oxidase has been reported in the system by Beresney, Funk and Crane[1], but its specific activities in intact and disrupted envelopes remain to be determined. It would be of interest to compare the activity of this enzyme with that of the NADH-cytochrome c oxidoreductase.

d) DNA Polymerase. The procedure whereby this enzyme might be demonstrated is not obvious. Incorporation of labelled DNA precursors into the envelope DNA would provide inconclusive evidence; on these grounds it would not be possible to distinguish between the polymerase and a DNA repair enzyme. Clear demonstration of the DNA polymerase would, however, provide crucial evidence in favour of the hypothesis that DNA initiation is synthesised at the envelope.

2. IONIC CONTENT

In chapter 8 the sodium, potassium, calcium and magnesium contents of intact and potassium-disrupted envelopes were compared. It was clear that disruption significantly altered the ionic content; if, as was argued in part II of the thesis, electrostatic bonds are of primary importance in the maintenance of envelope integrity, these changes in envelope ion levels may be closely related to the molecular nature of the loss of integrity.

It would be interesting to compare the changes brought about by disrupting the system with potassium ions with the changes brought about by other means of disruption, e.g. changes of pH, freezing and thawing or brief DNase treatment. If the effects on ion levels of these different treatments are significantly different, this might imply that the molecular natures of the morphological disruption techniques are also different.

The possible presence of cytochromes and cytochrome oxidase in the envelope also make it worthwhile to examine the iron and copper content of the system. This may, in combination with values for the specific activities of the electron transport enzymes, give a measure of the content of electron transport intermediates in the nuclear envelopes compared to, for example, mitochondria.

3. PROTEINS

The growing body of information concerning the lipid content of the nucleus[2] and nuclear envelopes[3,4] is not matched by information concerning the protein content. Preliminary investigations in this laboratory have shown that 0.5 mM sodium EDTA extracts some 30% of the envelope protein at pH 7.5 and 60% at pH 9.0 (pH in each case adjusted with NaOH). The bulk of the envelope lipid is also extracted by this treatment. DNase also solubilises some 60% of the protein, 0.25 M KCl some 40%, but acetic acid[5] and 0.02 M KCl less than 10%. This last result is interesting, given the dramatic effects of 0.02 M KCl on envelope morphology. It would appear that this type of morphological destruction may have to be interpreted in terms of rearrangement in the envelope of certain component macromolecules, rather than solubilisation of anything other than a considerable fraction of the DNA. At least 90% of the envelope protein can be solubilised by butanol extraction[6] in the presence of 0.5 mM EDTA, pH 7.5-8.0.

The term solubilised, as used above, means that the protein remains in the supernatant after centrifugation at 100,000 X g for 1 hour. However, on polyacrylamide gel electrophoresis, the bulk of the protein solubilised by these procedures remains at the origin under a wide range of pH and ionic strength conditions, and in both the presence and absence of EDTA. Only under the conditions described by Lenard[7] does the entirety of the protein migrate into the gel and separate into clear bands, which, after staining, appear to be about 12-15 in number. The band patterns of the proteins extracted by all the above procedures seem very closely similar, and similar in number and distribution to the pattern produced when entire envelopes dispersed in SDS are run on gels.

Much more elaboration of this work is needed before meaningful conclusions about the envelope protein can be drawn, but the information given above indicates that, while the envelope protein shows a considerable measure of heterogeneity and promises to be difficult to handle (the precipitation of the protein at gel surfaces implies a marked tendency to aggregate), at least a number of useful solubilisation procedures can be employed.

THE PORE COMPLEX

The pore complex has been described in ultrastructural terms (see chapter 1) in remarkable detail, and some portions of it are probably associated with highly specific functions; the central tubule, for example, is probably important in the transport of RNA from nucleus to cytoplasm. Electron microscopy can be applied to monitor the integrity of the pore complex under a range of conditions. There are indications that the structure can be disaggregated in stages. Fairly vigorous mechanical treatment, for example repeated passage through a hypodermic needle, appears to remove the central tubule;

RNase and 0.04 M NaCl appear to remove the amorphous material from the periphery of the annulus. By extension of such techniques, it is possible that a more or less comprehensive demolition of the pore complex can be achieved, removing one ultrastructurally definable portion at a time by careful manipulation of the conditions.

A technique for the isolation of the pore complex would, therefore, open up a research project with interesting and important implications. Removal of one ultrastructural component coupled with thorough studies of the change in composition of the remainder might ultimately result in a complete description in molecular terms of a morphologically defined entity. The significance of this for cell biology is evident, given that the pore complex most probably forms the site of transport of RNA and ribosomal precursors from nucleus to cytoplasm and the site of attachment of RNA.

No method is as yet available for the isolation of the pore complex. In searching for a method, however, advantage might be taken of the following facts:

a) Lipid does not appear (see part II) to be critical in the maintenance of morphological integrity of the envelope, i.e. in the organisation of the pore complex.

b) Very brief (1-3 seconds) sonication of the isolated envelope disrupts more of the membranous part of the system than the pore complexes, implying that, while the latter are very sensitive to changes of physico-chemical conditions, they are mechanically more robust than the rest of the envelope.

Moreover, since (see chapter 6) the conditions under which the pore complex is destabilised are well understood, these conditions can be avoided in the pore complex isolation procedure.

In this thesis, speculations as to the involvement of the nuclear envelope in mitosis, RNA synthesis, and other processes critical for cell function have been generated, and several aspects of its structural relationships and interdependence with other macromolecular systems have been clarified. It now remains to develop these findings, both theoretical and experimental, along lines such as those suggested in this last chapter. It is probable that the growth of ideas in this field will depend on the accretion of knowledge about the composition of the envelope and the details of its enzymic functions.

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A Negative Staining Study of Human Erythrocyte Ghosts and Rat Liver Nuclear Membranes

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An electron microscopic study of membrane features has been performed using negative staining. Hemoglobin-free human erythrocyte ghosts and membranes from rat liver nuclei have been observed in the electron microscope after negative staining with ammonium molybdate and the uranyl acetate–oxalic acid complex, both at pH 7.0.

The surfaces of partially empty nuclear envelopes have been shown to be covered with pores approximately 500 Å in diameter and surrounded by annuli approximately 250 Å wide. Many of the annuli appeared to be composed of eight subunits. Individual annuli have been found to separate themselves from the overall membrane sheet in regions where the membranes have undergone disruption.

Hemoglobin-free human erythrocyte ghosts have been shown to possess membrane holes or pits, approximately 140 Å in diameter. These pits are very similar to those formed by complement hemolysis, but are fewer in number and may be slightly larger. The erythrocyte pit structures have much smoother edges than the nuclear pores and do not appear to be composed of subunits, though this cannot be ruled out.

Apart from the pit structures the surface of the erythrocyte ghost has an overall fine structure. Within this fine structure individual protein molecules have been located, though it has very often been difficult to obtain sufficient contrast around the molecules owing to the double layer of membrane beneath them on the specimen grid. The uranyl complex provided a superior contrast to ammonium molybdate at this order of resolution, though it tended to impart a more granular texture to the electron images than did ammonium molybdate.

The results presented are discussed in relation to the possibilities and limitations of the negative staining method when applied to membrane systems.

In the present investigation, membranes obtained from human erythrocyte ghosts and rat liver nuclei have been studied by negative staining with ammonium molybdate and the uranyl acetate–oxalic acid complex.

It has been proposed independently by Muscatello and Horne (*22*) and Munn (*21*) that ammonium molybdate has desirable negative staining properties for the study

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of membrane systems. Mitochondrial and microsome preparations were investigated and a good preservation of membrane structure claimed. A negative staining study of low molecular weight proteins has been performed by Mellema *et al.* (*19*) using the uranyl acetate–oxalic acid complex which can be brought to neutral pH with ammonia without precipitation. This stain, which offers excellent electron contrast, has not yet been used to any extent on membrane systems.

In order to reveal detail on or within a membrane surface, a negative stain must be able to surround or penetrate to some degree any fine structure that is present. While penetrating a membrane surface, a negative stain may structurally alter components of the surface and thus create artifacts. A comparative study using different negative stains may show that some stains cause more membrane disruption than others (*12*, *21*, *22*). At the last resort it may, however, become impossible to rule out the possibility that a negative stain which is considered to have desirable staining properties is not causing changes within a membrane at the molecular level. Such changes could occur either before or during the drying of the stain–membrane mixture on the specimen grid. With these reservations in mind it is nevertheless generally accepted that negative stains have the ability to preserve and reveal structural detail within biological material.

MATERIALS AND METHODS

Preparation of human erythrocyte ghosts. Human erythrocyte ghosts were prepared by modification of previously published methods (*13*, *14*). Seven-day-old packed erythrocytes (group O +ve, in acid–citrate–dextrose) were obtained from the Blood Transfusion Unit of the Royal Infirmary, Edinburgh. The erythrocytes were washed three times in isotonic phosphate buffer (pH 7.0) at 4°C, with removal of the supernatant and buffy coat by aspiration. Hemolysis was then brought about by the addition of 5 volumes of 0.01 *M* phosphate buffer (pH 7.4) at 4°C. The ghosts were collected by centrifugation at 12 000 rpm (23 000 *g*) in the M.S.E. 6 × 250 ml angle rotor for 30 minutes. After 6 or 7 washes in the same buffer, ghosts of creamy-white coloration were obtained which appeared intact in the electron microscope by negative staining (*14*).

Preparation of rat liver nuclei. Nuclei were prepared from rat liver by modification of the methods of Sporn *et al.* (*26*), and Widnell and Tata (*28*). The livers of four overnight starved rats were removed as quickly as possible after sacrificing the animals and placed in ice-cold homogenizing medium (0.3 *M* sucrose and 0.003 *M* $MgCl_2$ in 0.03 *M* phosphate buffer at pH 6.1) and were minced for 2–3 minutes, with a scalpel. All subsequent operations were

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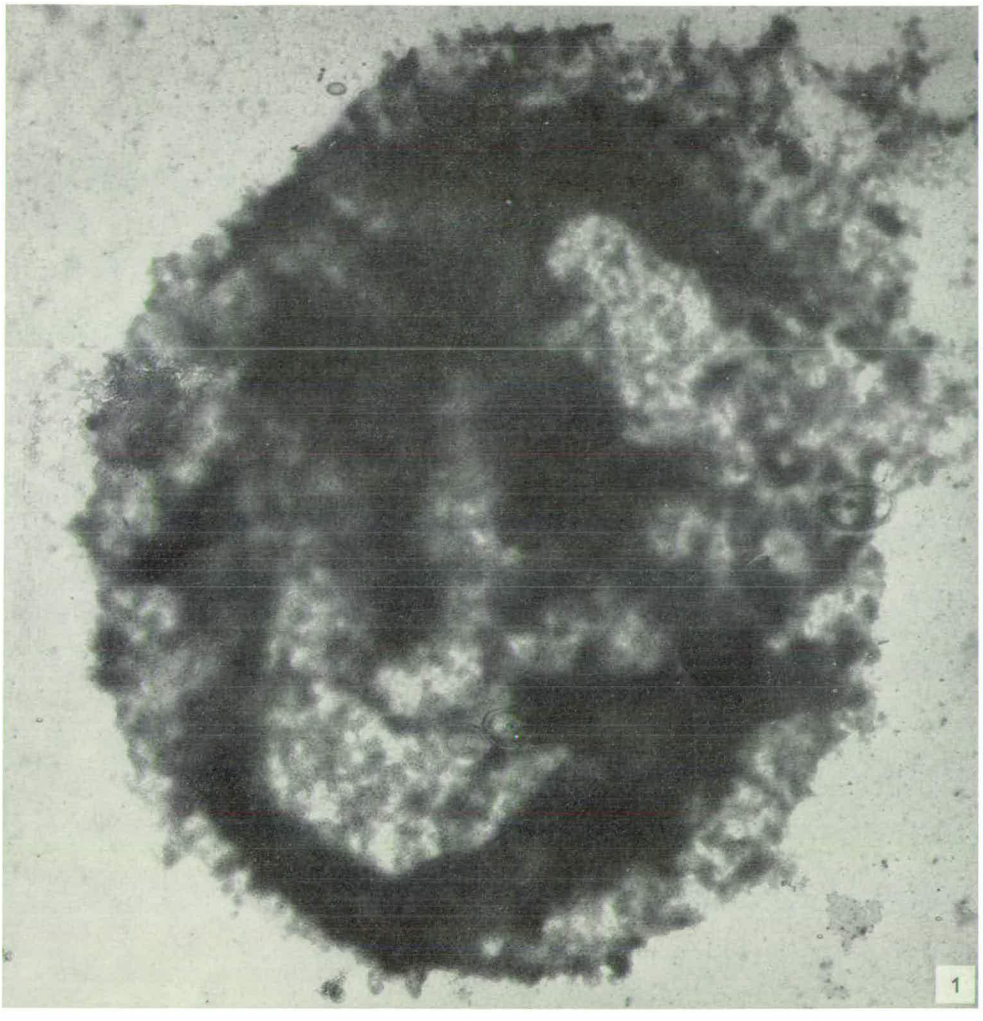
FIG. 1. A typical nuclear ghost. The large circular structures are flaws in the carbon backing film. Negatively stained with 2 % ammonium molybdate (pH 7.0). × 10,000.

FIG. 2. Part of a "nuclear ghost" that has undergone disruption. Negatively stained with 2 % ammonium molybdate. × 10,000.



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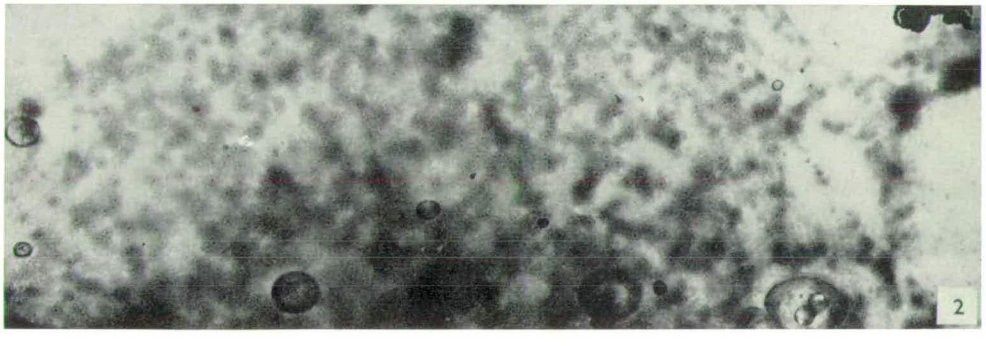

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carried out at 0–5°C. The fluid was then decanted and the minced liver resuspended in fresh homogenizing medium (5 ml/g tissue). The suspension was homogenized in a Potter-Elvehjem homogenizer with a loose-fitting pestle rotated at about 1000 rpm. Four up-down movements of about 30 seconds each were used. After filtration through 8 thicknesses of cheese cloth the homogenate was centrifuged at 1600 rpm (approximately 600 *g*) in the 8 × 50 ml swing-out rotor of the M.S.E. Mistral 4L centrifuge for 10 minutes. The pellet was resuspended in 0.03 *M* phosphate buffer (pH 6.1) containing 0.003 *M* $MgCl_2$, and sucrose added to 2.2 *M* concentration in centrifuge tubes. It was then centrifuged for 90 minutes at 25 000 rpm (55 000 *g*) in the Spinco No. 30 rotor, and the pellet of nuclei resuspended in the homogenizing medium after removal of the supernatant. The nuclei were further purified by 4 or 5 washes in the homogenizing medium, with centrifugation at 1600 rpm for 10 minutes each time.

A limited degree of nuclear disruption was brought about by resuspending the nuclei after the final wash in 0.01 *M* phosphate buffer (pH 6.1) which contained 0.002 *M* $BaCl_2$. This salt presumably does not have the same nuclear stabilizing power as $MgCl_2$; it was also found to act successfully as a bacteriostatic. After being stirred magnetically for 16–20 hours at 5°C samples were taken for electron microscopy. Negative staining revealed that approximately 5 % of the nuclei had lost a substantial portion of their contents.

Negative staining solutions. Ammonium molybdate was dissolved in water and the pH adjusted to 7.0 with ammonium hydroxide. The volume was then adjusted to give a 2.0 % (w/v) solution. The uranyl acetate–oxalic acid complex was prepared according to Mellema *et al.* (*19*). The pH was adjusted to 7.0 with ammonium hydroxide and the volume finally adjusted to give a solution 2.0 % (w/v) in uranyl acetate and 0.08 *M* in oxalic acid. Sodium phosphotungstate was prepared by dissolving phosphotungstic acid in distilled water and adjusting the pH to 7.0 with sodium hydroxide. The volume of the solution was then adjusted to give a solution 2.0 % (w/v) in phosphotungstate.

Negative staining technique. Single drops of the membrane suspensions were picked up on carbon-coated specimen grids from a strip of Parafilm. Most of the fluid was then drawn off by touching the side of the grid with a filter paper and a drop of the negative staining solution likewise picked up. The excess stain was then drawn off with a filter paper and the thin film of stain and membrane material allowed to dry. The procedure was carried out at room temperature.

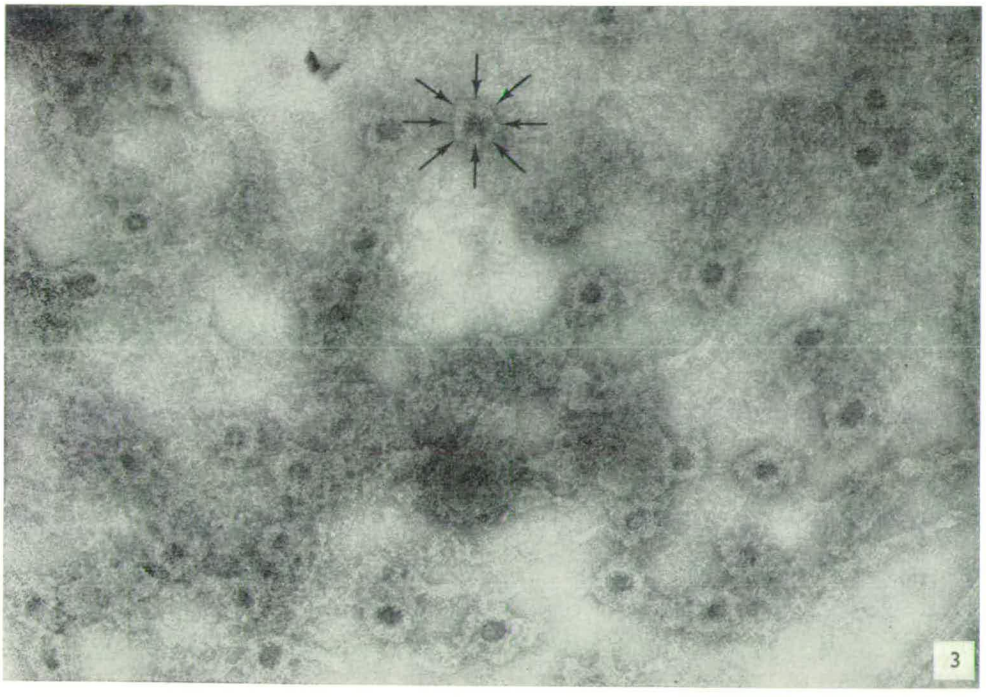
Electron microscopy. Electron micrographs were taken with an AEI EM 6B, operating at 60 kV with a 50-μ objective aperture. The magnification scale of the microscope was calibrated using a carbon grating containing 2160 lines/mm. Ilford $3\frac{1}{4} \times 3\frac{1}{4}$ inch plates, Special Lantern Contrasty, were used in the microscope camera.

FIG. 3. A higher magnification of a disrupted region of "nuclear ghost." Negatively stained with 2 % ammonium molybdate. Arrows indicate the subunits of a pore annulus. Some of the pores have electron transparent material at their centers. × 50,000.

FIG. 4. Part of a "nuclear ghost." Negatively stained with a 2 % solution of the uranyl acetate–oxalic acid complex (pH 7.0). × 30,000.

FIG. 5. Part of a thin-sectioned nuclear ghost. The plane of sectioning is tangential to the membrane surface. Positively stained nuclear pores are visible (arrowed). × 50,000.

FIG. 6. Part of a thin sectioned nuclear ghost showing the double nuclear membrane (*ONM*, outer nuclear membrane; *INM*, inner nuclear membrane). Contamination is present on both membranes. × 50,000.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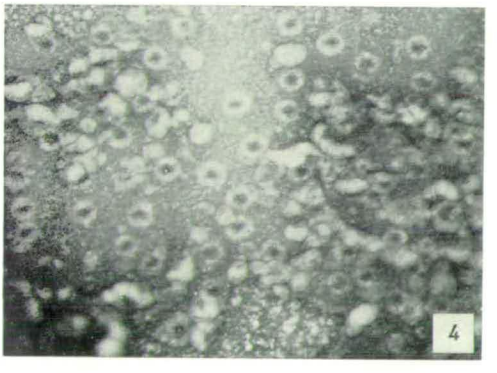
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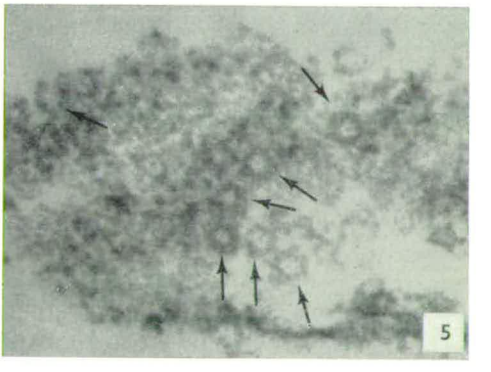
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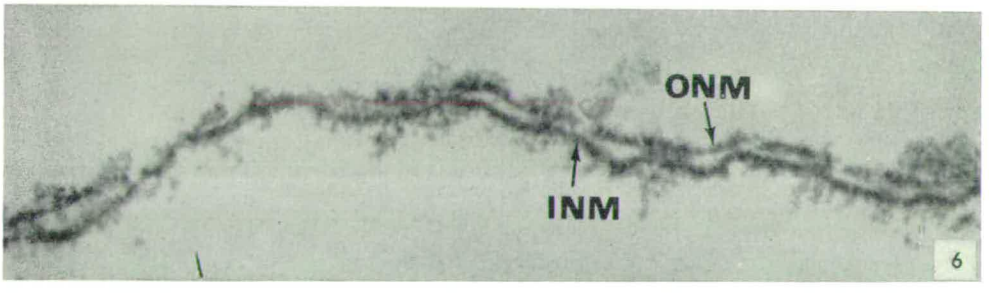
ONM
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OBSERVATIONS

Nuclear membranes

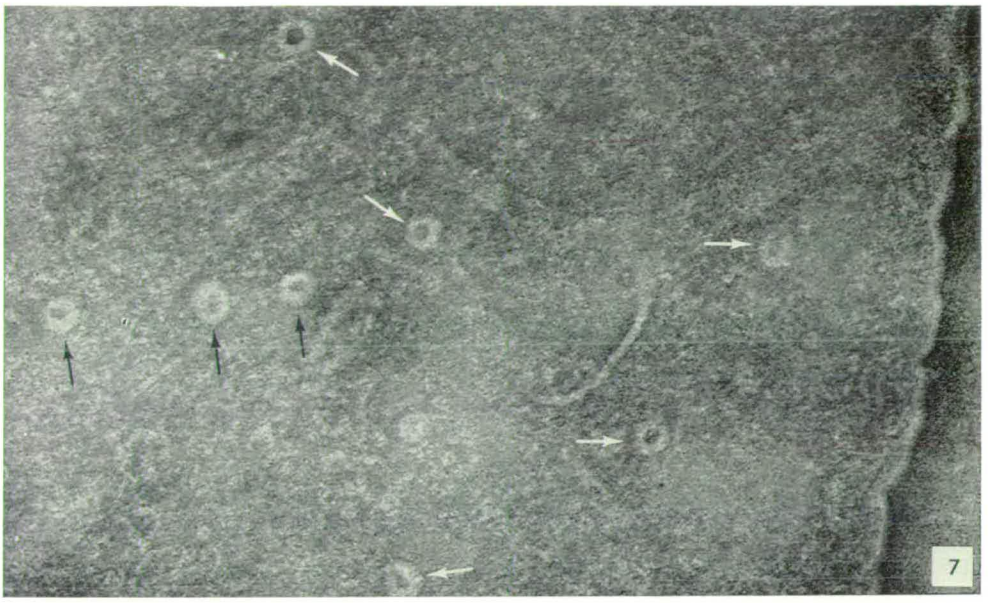
The small percentage of nuclei which were observed in the electron microscope to have lost a considerable portion of their contents were selected for this study. These "nuclear ghosts" were easily distinguished from intact nuclei, which were electron opaque bodies showing no surface detail by negative staining. The relatively empty "nuclear ghosts" and torn membrane sheets revealed membrane features even at electron optical magnifications as low at 5000 diameters. Figure 1 shows a typical "nuclear ghost" negatively stained with ammonium molybdate. In regions where the electron beam has penetrated the membrane material, the nuclear pores are clearly visible. These pores consist of electron transparent annuli filled with electron opaque stain. A smaller amount of stain surrounds the annuli. The annuli have an inner diameter of approximately 500 Å and an outer diameter of approximately 1000 Å.

In places the nuclear membranes appear to be undergoing fragmentation. This resulted in the isolation of the annuli, which must have a greater structural rigidity than the surrounding membrane (Fig. 2). This disruption was probably caused by the spreading forces applied to the nuclear membrane at the time the excess fluid was drawn off the specimen grid. At higher magnifications the annuli of the nuclear pores often appeared to be composed of 8 or 9 subunits (Fig. 3) in agreement with the observations of Gall (*9*) and Franke (*7*, *8*). The annuli sometimes appeared to be distorted. This again may be due to the spreading or disruption forces applied during the preparation of the negatively stained specimens. Essentially similar results were obtained using the uranyl acetate–oxalic acid complex (Fig. 4), but sodium phosphotungstate revealed the nuclear pores less clearly.

When thin-sectioned, the nuclear membranes were shown to be double and to have many positively stained annuli showing in regions where the plane of sectioning was tangential to the membrane surface (Figs. 5 and 6). These observations supported the negative staining study and indicated that on the negatively stained "nuclear ghosts" there must very often have been at least four layers of membrane, except where the membranes had undergone disruption.

Erythrocyte ghosts

Hemoglobin-free human erythrocyte ghosts were studied by negative staining with ammonium molybdate and the uranyl acetate–oxalic acid complex. These ghosts appeared as intact membrane sacs which had collapsed onto the carbon backing film. They thus presented a double layer of membrane material through which the electron beam had to penetrate. Figure 7 shows part of a typical erythrocyte ghost negatively



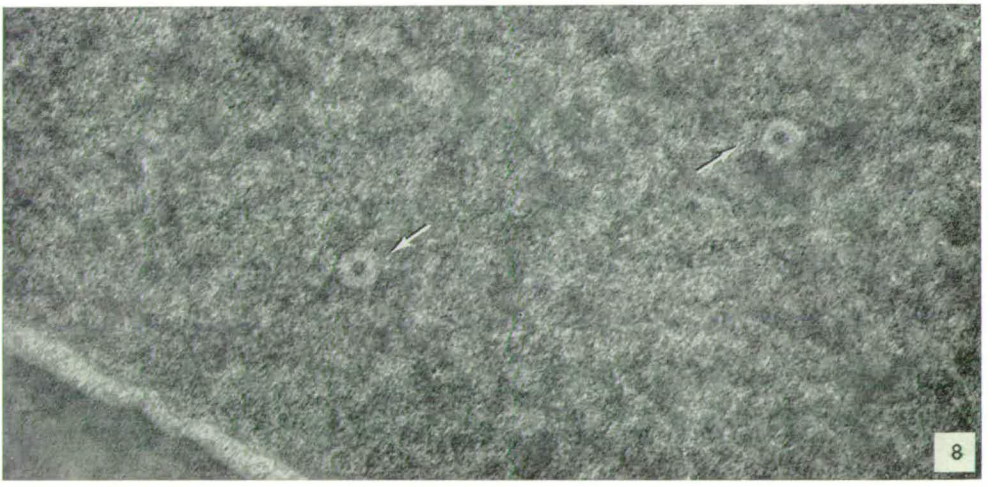


FIG. 7. Part of the surface of a human erythrocyte ghost. Negatively stained with 2 % ammonium molybdate. The arrows indicate pit structures. × 150,000.
FIG. 8. Part of the surface of a human erythrocyte ghost. Negatively stained with a 2 % solution of the uranyl acetate–oxalic acid complex. The arrows indicate pit structures. × 150,000.

stained with ammonium molybdate. The membrane surface contains many electron transparent rings or pitlike infoldings into which the negative stain has penetrated. These pits have an outer diameter of approximately 300 Å and an inner diameter of approximately 140 Å. They are randomly dispersed over the membrane surface and are found in very variable numbers on any one erythrocyte ghost. Sometimes the central hole of the pits appears less distinct. This may be because these pits are very slight infoldings containing little stain.

When negatively stained with the uranyl acetate–oxalic acid complex (Fig. 8), the pitlike structures appear similar to those observed with ammonium molybdate staining. The pitlike structures have also been observed with sodium phosphotungstate staining (*11*), but this stain tends to cause a rapid disruption of the nonfixed erythrocyte membrane, as suggested by Haggis and Harris (*12*).

Detail of the erythrocyte ghost surface

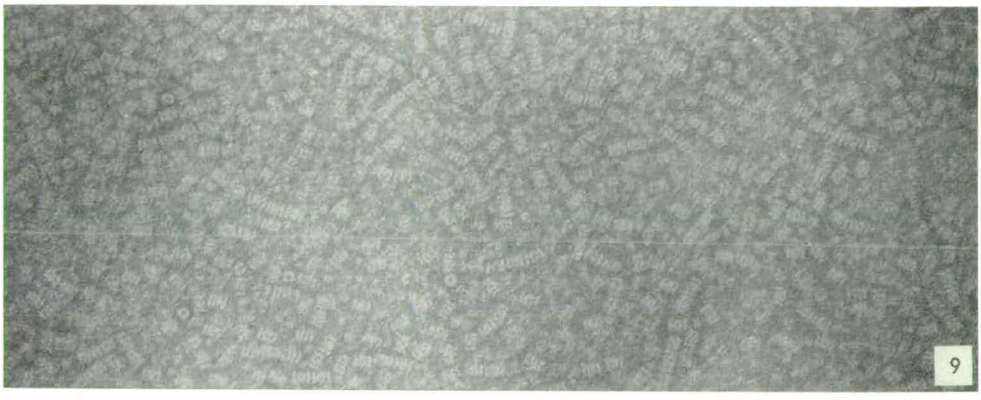
The membrane surface surrounding the pit structures (Figs. 7 and 8) has an unevenness of texture that makes it readily distinguishable from the carbon background. A survey of this surface has been made in an attempt to locate the hollow cylinder protein molecules previously isolated from erythrocyte ghosts (*13–15*). These molecules can be released from intact erythrocyte ghosts when they are made to undergo fragmentation by distilled water dialysis followed by a freeze-thaw treatment. Figure 9 shows a representative negatively stained field containing the isolated and concentrated hollow cylinder protein. Most of the molecules are oriented on their side, giving rectangular profiles (approximately 170 Å by 130 Å) with four cross striations. When oriented on their ends they appear as rings (approximately 130 Å in external diameter).

If located on or in the erythrocyte membrane, it is likely that these molecules will be revealed less clearly in the electron microscope than when they are in the isolated state. The structures indicated by the circles (Figs. 10–12) have rectangular and ringlike profiles and may represent hollow cylinder protein molecules situated on the membrane surface. Some individual molecules have been observed on the background (Figs. 10 and 11, arrowed) so it cannot be stated without reservation that the particles seen on the membrane surface are in their true locations. They may have been released by the negative stain and have settled randomly on the membrane before the stain dried.

DISCUSSION

The nuclear membrane

Negative staining with ammonium molybdate at pH 7.0 has been shown to reveal clearly the pore complexes situated in rat liver nuclear membrane. The uranyl acetate–oxalic acid complex at pH 7.0 was also found to be a successful stain though it prod-



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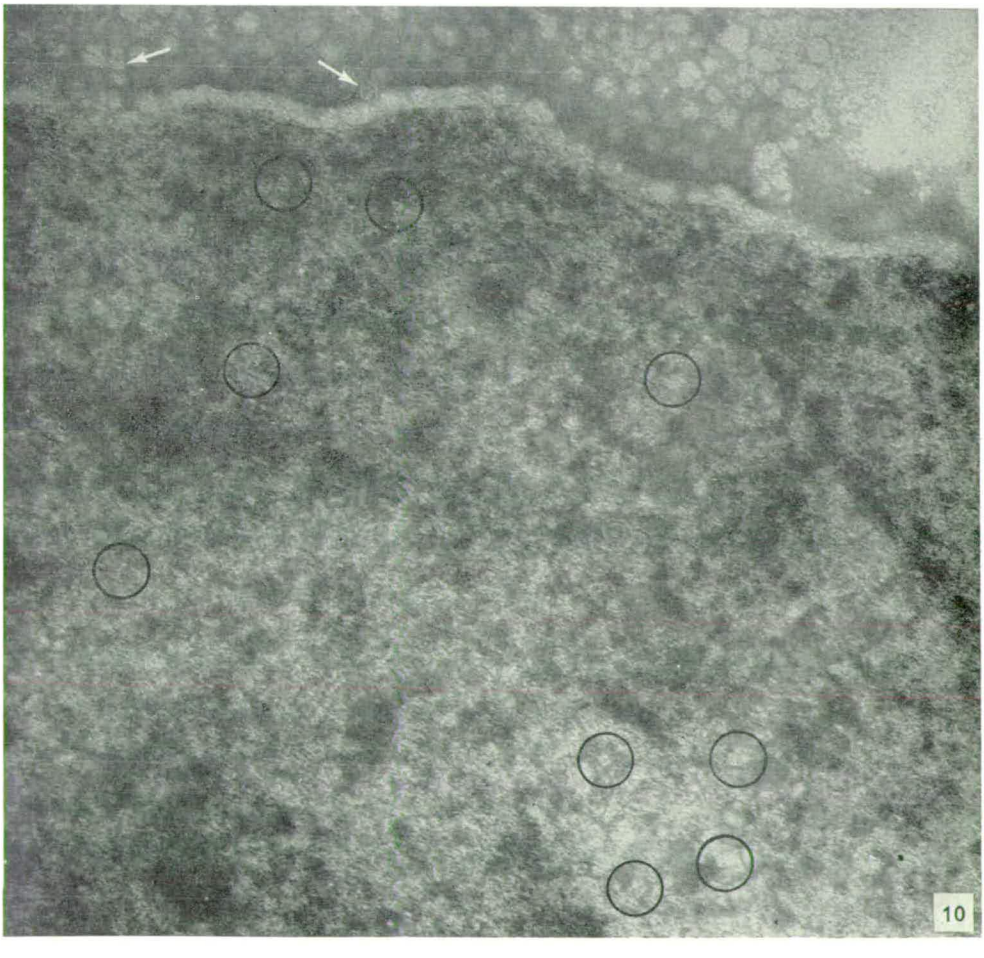

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uced a more granular background. The annuli of the nuclear pore complexes tend to be broader and more irregular than those observed by other workers, who used phosphotungstate as the negative stain, usually following osmic acid fixation (*7–9*, *30*, *31*). The octagonal symmetry of the annuli proposed by Gall (*9*) has been supported by the results presented in this study. The "subunits" of the annuli are approximately 250 Å in diameter, and their edges appear to be continuous with the surrounding membrane. The fact that the annuli are able to separate themselves from the surrounding membrane (Figs. 2 and 3) does suggest, however, that they have a greater structural rigidity than the membrane. This apparently mechanical disruption of the nuclear membrane by the negative staining procedure was also observed by Gall (*9*).

The nuclear pores trap a considerable quantity of negative stain which makes them stand out very clearly. Some of the pores have central electron transparent spots (Fig. 3), which is in agreement with thin-sectioning studies (*18*, *25*) that suggested the existence of material within the nuclear pore. The permeability studies of Wiener *et al.* (*29*) strongly suggest that some form of barrier exists within the nuclear pores. Yet on the other hand an accumulating amount of evidence suggests that there may be nuclear-cytoplasmic transport of material via the nuclear pores (*25*). When nuclear membrane preparations containing less nucleoprotein contamination are obtained, the negative staining technique should be able to resolve more detail within the nuclear pore complexes than has been possible in this study. It is probably reasonable to consider that the nuclear pore complexes perform important cellular functions for which they are structurally specialized.

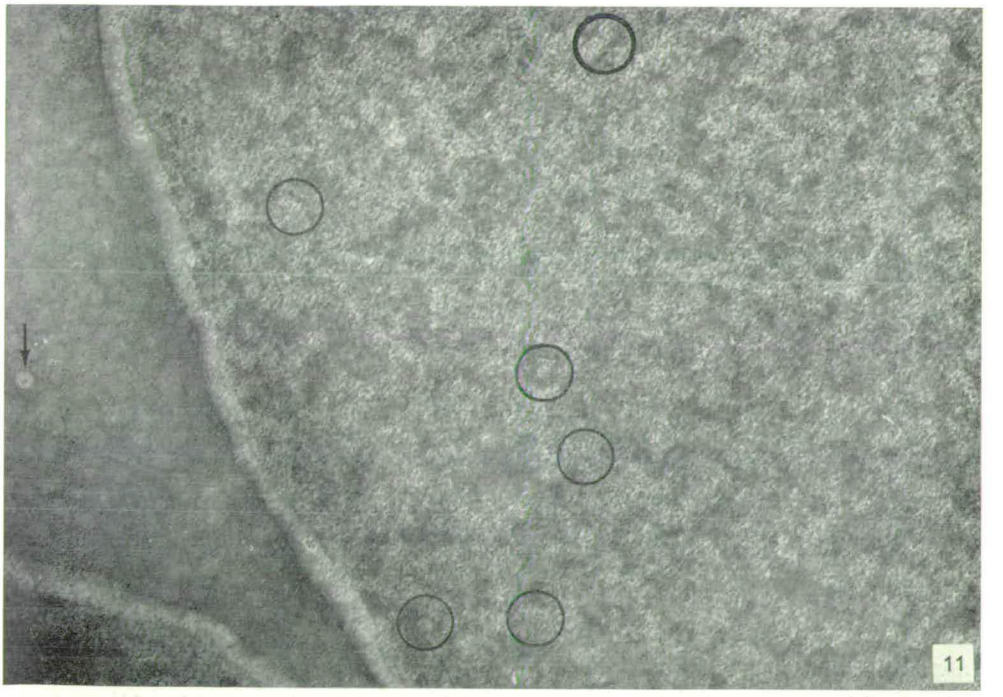
The erythrocyte ghost

Most previous studies on erythrocyte ghosts by negative staining have shown the membrane surface to be relatively smooth and featureless (*12*, *22*). Nevertheless, Haggis (*11*) has shown that erythrocyte ghost fragments negatively stained with sodium phosphotungstate possess pit structures similar to those observed on the surface of intact erythrocyte ghosts in the present study. After treatment with the plant glycoside saponin, erythrocyte ghosts have been shown by negative staining to be covered with an almost hexagonal array of 85 Å holes (*6*). This observation, however, was very rapidly explained by Bangham and Horne (*2*) and Glauert *et al.* (*10*),

FIG. 9. A representative field of the isolated and concentrated hollow cylinder protein from human erythrocyte ghosts. Negatively stained with a 2 % solution of sodium phosphotungstate (pH 7.0). × 150,000.

FIGS. 10 and 11. Parts of the surfaces of a human erythrocyte ghost. Negatively stained with a 2 % solution of the uranyl acetate–oxalic acid complex. The circles indicate structures on the membrane surface closely resembling the hollow cylinder protein molecules. The arrows indicate free hollow cylinder protein molecules on the background. × 150,000.

FIG. 12. Part of the surface of a human erythrocyte ghost. Negatively stained with 2 % ammonium molybdate. The circles indicate structures on the membrane surface closely resembling the hollow cylinder protein molecules. The arrows indicate pit structures. × 150,000.



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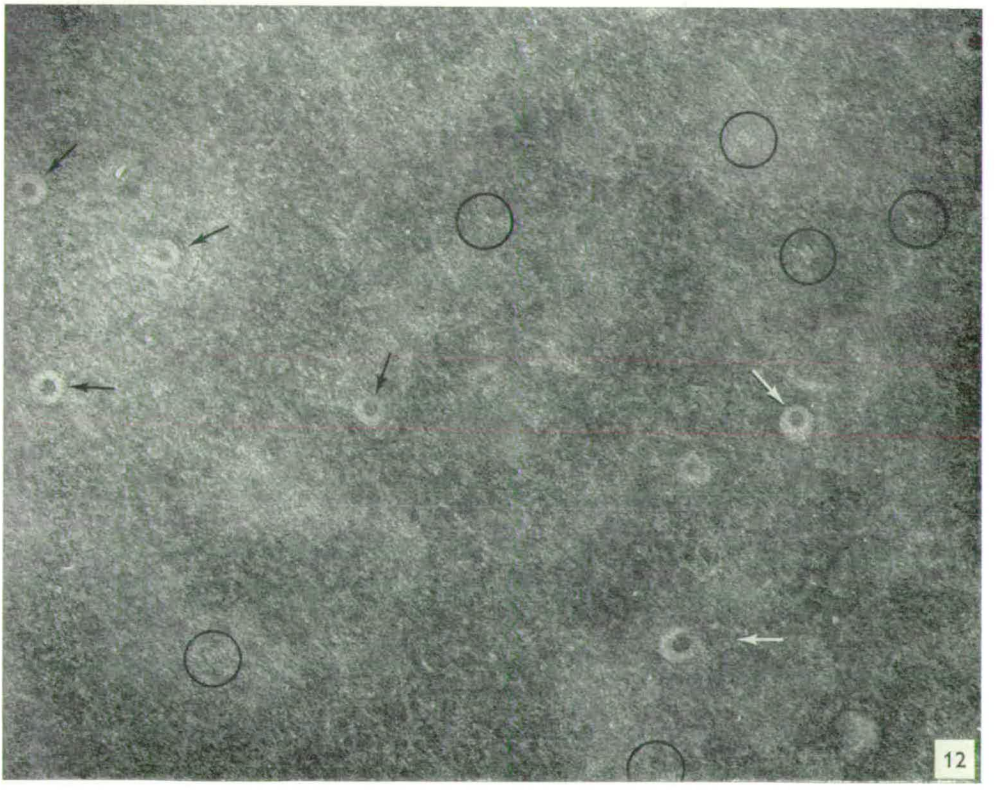

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who showed the holes to be artifacts produced by the interaction of saponin with the membrane lipids.

Complement hemolysis has also been shown to produce pitlike lesions 80–100 Å in diameter in the erythrocyte membrane (*16*). These pits have also been interpreted as localized changes within the lipid phase of the membrane. The complement-induced membrane pits are very similar to the pits observed on untreated intact human erythrocyte ghosts when negatively stained with ammonium molybdate or the uranyl acetate–oxalic acid complex, but are present in much greater numbers and may be slightly smaller. It is possible that the repeated washings in hypotonic buffer used to obtain hemoglobin-free ghosts may have produced membrane lesions and that the pits on untreated ghosts are membrane artifacts. Because of the close similarity between the two types of membrane pits, it cannot at the moment be claimed that the action of complement is essential for their formation, as claimed by Humphrey and Dourmashkin (*16*) and Coombs and Lackmann (*5*).

The holes or pits observed on the surface of the untreated erythrocyte ghost differ in several respects from the nuclear pore complexes. First, they are considerably smaller, and second, they have very smooth contours. The second difference tends to suggest that the erythrocyte ghost pits are not composed of subunits, though this possibility cannot be ruled out. The variable edge thickness of the erythrocyte ghost pits is also a feature not observed for the nuclear pore annuli. This suggests that the erythrocyte ghost pits may represent localized infoldings of the membrane rather than structures standing up on the membrane surface.

Haggis (*11*) postulated that the pits he observed on ghost fragments might be places where stromalytic outfoldings from the membrane had broken off. The authors consider this to be unlikely since no free stromalytic tubes have been observed on the background adjacent to intact ghosts showing these pits. Also, varying lengths of stromalytic tube have been shown by Baker (*1*) to be left attached to erythrocyte ghosts negatively stained with sodium phosphotungstate.

Fine detail of the erythrocyte ghost surface

Both the negative stains used throughout this study have shown that the surface of the erythrocyte membrane has a detailed fine structure. This fine structure does not appear as an ordered array of subunits, but as an overall random unevenness of texture. It has been possible, however, to locate, with reasonable accuracy, individual particles which closely resemble the hollow cylinder protein molecules previously isolated from erythrocyte ghosts (*13–15*). The clarity of the particles on the membrane surfaces is not as great as when they are in the isolated state. The electron micrographs presented do nevertheless show clearly that the surface of the erythrocyte ghost is not featureless. It is probable that there are a large number of proteins (e.g., blood

group antigens and enzymes) that are located on or within the outer surface of the erythrocyte membrane. These proteins may even be organized in characteristic spatial arrangements, as suggested by Johnson (*17*) for the enzymes located on the surface of hamster intestinal microvilli. Proteins on or in the surface of the erythrocyte ghost have beneath them two layers of membrane which will reduce the ability of a negative stain to contrast the proteins. Thus the difficulty regarding the precise location of the protein under consideration by negative staining can be readily appreciated. The fact that some free molecules can be observed on the carbon background adjacent to the erythrocyte ghosts may be indicative of their having been released from the membranes during the negative staining procedure.

Several groups of workers have been able to show membrane-associated molecules or particles by negative staining (*3*, *4*, *17*, *23*, *24*, *27*). Humphrey and Dourmashkin (*16*) were able to visualize a 19 S hemolytic antibody at the edge of sheep erythrocyte ghost fragments, but they stated that it was invisible on the surface of the membranes. The hollow cylinder protein from erythrocyte ghost (22.5 S) has a very easily recognizable rectangular profile when lying on its side and is thus brought just within the range of visualization by negative staining when situated on the erythrocyte membrane surface. The clarity with which membrane associated molecules can be visualized by negative staining depends primarily on the degree to which the particle stands up from the membrane surface and can accumulate stain around itself, and, second, on thickness of the membrane layer or layers beneath the particle.

The efficiency of the two negative stains employed throughout this study has been investigated at different electron optical magnifications. At lower magnifications both ammonium molybdate and the uranyl complex provide adequate contrast. At higher magnifications the uranyl complex appears to afford a superior contrast to ammonium molybdate, but it does at the same time give a more granular texture to the electron image.

In conclusion it can be said that the results obtained using ammonium molybdate are in general agreement with those of other workers (*21*, *22*). Negative staining has been shown to provide a successful means of studying features of membrane material over the entire range of electron optical magnifications now available. At high magnifications negative staining has the ability to reveal detail on or within cellular membranes at the molecular level.

The authors wish to acknowledge the fact that electron microscopic facilities were made available by Professor W. E. Watson, Department of Physiology, University of Edinburgh. They also wish to thank Mr. J. N. Brown for the skillful technical assistance he provided. The helpful comments made by Dr. A. H. Maddy, Department of Zoology, University of Edinburgh, throughout the preparation of the manuscript are gratefully acknowledged. P.A. is a recipient of an M.R.C. training grant.

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The isolation of the envelopes of rat liver nuclei

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SUMMARY

1. A strict morphological definition of the nuclear envelope is established, and a procedure for the isolation of envelope fragments consistent with this definition is described.

2. The composition of the isolated envelope (% by weight) is: protein 64%; lipid 23%; DNA 8%; RNA 5%.

3. The nuclei and envelopes contained little or no detectable succinoxidase, 5′-nucleotidase or glucose-6-phosphatase activity.

4. It is demonstrated that the application of techniques reducing the envelope DNA content to significantly less than 8% leads to a loss of morphological integrity of the system.

5. It is concluded that DNA is probably an essential structural component of the nuclear envelope.

Available information concerning the nuclear envelope of mammalian liver suggests no characteristic functional property whereby isolated preparations of the envelope can be recognised as such. However, electron microscopy reveals the following characteristic morphological features: (a) The envelope is a double-membrane system; (b) under certain conditions of negative staining, some 20–30% of the surface can be seen to be occupied by "pore complexes"[2]. The latter are complicated structures comprising an octagonally symmetric annulus[3], a "central spot"[4], and occupying the space between these possibly a diaphragm[5] or struts[6]. It follows that any preparation of nuclear envelope is satisfactory if and only if it is a double membrane, and 20–30% of its surface consists of pore complexes with the above-mentioned ultrastructural features. Only when this criterion has been satisfied can consideration reasonably be given to the "purity" of the preparation; this may be represented, for example, as the minimum attainable DNA/lipid ratio (assuming most of the nuclear lipid to be associated with the envelope). Material of

membranous appearance, prepared from nuclei and having a low DNA content, does not constitute a satisfactory nuclear envelope preparation unless it satisfies the criterion of morphological integrity.

Nuclei were isolated by a modification of the methods of Widnell and Tata[7] and Sporn *et al.*[8]. Three male albino rats (150–200 g) were killed by a blow on the head, and the livers excised as quickly as possible and placed in ice-cold homogenising buffer (0.32 M sucrose–0.03 M sodium phosphate buffer, pH 6.1–3 mM $MgCl_2$). After the liver had been minced with scissors for 1–2 min, the fluid was decanted and replaced by 80–90 ml fresh homogenising buffer. The suspension was homogenised in a Potter–Elvehjem homogeniser with a 0.5 mm clearance, the pestle being rotated at 3000 rev./min. Five to six up-down movements of an average of 20–30 sec each were used, care being taken to ensure that the temperature did not rise above 5°. The homogenate was filtered through eight thicknesses of cheesecloth and centrifuged at 800 X *g* for 10 min. The supernatant was carefully decanted and the pellet resuspended in 4 vol. of dense sucrose medium (2.3 M sucrose–0.03 M sodium phosphate buffer, pH 6.1–3mM $MgCl_2$). This suspension was layered over 10 ml of the same dense sucrose medium in 35-ml capacity tubes and centrifuged at 60 000 X *g* for 60 min. The pellet of nuclei was resuspended in 5 ml homogenising buffer and centrifuged at 800 X *g* for 10 min; this washing was repeated 2–3 times.

Electron and phase contrast microscopy of the nuclei showed no contamination other than traces of collagen. Succinoxidase, assayed by the method of King[9], had 3% of the activity of the total liver homogenate. 5′-Nucleotidase, assayed by the method of Emmelot and Bos[10], and glucose-6-phosphatase, assayed by the method of Swanson[11], had no detectable activity. It was concluded that microsomal, mitochondrial and plasma membrane contamination of the nuclei was minimal.

Homogenisation, passage through a hypodermic needle, freeze–thawing and shearing in a Waring blender failed to lyse the nuclei; these observations are consistent with those of Kashnig and Kaspar[12]. Osmotic shock had no effect if the magnesium concentration was greater than 0.2 mM. The nuclei were disrupted by sonication after suspension at a concentration of about 5 mg protein/ml in 5 ml 0.02 M Tris–HCl buffer, pH 7.5, containing 1 mM $MgCl_2$. (The concentration of nuclei in the suspension is not critical). 2 X 20 sec sonication at 1 μm amplitude, 21 kHz, in a M.S.E. ultrasonicator with a 0.5 cm probe was used at 0°. The suspension was then diluted with an equal volume of ice-cold water and centrifuged at 3000 X *g* for 10 min; intact nuclei, nucleoli, collagen and dense chromatin were pelleted. The supernatant was centrifuged at 30 000 X *g* for 30 min; the pellet of crude nuclear envelopes was rinsed with and resuspended in distilled water. Electron microscopy, using 2.0% ammonium molybdate at pH 7.0 (ref. 13), revealed on the envelope fragments pore complexes of the expected structure and frequency. There was little membrane vesicle formation (see Fig. 1).

The envelopes were purified by layering on a 5-ml sorbitol gradient, 1.0–5.0 M in distilled water, and centrifuging for 16 h at 25 000 rev./min (50 000 X *g* average) in the SW 39 rotor of a Spinco L centrifuge at 0–5°. The envelopes were recovered from a narrow band with specific gravity 1.27. Centrifugation for up to 64 h did not alter the position of this band; it therefore represented the equilibrium position of the envelopes on the gradient. There was no pellet, but much dissolved material remained in the upper part of the gradient. The envelopes, after 24 h dialysis against water, showed the same morphological integrity as the crude envelopes.

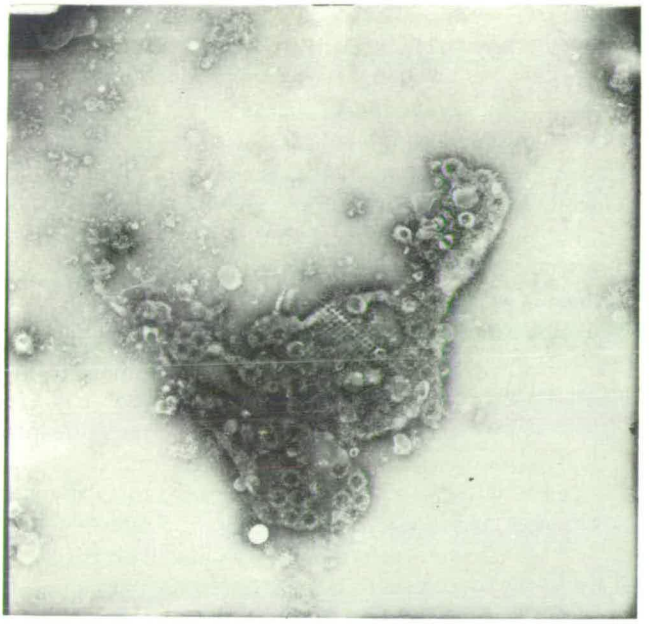

Fig. 1. Nuclear envelope fragment. Fragment of nuclear envelope prepared by the procedure described in the text. Negatively stained with 2% ammonium molybdate, pH 7.2. Magnification, x 25 000 diameters.

The most significant features of this preparation procedure are: (a) The absence of K^+ from all media; (b) low ionic strength throughout; (c) No DNAase treatment of the envelopes. Any variation in these three criteria leads to loss of morphological integrity. However, K^+, high ionic strength and DNAase have been employed by other groups in the isolation of nuclear envelopes. Berezney *et al.*[14], Kashnig and Kaspar[12] and Franke *et al.*[15] all used high ionic strength media; the latter two groups used high K^+ concentrations, and the first used a prolonged exposure to DNAase. Attempts to repeat these procedures in this laboratory have yielded unsatisfactory results. The method of Kashnig and Kaspar[12] gives a tangled mass of membranous vesicles essentially free of pore complexes. The method of Franke *et al.*[15] gives highly vesicularised material with few pore complexes, most of which lack the characteristic structural features. The method of Berezney *et al.*[14] gives envelopes in which pores are visible, but in general the annulus and central spot are lacking. All these envelope preparations are of low DNA content.

The approximate composition of the envelopes obtained from the sorbitol gradient was as follows: 8% by weight DNA (assayed by the method of Giles and Myers[16]); 5% RNA (assayed according to Schneider[17]); 23% by weight lipid (measured as phosphate by a slight modification of the method of Bartlett[18]). The remaining 64%, after enzymatic removal of the nucleic acids and extraction with chloroform–methanol (2:1, v/v) to remove lipids, was protein. The most significant difference between this composition and that of envelopes prepared by other groups is the relatively high DNA content, which may account

for the relatively high apparent density.

The pore complexes were rapidly destroyed by treatment with KCl ($\geqslant 0.01$ M) or NaCl ($\geqslant 0.25$ M), or by exposure to any high ionic strength medium irrespective of the ions used. The most significant fact to emerge from these observations is the extreme sensitivity of the envelope structure to K^+. Concommitant with the destruction of the pore complexes was the release of outer and inner nuclear membrane material in the form of minute single-membrane vesicles, of diameter generally less than 100 nm. Inner and outer membrane could not, of course, be distinguished in this highly fragmented form. At the same time, there was a reduction of around 40% in the DNA content of the remaining solid material (*i.e.* the material which could be recovered by centrifugation at 30 000 X *g* for 30 min). It was concluded either (1) that DNA is an essential structural component of the nuclear envelope, being probably associated with the pore complexes, so that removal of DNA from the system causes morphological destruction; or (2) that high ionic strength and K^+ treatments destroy the envelope structure directly, and in the process release any attached DNA.

To distinguish between these possiblities, the envelopes from the sorbitol gradient were treated with 10 μg pancreatic DNAase/mg protein in the presence of 5 mM Tris–HCl, pH 7.5, containing $1 \cdot 10^{-4}$ M $MgCl_2$, at room temperature for 10–15 min. This treatment destroyed the pore complexes and led to the formation of minute single-membrane vesicles from the remainder of the envelope. DNAase inactivated with $1 \cdot 10^{-4}$ M EDTA, or the products of DNAase digestion of chromatin, had no effect on the morphology of the envelope. The DNAase was shown to be essentially protease and lipase free by the following procedure. Erythrocyte membranes, prepared by the method of Maddy[19], were suspended in 5 mM Tris–HCl–$1 \cdot 10^{-4}$ M $MgCl_2$, pH 7.5, containing 0, 5, 10 or 20 μg pancreatic DNAase/mg protein. After incubation for 1 h at room temperature and centrifugation at 40 000 X *g* for 30 min, the absorbance in all supernatants was the same. This demonstrated that the enzyme was not catalysing the release of protein or peptides or scattering material, from a nucleic-acid-free membrane. It is concluded that removal of DNA leads to morphological disruption of the whole envelope, which implies that DNA is an essential structural component of the system.

The destruction of morphological integrity by K^+, high ionic strength or DNAase treatment results in material which does not meet the primary criterion for a nuclear envelope preparation cited at the beginning of this report. It is reasonable to interpret all these effects as involving primarily the removal of DNA, and postulating that the pore complexes are stable structures only in the presence of DNA. When the pore complexes are destabilised by the DNA removal, the two membranous components of the envelope separate and fragment. Thus DNA is, on this interpretation, required to maintain the morphological organisation of the whole envelope system; it cannot be said to stabilise either one or the other of the component membranes.

The finding that nuclear envelopes are associated with significant amounts of DNA is consistent with the results of electron microscopic studies which have revealed apparent structural linkages between envelopes and chromatin fibrils in a range of mammalian and avian cells[20–22]. It is possible that this association has significance in DNA replication[23,24]

More detailed investigations of the nucleic acid components of the nuclear envelope, and of the ionic sensitivity of the morphology of the system, are in preparation.

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