Techniques of Biochemical and Biophysical Morphology

VOLUME THREE

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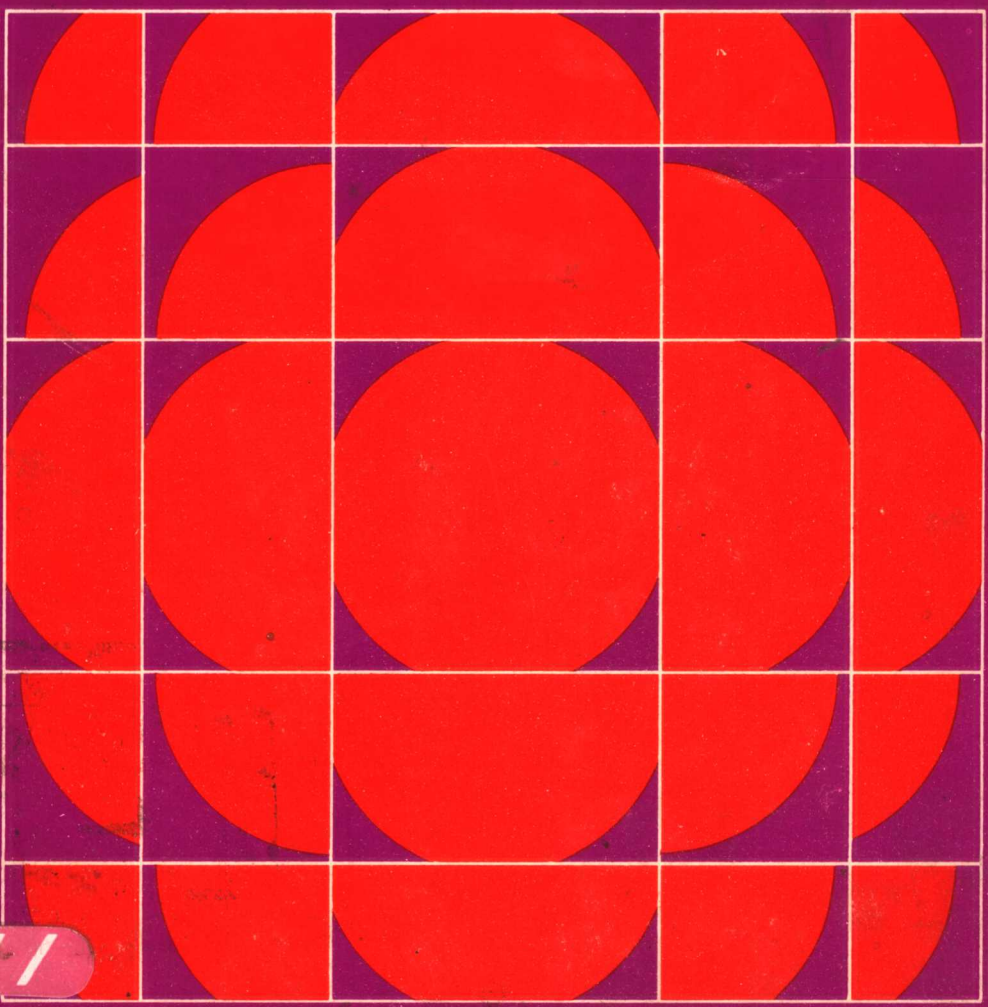

TECHNIQUES OF
BIOCHEMICAL AND
BIOPHYSICAL MORPHOLOGY

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CONTENTS

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Nuclear Envelope Isolation

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I. INTRODUCTION

Interest in the nuclear envelope has considerably increased in recent years, probably as a result of the formulation of some specific questions of envelope function and biochemistry (for reviews see refs. 1–4). One approach to answering these questions has been by direct biochemical analysis of the envelope—an approach that obviously demands the isolation of suitable quantities of pure envelopes. Since about 1969 a variety of procedures have been developed to meet this demand, so that today there are at least 10 that could plausibly be used for obtaining envelopes on a large scale from, for instance, rodent liver.

Some particular difficulties arise with these isolation procedures, quite apart from the important problems of envelope yield, purity, and preservation. Firstly, the envelope represents a small proportion of the total cellular lipid and protein, typically for rat hepatocytes, about 2 and 1%, respectively [a partial and useful exception is provided by the avian erythrocyte, in which the plasma membrane and nuclear envelope are the only major membrane systems (5)].

Secondly, the envelope is in places continuous with the endoplasmic reticulum, and finally, chromatin is apparently attached to the inner nuclear membrane. Thus there are particular dangers that isolated envelopes may be contaminated by microsomes or chromatin. For these reasons there is a tendency for envelope-isolation procedures to be tedious, involving large quantities of starting material and many steps and often taking 24 hr to provide purified envelopes from an animal. As a result, envelope purity too often comes at the expense of loss of morphology and enzymatic activities.

From this point of view, no envelope-isolation procedure so far developed is without disadvantages, and in the absence of direct experimental comparison of the success of the different procedures, it is impossible to say certainly which might be most useful for a particular purpose. However, using the information available on the different envelope preparations and the procedures used to obtain them, we attempt to draw some guidelines. As a first step, we consider the available criteria for the integrity and purity of isolated envelopes and then, using these criteria as a background, we survey the existing isolation techniques. Finally, we select three procedures that can be recommended on the basis of this survey for more detailed description.

II. CRITERIA FOR PRESERVATION AND PURITY OF ISOLATED NUCLEAR ENVELOPES

1. Preservation

Many features of an isolated envelope preparation give some indication of their state of preservation. Of these, morphology is of paramount importance because not only is it possible to compare the morphology of isolated envelopes directly with that of those in intact tissue, but also an overall view of the preservation of the envelopes is obtained. The chemical and biochemical composition of isolated envelopes gives less information than morphology because usually it cannot be compared with the intact tissue. However, in situations where a certain component is accepted as being present in the envelope (e. g., glucose-6-phosphatase, see Section II.1.B) but is found to be absent in envelopes made by certain procedures, this can be taken as a sign that these envelopes are not well preserved.

A. MORPHOLOGY

In the absence of suitable chemical or biochemical markers (see Section II.2), the only way to recognize a nuclear envelope preparation as such is by its distinctive morphology and, in particular, by the presence of nuclear pores. Extensive electron microscopic studies (e.g. refs. 6 and 7) have shown the nuclear envelope to consist of a pair of separate membranes, running roughly in

parallel over the nuclear surface. The outer membrane is usually ribosome studded, while the inner one makes close contact with peripheral heterochromatin. At intervals the two membranes fuse at nuclear pores, of which there are approximately 5000 per diploid rat hepatocyte nucleus. These features are shown in Figure 1. At higher power (Figs. 1*b*, 1*c*, and 1*d*) cross and tangential sections reveal further details of the nuclear pores, which according to the model of Roberts and Northcote (7) consist of three rings of eight granules: one set of annular granules on the cytoplasmic side of the envelope, a further set on the nucleoplasmic side, and a third set of peripheral granules between the other two. Often the central channel of the pore is partially plugged by a central granule. The structure of the nuclear pore is essentially invariant among various eukaryotes (6), although the precise dimensions do vary somewhat. The outer diameter of the annulus is usually in the range 80–120 nm and the

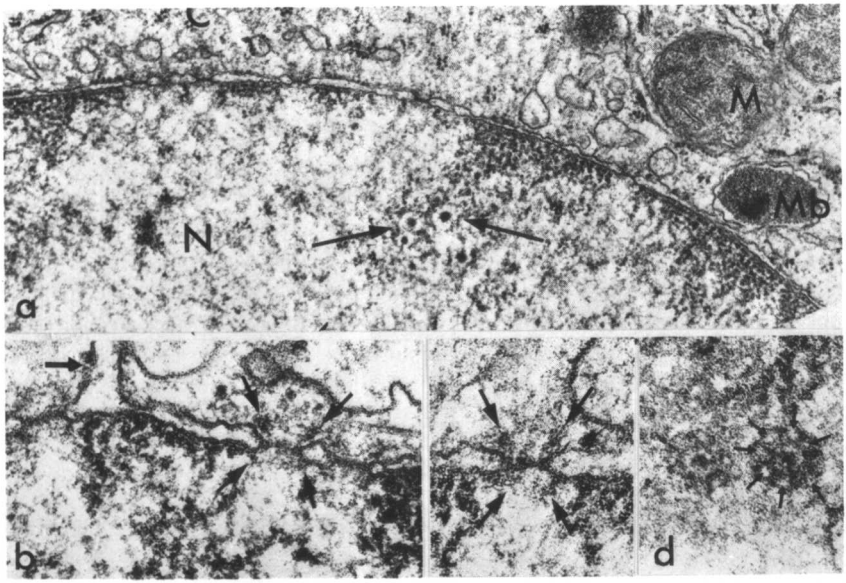

Figure 1. Electron micrographs of stained ultrathin sections of intact rat liver tissue. A survey of the nuclear periphery is shown in (*a*) (×30,000 diameters) in which N indicates the nucleus, M a mitochondrion, and Mb a microbody, and the arrows point to two perichromatin granules. The two membranes of the envelope are visible, as are nuclear pores in section and chromatin in patches on the inner membrane, between pores. Nuclear pores are shown at higher power in *b* (×70,500 diameters), *c*, and *d* (both ×94,000 diameters). In *b* and *c*, both cross sections, the annular granules are indicated by the sets of four arrows, while in the grazing section (*d*), they are shown by eight arrows. Finally, the thick arrow in *b* indicates a region of continuity between the outer nuclear membrane and the endoplasmic reticulum. Micrographs by courtesy of W. W. Franke and J. Kartenbeck.

inner in the range 40–70 nm. It should be noted, however, that some authors measure instead the diameter of the hole in the membrane, regarding the annulus as partially blocking this hole and also overlapping it on the outside. Thus a range of values intermediate between the inner and outer annular diameters is obtained.

While the pore ultrastructure is certainly very distinctive, it is not unique to the nuclear envelope, identical structures being found in the annulate lamellae (8). This membrane system is most commonly found in oocytes and embryonic and transformed cells but is rare or absent in adult somatic cells. Therefore in liver and many other tissues, nuclear pores can be regarded as definitive nuclear envelope markers, and the preservation of their ultrastructure should be a major objective of any envelope-isolation procedure.

B. ENZYMOLOGY

It is now widely accepted that a large group of microsomal enzymes, such as glucose-6-phosphatase, ATPase, NADH-cytochrome *c* reductase, and NADPH-cytochrome *c* reductase, are also integral components of the rodent liver nuclear envelope (2,4). This belief rests on their presence in many preparations of isolated envelopes and also in some cases on histochemical studies. However, in the case of glucose-6-phosphatase, this activity is virtually absent from envelopes isolated by certain procedures (9,10) and so it must be concluded that here the enzyme was lost or inactivated during isolation—in either case an indication that the envelopes are not well preserved. Certain nuclear enzymes, such as DNA polymerase (11,12), RNA, and poly A polymerases (12a), and also several mitochondrial proteins (see Section II.2) are also present in some envelope preparations, but their relationship to the envelope is not clear enough to justify their use as a criterion for the preservation of isolated envelopes.

2. Purity

Gross contamination of isolated envelopes by components of other membrane systems or chromatin can usually be checked by combinations of electron microscopy, chemical analysis, and enzyme markers. Chemical analysis of isolated envelopes reveals the expected presence of protein, phospholipid (200 to 400 μg/mg protein), RNA, and small amounts of DNA. Gross contamination of the envelopes by chromatin, for instance, is shown by a high recovery of nuclear DNA in the envelope preparation and a high DNA-to-protein ratio (e. g. see ref. 13). Suitable enzyme markers exist for detecting severe contamination by components such as mitochondria and plasma membranes (14), but it should be noted that there is at present no marker suitable for differentiating

between nuclear envelopes and the endoplasmic reticulum, nor indeed is there a unique nuclear envelope marker (4).

Great difficulties arise, however, in deciding whether or not a minor component, which is known to be present in abundance in other parts of the cell, is in fact a contaminant when found in isolated envelopes. This situation holds for some respiratory proteins and for the DNA recovered in many envelope preparations. Much controversy has been aroused by these questions, which therefore deserve further consideration.

It is a common experience that low levels of cytochromes $a + a_3$ and cytochrome oxidase can be recovered in isolated nuclear envelopes (10,15–17). Since even lower levels of succinate dehydrogenase and other mitochondrial marker enzymes are found in the envelopes, it is concluded that the presence of some of the first-mentioned proteins cannot be explained by mitochondrial contamination. This conclusion rests on two major untested assumptions, namely that the marker enzymes are at least as stable to the isolation procedure as are the proteins of interest and that the two groups of proteins do not become physically separated at some stage during isolation, with the cytochromes preferentially associating with the envelopes. When cardiolipin, taken as a chemical marker for mitochondria, is analyzed in isolated envelopes (18), levels of mitochondrial contamination are indicated sufficient to explain the observed quantities of respiratory proteins, which suggests that one or other of the above assumptions is incorrect. In the absence of more striking differences in the properties of the mitochondrial and envelope cytochrome oxidase activities (16,18), the presence of this activity in isolated envelopes seems likely to be a result of mitochondrial contamination.

In a similar fashion, small amounts of DNA are also recovered in envelope preparations (except for ref. 19). Here the question has resolved into two parts: firstly, whether any DNA is associated with the isolated envelopes as a result of a previous *in vivo* association and secondly, whether newly synthesized DNA is preferentially localized in the envelope fraction, that is, whether DNA synthesis occurs at the envelope. The second part can now be answered in the negative (e.g. see refs. 2 and 20), and it must be concluded that in certain envelope preparations newly replicated DNA preferentially and artifactually associates with the envelope during isolation (21–23). Available evidence (2–4) suggests that the first part of the question should be answered in the affirmative and the problem becomes one of estimating how much of the DNA associated with isolated envelopes is in fact a contaminant. An attempt has been made at doing this by means of reconstitution experiments (11), but these cannot be wholly satisfactory. However, these experiments and studies on the properties of the envelope-associated DNA [late replication and moderate enrichment in repetitive sequences (24–26)] do suggest that if appropriate precautions are taken,

most of the envelope-associated DNA is not a contaminant. Further confirmation of this point is essential, however, and it is probably not sufficient to show that the envelope-associated DNA is resistant to high-salt extraction (25), since nucleic acids can associate with membranes *in vitro* in a way resistant to dissociation by 4*M* CsCl (27).

III. SURVEY OF PROCEDURES USED FOR ISOLATING NUCLEAR ENVELOPES

From a survey of the published procedures for the large-scale isolation of nuclear envelopes, it is apparent that most of them employ combinations of a rather limited range of basic steps. It is easier to discuss this group first, mainly in terms of the various steps used, rather than detailing each procedure separately. Following this we mention some other procedures that employ more diverse steps, some of which are quite novel.

1. Main Group of Procedures

In general the initial step towards nuclear envelope isolation is the preparation of the corresponding purified nuclei (an exception is provided by ref. 28). During this process the envelope is separated from about 99% of the other cellular membranes and is then the major membranous component of a well-purified nuclear fraction. At this stage it is fairly easy to separate nuclei from potential contaminants of the final envelope preparation, such as mitochondria and microsomes, since nuclei and these components differ considerably in size and density. However, after the next stage, nuclear disruption, the released envelope fragments and the other membranes differ much less in physical properties and it is correspondingly more difficult to separate them. Accordingly, it is advisable to obtain a nuclear preparation as pure as possible before proceeding any further. For this purpose isolation procedures using dense sucrose (2.2*M*) are most suitable and these have been extensively reviewed elsewhere (29,30).

The subsequent steps in envelope isolation (starting from purified nuclei) fall into two broad groups. As a first step the nuclei are disrupted; this can be by sonication, ionic conditions, or DNAase digestion. Then the envelope fragments are separated from other debris by procedures involving rate or isopycnic centrifugation or extraction with high salt concentrations. Table I shows how these steps have been combined in a number of isolation procedures.

Disruption of nuclei by sonication, a method used by many workers, results in severe fragmentation of the nuclear envelope, so that the largest pieces may be no bigger than 1 μm^2 and have fewer than 10 nuclear pores (9). These pieces often appear as single-membraned vescicles. It is also very possible, though not proven, that sonication may contribute to the contamination of

TABLE I

Procedures Involved in the Isolation of Nuclear Envelopes from Isolated Nuclei

Material	Disruption of Nuclei			Purification of envelope fragments			Authors
	Sonication	Ionic conditions	DNAase digestion	Rate centrifugation	Isopycnic centrifugation	High salt	
Liver, ascites cells	+				+		Zbarsky et al., 1969 (10); Zbarsky 1972 (31)
Liver	+				+	+	Kashnig and Kasper, 1969 (19)
Liver			+		+	+	Berezney et al. 1970, 1972 (32,33)
Liver, avian erythrocytes	+				+	+	Franke et al., 1970 (9); Zentgraf et al., 1971 (5)
Liver		+	+	+			Kay et al. 1971, 1972 (24,11)
Liver	+				+		Agutter, 1972 (34)
Thymus			+	+	+	+	Matsuura and Ueda, 1972 (17)
Liver		+			+	+	Monneron et al., 1972 (35)
Liver, hepatoma		+		+	+		Price et al., 1972 (28)
Prostate	+				+	+	Moore and Wilson, 1972 (36)
Pea	+				+		Stavy et al., 1973 (37)
Tetrahymena pyrimiformis		+		+		+	Nozawa et al., 1973 (38)

certain envelope preparations by newly replicated DNA (21–23) and to the destruction of envelope-associated glucose-6-phosphatase in others (9,10). For these reasons sonication is best reserved as a last resort for nuclear disruption, to be used if milder methods fail.

The use of appropriate ionic conditions or DNAase digestion, alone or in combination, to disrupt nuclei offers more hope for the preservation of the envelope than does sonication, since physical stress is largely avoided. Two types of ionic condition have been used for this purpose. In the first the nuclei are extracted with a high salt concentration [0.5 to 1*M* (35,38)] that solubilizes most of the nucleoplasm, but not the nucleoli. Alternatively, in slightly alkaline conditions and at low magnesium ion concentrations [pH > 8.0, $[MgCl_2] < 0.2mM$ (11)] chromatin is released from the nuclei and the nucleoli are destroyed (11,28). In either case the result is the formation of nuclear ghosts, possibly entrapped in a chromatin gel. While disruption of nuclei by high salt certainly has advantages compared to sonication, it does have the drawback that is is likely to remove many components ionically associated with the envelope. As judged from published electron micrographs (35,38), this group of components includes many present in the nuclear pore, since the pores are rather ill-defined after high-salt treatment. As the nuclear pore is likely to be a focus of interest in the future, this drawback may be serious. However, for the study of the remaining proteins this method is just as good as those using conditions of low ionic strength.

DNAase 1 digestion of nuclei has also been used in two fashions for nuclear disruption. Extensive digestion [e.g., 100 μg/ml of DNAase 1 for 12 hr (32)] results in the solubilization of the nucleoplasm, leaving nuclear ghosts and remnants of nucleoli (17,32,33). Alternatively, brief digestion (1 μg/ml of DNAase 1 for 35 min) has been used in conjunction with suitable ionic conditions and here the nucleoli are also destroyed (11,24). Although very mild, these procedures do have potential disadvantages in that chromatin may reprecipitate during digestion and the envelope-associated DNA is likely to have many single-stranded nicks. The first problem can with care be circumvented (11), but for the latter this may not be possible, so that difficulties can arise in the detailed characterization of the envelope-associated DNA (26).

The above nuclear disruption methods have been discussed in some detail, since they are probably of prime importance to the success of the whole isolation procedure; less, however, needs to be said about the subsequent separation of the envelope fragments from other nuclear components. High-salt extraction, usually in conjunction with isopycnic centrifugation, is widely used to remove contaminating chromatin from the envelope. In this it is effective, sometimes to the point of removing all the DNA (19), and as previously mentioned, high-salt extraction is also likely to remove normal components of the envelope. Isopycnic banding normally in sucrose, though sometimes in sorbitol (34) or

CsCl (25,36) density gradients, has proved to be a very useful way of separating envelope fragments from the much denser chromatin or nucleoli. It is not clear, however, that this procedure necessarily separates the envelope from all other membranes [see the comments of Franke et al. (9), and for the dangers of membrane fusion, see ref. 39] and this possibility reemphasizes the advisability of separating the nuclei from other membranes as far as possible before nuclear disruption. Finally, rate centrifugation has also been used in some cases where sonication has been avoided, since here the envelope fragments are much larger than other nuclear debris (except possibly nucleoli) and can therefore be centrifuged away from them.

2. Other Procedures

Two further methods of nuclear disruption deserve consideration, namely the use of polyanions and of detergents. In an interesting earlier study Bach and Johnson (40) extracted isolated nuclei with DNA and obtained a lipid-rich fraction which they assumed to be derived from the nuclear envelope. Unfortunately, this claim was not assessed by electron microscopy; however, more recently Bornens (41) has studied the action of other polyanions on isolated nuclei and has shown by microscopy that heparin treatment does indeed disrupt the nuclei and that well-preserved envelopes can be pelleted from the lysate. Development of this approach should yield another mild procedure for envelope isolation.

Detergents can also be used for nuclear disruption and it has been suggested by some workers that appropriate mild detergent treatment (e.g., 1% Triton X-100) of isolated nuclei can be used to selectively remove the outer nuclear membrane (42–44). Certainly after this treatment the nuclei appear substantially intact by electron microscopy, but are lacking the outer membrane. However, this observation is not sufficient to preclude the possibility that other nuclear components have also been solubilized by this procedure; indeed the lipid content of the extracted nuclei does suggest that inner membrane lipids are also extracted (2). Since the extracted material is largely solubilized, this possibility is exceedingly hard to evaluate by microscopy and so this use of detergents remains questionable.

In addition to the large-scale procedures so far discussed, it is also possible to manually isolate single nuclear envelopes from isolated amphibian oocyte nuclei and probably other giant nuclei. This procedure stems from the early work of Callan and Tomlin (45) and can produce pure and extremely well-preserved envelopes, ideal for electron microscopy (46–48). Unfortunately, only minute quantities of material can be isolated like this and these are generally too small for normal biochemical analysis. However, Scheer (49) has shown that with sufficient patience in the isolation stages, enough material can be accumulated

for semi-micro RNA analysis and probably other semi-micro techniques. Where such techniques are available, this method has considerable promise.

IV. SELECTED PROCEDURES FOR NUCLEAR ENVELOPE ISOLATION

The three recommended procedures described here are to some extent complementary and a choice between them depends largely on the purpose for which the envelopes are required. The method of Kay et al. (11,24) is rapid and mild, gives well-preserved envelopes, is suited to investigations of envelope enzymes and some feature of envelope-associated DNA, and may be a good starting point for studies of the nuclear pore and envelope-associated RNA. The procedure of Monneron et al. (35) is also rapid and is probably most suited to investigations of "intrinsic" components of the envelope, i.e., those not extracted by 0.5*M* $MgCl_2$. Finally, the hand-isolation procedure of Scheer (49), which is limited to giant nuclei as a source of material, is to be recommended for electron microscopic studies of the envelope and for use in conjunction with suitable semi-micro methods of analysis. More extensive characterization of the envelopes isolated in these ways may be found in the original papers.

1. Method of Kay, Fraser, and Johnston, 1972 (11)

Nuclei are isolated from rodent liver homogenates by a modification of the Widnell and Tata (50) procedure, as previously described (11). The steps in the envelope isolation from the nuclei pelleted through dense sucrose are summarized in chart 1. Table II.

The dense-sucrose nuclear pellets are resuspended in about 50 ml of 0.25*M* sucrose, 1m*M* $MgCl_2$ brought to pH 7.4 with $NaHCO_3$, and are then centrifuged at 700*g* × 5 min. This final nuclear pellet is drained and the centrifuge tube wall carefully wiped dry. For each 4 g of liver from which the nuclei were isolated, 1 ml of ice-cold 0.1m*M* $MgCl_2$ is added and the nuclei are resuspended by gentle pestling and vortex mixing. DNAase 1 (electrophoretically pure, 100 μg/ml fresh stock solution in water) is immediately added to 5 μg/ml, followed by 4 volumes of 10m*M* Tris-HCl, 0.1m*M* $MgCl_2$, 5m*M* 2-mercaptoethanol (which should be added fresh on the day of the experiment), 10% w/v sucrose, pH 8.5. After a further vortex mixing, DNAase 1 digestion is allowed to proceed for 15 min at 22°C before being terminated by the addition of an equal volume of ice-cold water. Crude nuclear envelopes are then pelleted from the lysate by centrifugation at 38,000*g* × 15 min in an angle rotor. The pellets are drained and resuspended in 5 volumes of 10m*M* Tris-HCl, 0.1m*M* $MgCl_2$, 5m*M* 2-mercaptoethanol, and 10% w/v sucrose, pH 7.4 by vortex mixing, DNAase 1 added to 1 μg/ml and the mixture is digested

TABLE II
Outline of the Isolation Procedure of Kay et al. (11)

Nuclear pellet from dense-sucrose centrifugation
1. Resuspend 0.25*M* sucrose, 1m*M* $MgCl_2$
2. Centrifuge 700*g* × 5 min

Supernatant discarded

Nuclear pellet
1. Resuspend in 0.1m*M* $MgCl_2$, 5 μg/ml DNAase 1
2. Add 4 vols. buffer pH 8.5
3. Digest 15 min at 22°C

Nuclear lysate
1. Add equal vol. ice-cold H_2O
2. Centrifuge 38,000*g* × 15 min

Supernatant discarded

Crude nuclear envelope pellet
1. Resuspend buffer pH 7.4, 1 μg/ml DNAase 1
2. Digest 20 min at 22°C
3. Add equal vol. ice-cold H_2O
4. Centrifuge 38,000*g* × 15 min

Supernatant discarded

Final nuclear envelope pellet

again at 22°C for 20 min. After this time digestion is terminated by dilution and centrifugation as before to give the final envelope pellet. The electron microscopic appearance of envelopes isolated in this way is shown in Figures 2 and 3. Starting from 100 g of liver (which can be obtained from about twelve 180-g rats), 250 to 300 mg of purified nuclear protein can be obtained in 2½ hr and these nuclei in turn yield about 25 mg of envelope protein after a further 80 min.

For some purposes it may be worthwhile to further purify the envelopes by isopycnic centrifugation in sucrose density gradients (24). For this purpose continuous or discontinuous sucrose gradients over the density range 1.16–1.24 g/cc are suitable. Envelopes are loaded over or under the density gradient and then centrifuged for about 12 hr at 50,000*g* in a swing-out head. When the gradient is made up in a buffer containing 10m*M* Tris-HCl, 10m*M* NaCl, 1m*M* EDTA, and 5m*M* 2-mercaptoethanol pH 7.4 the envelopes band in a density range of 1.17 to 1.19 g/cc as shown in Figure 4. The recoveries of DNA, RNA, and protein in this zone are good, but there are some losses of phospholipid.

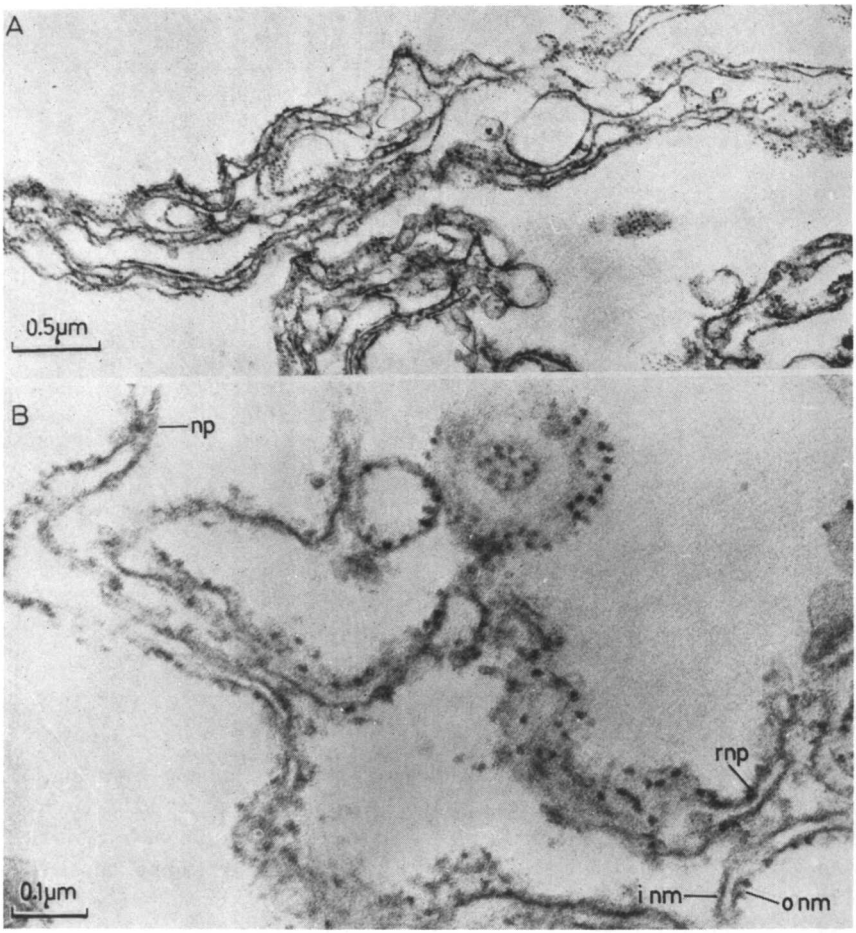

Figure 2. Electron micrographs of stained ultrathin sections of nuclear envelopes isolated from rat liver by the method of Kay et al. (11). (*A*) (×23,700 diameters) shows a representative field of envelopes which are seen at higher power in *B* (×91,000 diameters). Notice the presence of both inner and outer nuclear membranes (inm and onm) with ribosomes (rnp) attached to the outer and amorphous material, probably chromatin, to the inner. Fairly well preserved nuclear pores (np) can also be seen in section. Reproduced from Kay et al. (11) by permission of FEBS.

2. Method of Monneron, Blobel, and Palade, 1972 (35)

For this method rodent liver nuclei are isolated by the procedure of Blobel and Potter (51) except detergents are not used. After the dense sucrose step of the nuclear isolation the nuclear pellets are resuspended in cold 0.25*M* sucrose—TKM (TKM is 50m*M* Tris-HCl, 25m*M* KCl, and 5m*M* $MgCl_2$, pH

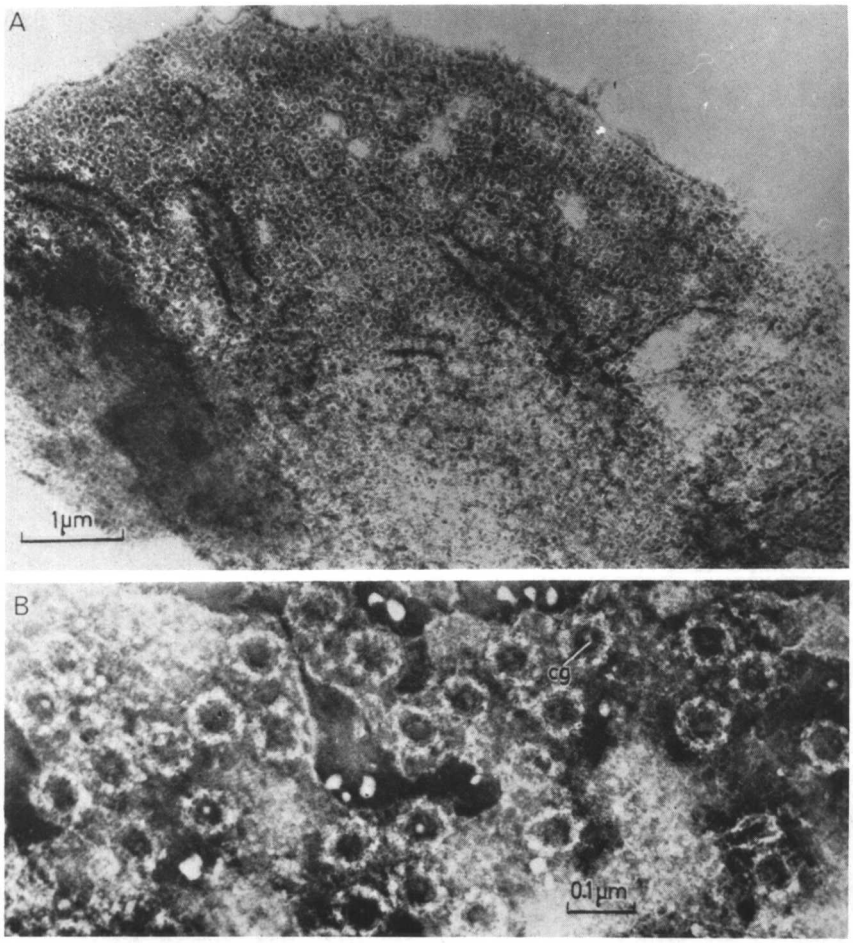

Figure 3. Electron micrographs of negatively stained nuclear envelopes, isolated as in Figure 2. In *A* (×13,616 diameters) a large field of nuclear pores is shown, and at high power in *B* (×91,100 diameters) the annuli and occasional central granule (cg) can be seen. Reproduced from Kay et al. (11) by permission of FEBS.

7.5) and then centrifuged at 1000*g* × 10 min. The resulting nuclear pellets can be used immediately for envelope isolation, or alternatively mixed with twice their volume of glycerol and stored at −20°C until required. In either case the nuclei are next resuspended in more 0.25*M* sucrose—TKM and the A_{260} of the suspension determined. The suspension is then distributed among centrifuge tubes to fit a high-speed swing-out rotor, so that each tube, when full, contains