BBA 75442

THE ISOLATION OF NUCLEAR MEMBRANE FROM A LARGE-SCALE PREPARATION OF BOVINE LIVER NUCLEI

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(Received February 13th, 1970)

SUMMARY

1. A procedure for the large-scale preparation of bovine liver nuclei is described.
2. Nuclear membrane was prepared by treatment of nuclei with deoxyribonuclease followed by extraction with a 0.5 M $MgCl_2$ solution.
3. An evaluation of mitochondrial and microsomal contamination involving chemical, enzymatic and ultrastructural analysis indicated only slight contamination in both nuclear and nuclear membrane fractions.
4. The total contaminating protein averaged 2.65 % for nuclei and 10.6 % for nuclear membrane.
5. Electron microscopy showed that the membrane fraction contained both outer and inner nuclear membranes.
6. The nuclear membrane fraction contained 74.6 % protein, 8.9 % RNA, 0.92 % DNA, and 13.7 % phospholipid. Recovery studies showed an average of 10.3 % of the nuclear protein, 23.9 % of the nuclear RNA, 0.27 % of the nuclear DNA, and 47.1 % of the nuclear phospholipid recovered in the membrane fraction.

INTRODUCTION

Although the nuclear membrane or envelope has been well studied structurally[1–5], a great deal of unanswered questions of fundamental importance remain concerning its functioning. Such aspects as the chemical composition and enzymatic activity of nuclear membrane, the role of nuclear membrane in nucleo–cytoplasmic exchanges, the biogenetic relationship between endoplasmic reticulum and nuclear membrane, the relationship between the outer and inner nuclear membranes, and the functioning of nuclear pores are all but impossible to investigate without direct methods of analysis. Until recently, lack of procedures for nuclear membrane isolation have prevented its biochemical and physicochemical analysis[6–8].

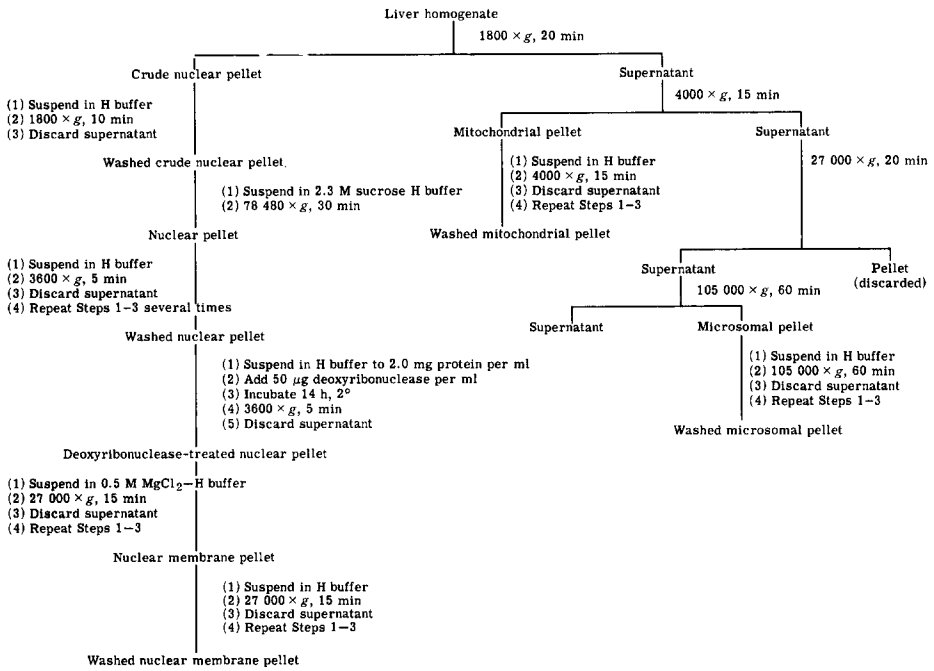
For the past 2 years we have been concerned with isolating nuclear membranes in amounts useful for comprehensive biochemical analysis. In this paper a procedure is described for nuclei isolation from bovine liver which combines high purity with the important advantage of being on a large scale. From this large-scale nuclear preparation, nuclear membranes were isolated. Rigorous criteria involving electron microscopic and enzymatic analysis were used in evaluating contamination in the nuclei and nuclear membrane fractions. Because of the large amount of membrane

obtained, this procedure provides practical means for biochemical fractionation studies of the nuclear membrane.

METHODS AND MATERIALS

Preparation of nuclei

A scheme summarizing the preparation of nuclei and membrane fractions is presented in Scheme 1. A bovine liver weighing approx. 10 pounds was obtained immediately after the animal had been sacrificed. Subsequent to the removal of the outer covering membrane and excessive connective tissue, the liver was cut into small cubes approx. 1 cm^3 and immediately placed in a solution of ice-cold homogenizing buffer (0.05 M Tris–HCl buffer (pH 7.5), 0.25 M sucrose, 0.025 M KCl, 0.005 M $MgCl_2$)[9] hereafter referred to as H buffer. The liver cubes were washed several times in H buffer, placed in a Waring blender in the ratio of 1 vol. of liver to 3 vol. of H buffer, blended for a 30-sec period at 19000 rev./min and then filtered through cheesecloth of two, four, and eight layers in rapid succession.



Scheme 1. The preparation of nuclei, nuclear membranes, mitochondria and microsomes from a bovine liver homogenate.

The filtered liver homogenate was then centrifuged in a high capacity (13 l) International centrifuge at 2000 rev./min (1800 × *g*) for 20 min. After decanting off the supernatant, the crude nuclear pellets were washed by resuspending in H buffer to a volume equal to one-half of the original filtered homogenate. Following centrifugation at 2000 rev./min for 10 min, the nuclear pellets were resuspended in homogenizing buffer which was 2.3 M in sucrose and filtered through twelve layers

of cheesecloth. After adjustment of the volume to three-tenths of the original filtered homogenate with 2.3 M sucrose H buffer, the suspension was centrifuged at 78480 × *g* for 0.5 h in the 30s rotor of the Spinco Model L-2 ultracentrifuge.

The separation after centrifugation is illustrated in Fig. 1. The fact that the side contamination band was always on the opposite site of the nuclear pellet made possible the collection of the nuclear pellets without contamination. To collect the nuclei, the centrifuge tubes were turned upside down and the contaminants removed by means of a spatula followed by swabbing with tissue paper and washing the inside surface of the tube and the nuclear pellet surface 3 times with H buffer. The nuclear pellets were then washed several times by resuspending them in H buffer and centrifuging at 3600 × *g* for 5 min. Visible contamination was removed with a spatula between each washing.

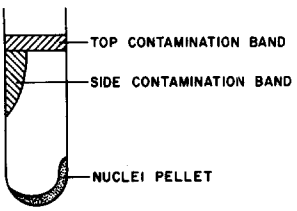


Fig. 1. Typical separation after high-speed centrifugation (78480 × *g*) of the crude nuclear fraction suspended in 2.3 M sucrose H buffer.

Preparation of nuclear membranes

The purified nuclear pellets were suspended in H buffer to a final protein concentration of 2.0 mg/ml. Deoxyribonuclease was added (50 μg/ml) and digestion was continued for 14 h at 2°. Following two washings with H buffer, the deoxyribonuclease-treated nuclei were resuspended in H buffer made 0.5 M in $MgCl_2$. This caused visible solubilization of much of the nuclear fraction. The suspension was centrifuged at 27000 × *g* for 15 min. After repeating the process of extraction with 0.5 M $MgCl_2$ H buffer and centrifugation, the nuclear membrane fractions were washed 2 times in H buffer and then resuspended in H buffer for immediate analysis or stored at −20° for later analysis.

This method for nuclear membrane isolation is similar to a procedure recently reported by UEDA *et al.*[7] for isolating a membraneous fraction from triton-treated calf thymus nuclei in which they used deoxyribonuclease followed by NaCl treatment.

Preparation of mitochondria and microsomes

Mitochondrial and microsomal fractions were prepared from the supernatant of the first centrifugation. Mitochondria were isolated by centrifuging the supernatant at 4000 × *g* for 15 min. The supernatant was centrifuged at 27000 × *g* for 15 min and the pellets discarded. The corresponding supernatant was centrifuged at 105000 × *g* for 1 h to isolate a microsomal fraction. Both the mitochondrial and microsomal fractions were washed 2 times by resuspending in H buffer and centrifuging the mitochondria at 4000 × *g* for 15 min and the microsomes at 105000 × *g* for 1 h.

Chemical analysis

DNA and RNA were separated according to MUNRO AND FLECK[10]. DNA was estimated by the diphenylamine reaction[11]. RNA was determined spectrophometri-

cally according to MUNRO AND FLECK[12]. Lipid was isolated by the method of FOLCH *et al.*[13]. Phosphorus analysis of the total lipid extracts was according to CHEN *et al*[14]. The phospholipid content was calculated by multiplying the determined number of μg of phosphorus by 25. Protein was estimated by a modified biuret procedure of YONETANI[15].

Enzyme assays

NADPH–cytochrome *c* reductase and succinate–cytochrome *c* reductase were assayed by following the reduction of cytochrome *c* at 550 mμ at 30° according to ERNSTER *et al.*[16]. The succinate–cytochrome *c* reductase assay was 5 mM in succinic acid. Succinoxidase was measured polarographically at 38°. The assay mixture contained in a total volume of 1.8 ml, 2 mg of cytochrome *c* and 66.7 μmoles of phosphate at a pH 7.5.

Electron microscopy

Specimens prepared for sectioning were prefixed in a 2.5 % glutaldehyde solution buffered in 0.1 M cacodylate (pH 7.5) and post-fixed in 1 % O_8O_4 in an acetate–veronal buffer according to PALADE[17]. Samples were dehydrated in acetone and embedded in Epon based on a procedure by LUFT[18]. Sectioned specimens were post-stained with either a saturated aqueous solution of uranyl acetate at 60° for 1 h followed by lead citrate according to REYNOLDS[19] for 15 min at room temperature or with 0.25 % lead citrate in 0.1 M NaOH for 3 min at room temperature. Samples negatively stained with 2 % phosphotungstate (pH 7.0 with KOH) were prepared according to the method of CUNNINGHAM AND CRANE[20]. Specimens were observed and photographed with a Philips EM 75, EM 200 or EM 300.

Materials

NADPH, cytochrome *c*, and succinic acid were purchased from Sigma Chemical Co. Deoxyribonuclease, DNA, and RNA were obtained from CalBiochem. All other chemicals were of reagent grade. Glass-distilled water was used exclusively.

RESULTS

Chemical composition of nuclei

The development of the mass preparation procedure for nuclei was monitored for purity by combined electron microscopic and biochemical techniques. Compositional studies can be a useful tool for indicating the purity of a sub-cellular fraction as well as being one of the basic means of characterization of a particular cellular structure. In this case, pure nuclear fractions have been shown to be low in phospholipid content and have a characteristic RNA/DNA ratio of approx. 0.10–0.20 (refs. 9, 21 and 22).

Compositional analysis of the nuclear fraction is presented in Table I with the assumption that % protein + % DNA + % RNA + % phospholipid = 100 %. The amount of phospholipid and the RNA/DNA ratio are similar to values reported for other purified nuclear preparations. This analysis therefore strongly suggests the high purity of the mass nuclei preparation.

TABLE I

CHEMICAL COMPOSITION OF BOVINE LIVER NUCLEI PREPARED BY A LARGE-SCALE PROCEDURE

Bovine liver nuclear preparation	*Protein* (%)	*DNA* (%)	*RNA* (%)	*Phospho-lipid* (%)	*RNA/DNA ratio*
1	65.0	29.6	2.8	2.6	0.095
2	69.2	25.5	2.3	3.0	0.090
3	71.0	22.8	3.0	3.2	0.131

Electron microscopic observations of nuclei

Study of many mass preparations revealed no whole cells and only very slight contamination with mitochondrial and endoplasmic reticular fragments. Fig. 2 illustrates the purity of the nuclear preparation as well as the relatively intact nature of the nuclei. The double-membraned nuclear envelope is clearly seen surrounding the nuclei. Intact nucleoli are commonly seen inside the nuclei. Fig. 3 is a higher magnification showing ribosomes attached to the outer membrane on its cytoplasmic side and the absence of ribosomes on the inner membrane. The two nuclear membranes appear to join together at the nuclear pores. Also in Fig. 3 is a portion of the nuclear envelope detached from the nucleus. The outer and inner membranes are still separated by the characteristic 150–200 Å wide perinuclear space, suggesting that the nuclear pore structures may play an important role in holding the two membranes in a double-membraned envelope structure. Fig. 3 also shows nuclear pore annuli which have detached from the membrane. These free nuclear pore structures appear similar to a structure observed by MAGGIO *et al.*[22] who suggested it may be an annulus displaced from the membrane.

Enzymatic evaluation of mitochondrial and microsomal contamination in nuclei

Both compositional and ultrastructural analysis indicated that cytoplasmic contamination was low. An attempt was made to quantitate the slight amount of mitochondrial contamination by assaying for succinoxidase and succinate–cytochrome *c* reductase. Fifteen separate nuclei mass preparations were assayed along with mitochondrial controls. The mitochondrial contamination was found to vary from 0 to 2.3 % mitochondrial protein with an average of 1.1 % using succinoxidase. With succinate–cytochrome *c* reductase, contamination varied from 0.8 to 1.5 % with an average of 1.3 %. Three typical experiments are presented in Table II. These results indicate a low degree of mitochondrial contamination as well as good correlation by two separate assays. It thus appears that the mitochondrial succinoxidase is not being inhibited by the nuclei.

This conclusion was further tested by means of mixing experiments. Succinoxidase activity was determined in nuclei, mitochondria and mixtures of nuclei and mitochondria. The specific activities of the mixed fractions were close to the calculated values obtained by adding the activities of the nuclear and mitochondrial fractions together (Table III). The low values obtained for succinoxidase in the nuclear fraction is therefore a reflection of the low degree of mitochondrial contamination.

Since glucose-6-phosphatase has been demonstrated both histochemically[23–25]

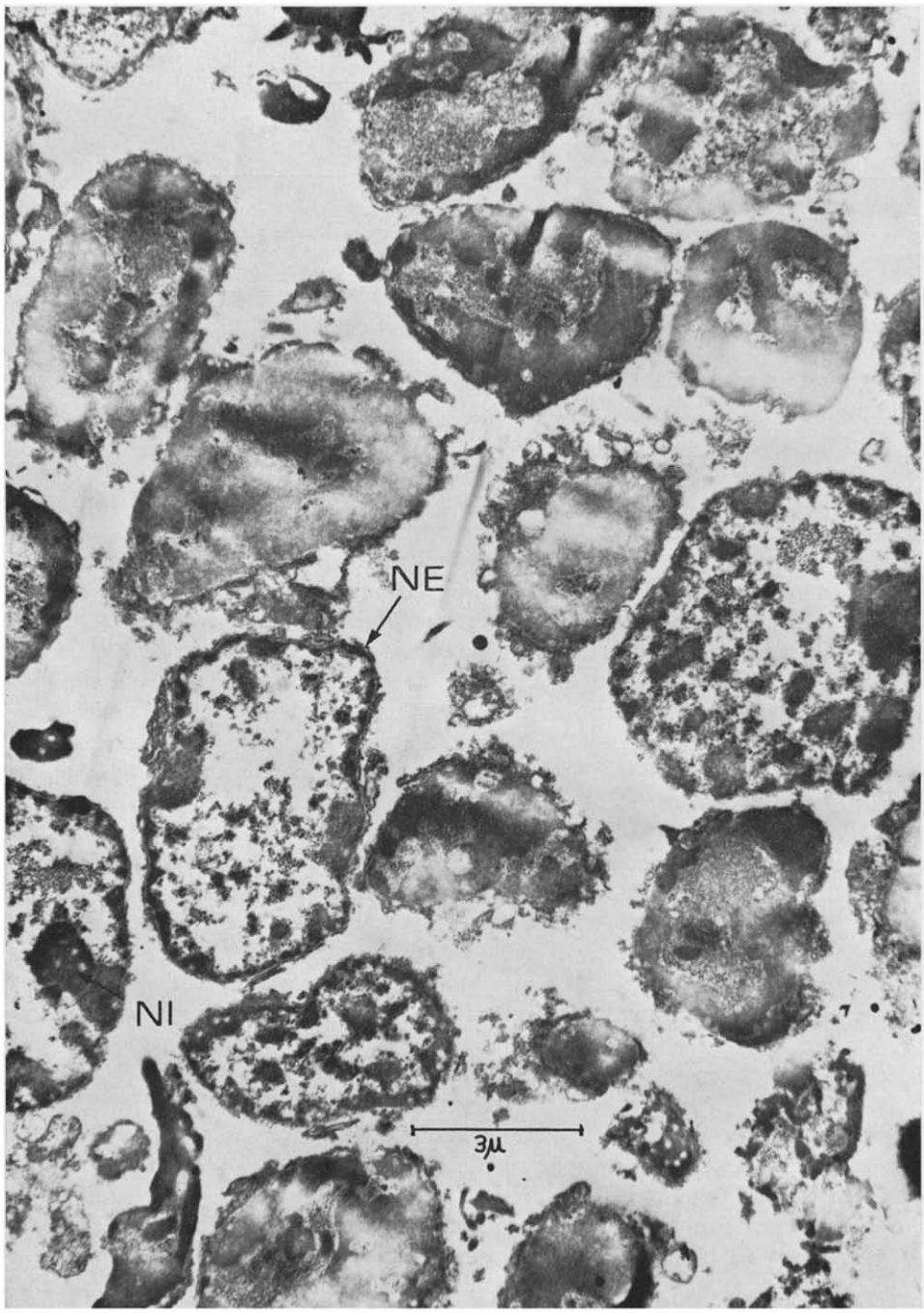


Fig. 2. Nuclei isolated in the large scale preparation. NI, nucleoli, NE, nuclear envelope. Magnification × 7000.

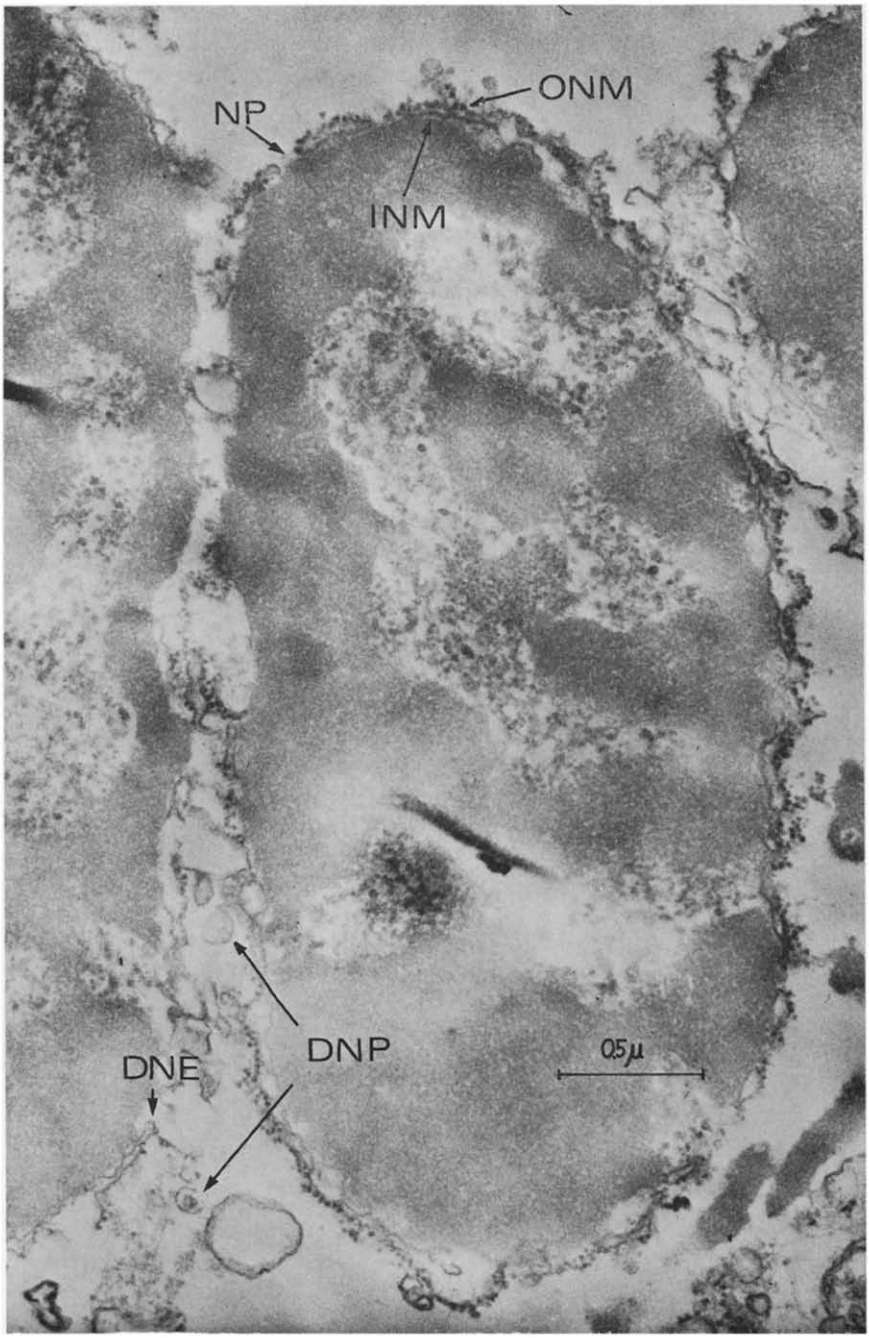


Fig. 3. Higher magnification of nuclei showing distinct inner membrane and outer membrane with attached ribosomes. Nuclear pores can be clearly seen. A portion of the nuclear envelope is detached from the nucleus. ONM, outer nuclear membrane; INM, inner nuclear membrane; NP, nuclear pore; DNP, detached nuclear pore; DNE, detached nuclear envelope. Magnification × 37000.

TABLE II

MITOCHONDRIAL AND MICROSOMAL CONTAMINATION IN THE NUCLEAR FRACTION

Activities for succinoxidase are expressed in μmoles O_2 per min/mg protein whereas succinate–cytochrome *c* reductase and NADPH–cytochrome *c* reductase activity are measured in μmoles cytochrome *c* reduced per min/mg protein. In calculating total percentage protein contamination the mitochondrial contribution represents an average of the succinate oxidase and succinate–cytochrome *c* reductase values.

Prep.	*Fraction*	*Succinoxidase activity*	*Mitochondrial protein in nuclear fraction* (%)	*Succinate–cytochrome c reductase activity*	*Mitochondrial protein in nuclear fraction* (%)	*NADPH-cytochrome c reductase activity*	*Microsomal protein in nuclear fraction* (%)	*Total protein contamination* (%)
1	Nuclei	0	0	0	0	0.00255	2.99	2.99
	Mitochondria	0.322		0.339				
	Microsomes					0.0853		
2	Nuclei	0.00231	1.29	0.00394	1.42	0.000897	1.12	2.48
	Mitochondria	0.179		0.277				
	Microsomes					0.0804		
3	Nuclei	0.00359	1.16	0.00452	1.76	0.00062	1.03	2.49
	Mitochondria	0.310		0.257				
	Microsomes					0.0604		

TABLE III

EFFECT OF NUCLEI ON SUCCINOXIDASE ACTIVITY IN MITOCHONDRIA

The mixed fraction consisted of equal protein amounts of mitochondria and nuclei. The calculated values for the specific activities of the mixed fraction were obtained by adding the activities obtained by the separate nuclear and mitochondrial fractions. Specific activities are expressed in μmoles O_2 per min/mg protein. Assays were performed in the presence of 2 mg cytochrome *c*.

Prep.	*Fraction*	*Succinoxidase activity value*	*Calculated activity*	*Difference in activity* (%)
1	Nuclei	0.00061		
	Mitochondria	0.0939		
	Nuclei-Mitochondria	0.040	0.0475	−15.7
2	Nuclei	0.00329		
	Mitochondria	0.243		
	Nuclei-Mitochondria	0.104	0.122	−11.7

TABLE IV

DISTRIBUTION OF MACROMOLECULES IN NUCLEAR MEMBRANE

Nuclear membrane fraction	*Protein* (%)	*RNA* (%)	*DNA* (%)	*Phospho-lipid* (%)	*Recovery nuclear protein* (%)	*Recovery nuclear RNA* (%)	*Recovery nuclear DNA* (%)	*Recovery nuclear phospholipid-P* (%)
1	70.9	10.3	1.12	17.8	12.1	28.0	0.31	54.5
2	77.3	8.5	1.30	12.9	10.1	22.0	0.38	43.0
3	80.1	8.6	0.40	11.0	9.3	21.2	0.14	42.3
4	70.0	8.3	0.88	12.9	9.8	24.3	0.24	48.6
Average value	74.6	8.9	0.92	13.7	10.3	23.9	0.27	47.1

and biochemically[8] to be concentrated in the nuclear envelope as well as the endoplasmic reticulum, it can not be used as a marker for estimating microsomal contamination. Studies of glucose-6-phosphatase activity in our laboratory lend support to these previous findings. Specific activities as high as 1.5 μmoles P_i per 10 min per mg protein are obtained in the nuclear membrane fractions where the microsomal fractions have a specific activity of approx. 4.5.

Another microsomal enzyme, NADPH–cytochrome *c* reductase, was found in nuclei and in nuclear membrane fractions in very low amounts. We therefore feel that it represents a better indicator of microsomal contamination in this case. Twelve separate nuclei mass preparations, together with microsomal fractions, were assayed for NADPH–cytochrome *c* reductase. Contamination measured in this manner varied from 0.0 to 3.0 % on a protein basis. Three typical experiments are presented in Table II. The total of microsomal and mitochondrial protein in the nuclear fraction is also presented in Table II. For three separate preparations percentages of 2.99, 2.48, and 2.49 were obtained.

Effect of deoxyribonuclease–$MgCl_2$ treatment on mitochondria and microsomes

Mitochondria and microsomes were treated with deoxyribonuclease and $MgCl_2$ under the exact same conditions as the nuclei for nuclear membrane isolation. The treatment had little effect on microsomal protein recovery (85–95 %) or recovery of NADPH–cytochrome *c* reductase (75–85 %). In contrast 50–75 % of the mitochondrial protein was not recovered in a pellet centrifuged at 27000 × *g* for 15 min. Similarly 50–80 % of the succinoxidase activity was not recovered in the pellet. It therefore appears that the deoxyribonuclease–$MgCl_2$ treatment serves the dual role of isolating nuclear membranes and purifying the membranes by solubilizing mitochondrial protein.

The distribution of macromolecules in the nuclear membrane fraction

The chemical composition of nuclear membrane along with the percent recovery of each macromolecule from the initial nuclear fraction is summarized in Table IV. The fact that an average of 47.1 % of phospholipid in the nuclear fraction is recovered suggests that a good deal of nuclear phospholipid is concentrated in the nuclear envelope. A detailed analysis of the lipids in nuclear membrane will be presented elsewhere[26]. In contrast, an average of only 0.24 % of nuclear DNA is recovered. This indicates very low contamination by chromatin material. The recovery of 23.9 % RNA in the membrane fraction is consistent with morphological observations of the nuclear membrane fraction which show the outer membrane covered with ribosomes. The remaining 76 % of the RNA, consisting of nucleolar and other nuclear RNA species, is recovered in the supernatant after $MgCl_2$ extraction. A high yield of nuclear membrane is suggested by the 10.3 % protein recovery and the high phospholipid recovery.

Electron microscopic analysis of nuclear membrane

Development of the procedure for nuclear membrane isolation was monitored by electron microscopic observations after various treatments. Use of the negative-staining technique was of invaluable aid in this respect as it allowed for the immediate observation of material in suspension. Fig. 4 shows the nuclear membrane fraction negatively stained with phosphotungstic acid. Nuclear membrane is readily distin-

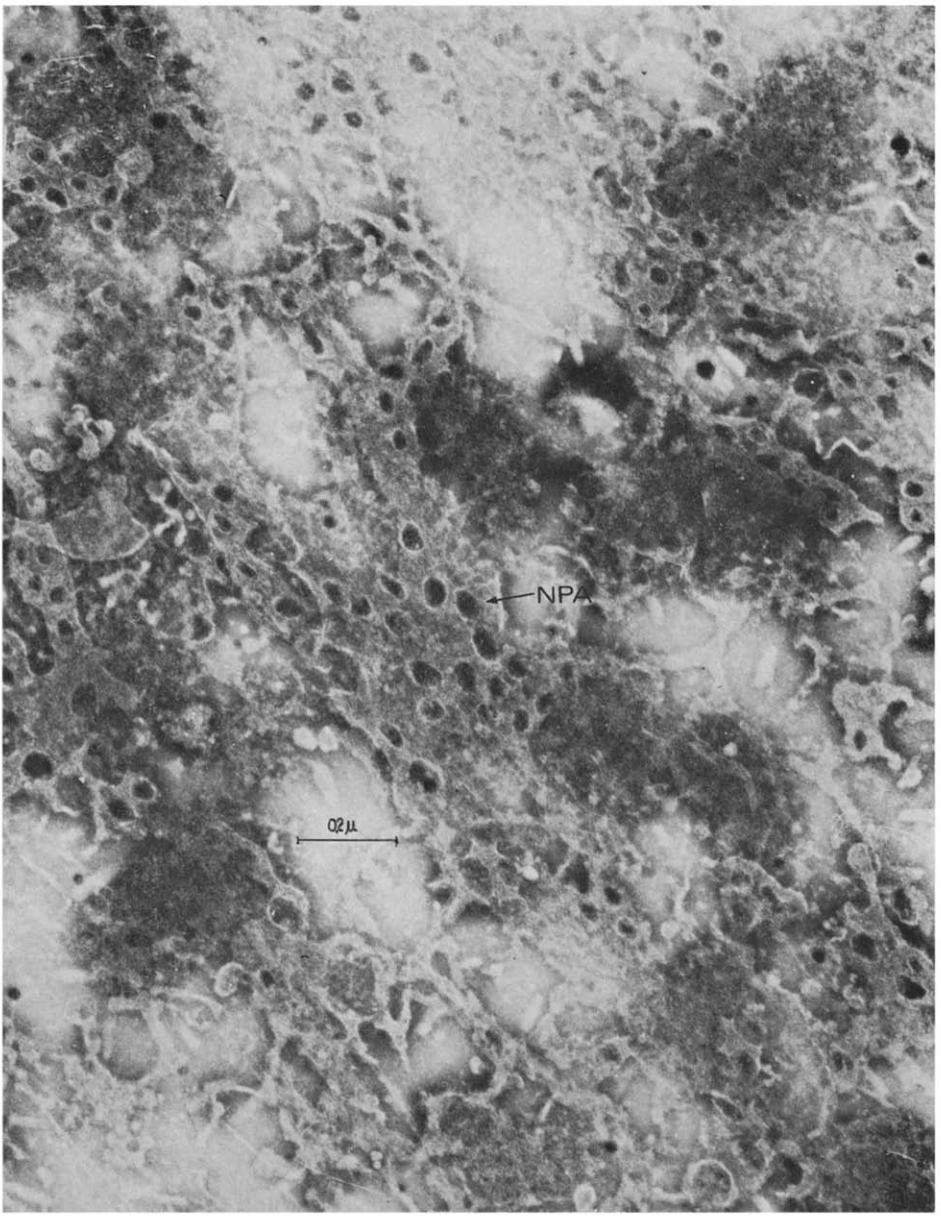


Fig. 4. Electron micrograph of nuclear membrane negatively stained with phosphotungstic acid. NPA, nuclear pore annuli. Magnification × 66300.

guished from other cellular membranes in negative staining by the characteristic 800–1200 Å diameter annuli or "doughnuts" which are the nuclear pore complexes seen in surface view. The preparation appears virtually free of mitochondrial and microsomal membranes.

Sections of the nuclear membrane preparations indicate its purity from con-

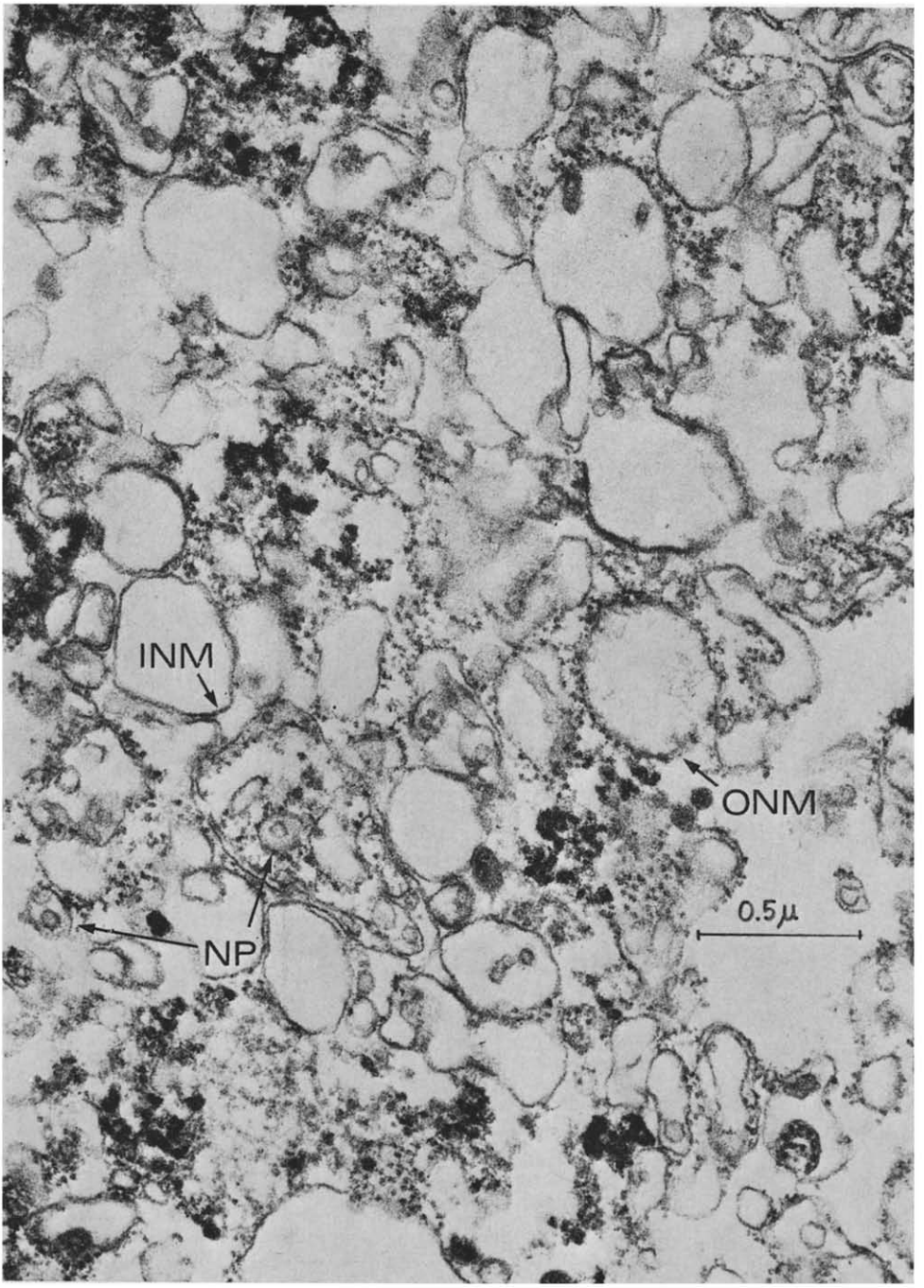


Fig. 5. Nuclear membrane fraction isolated from large scale nuclear preparation treated with deoxyribonuclease followed by $MgCl_2$. ONM, outer nuclear membrane with attached ribosomes; INM, inner nuclear membrane; NP, nuclear pore. Magnification × 45000.

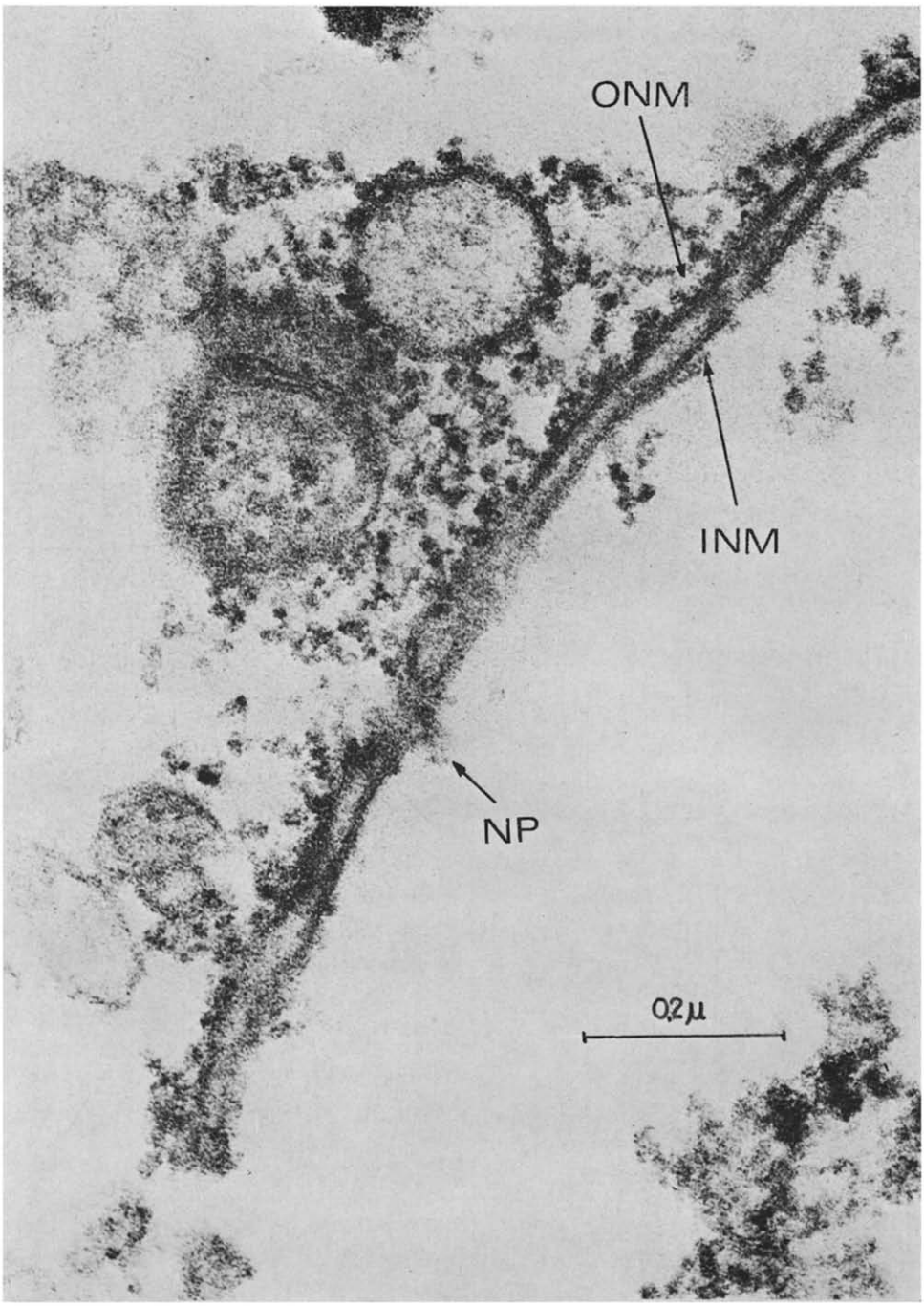


Fig. 6. Higher magnification of the nuclear membrane fraction. ONM, outer nuclear membrane; INM, inner nuclear membrane; NP, nuclear pore. Magnification × 137000.

TABLE V

EVALUATION OF MITOCHONDRIAL AND MICROSOMAL PROTEIN IN THE NUCLEAR MEMBRANE FRACTION

Activities for succinoxidase are expressed as μmoles O_2 per min/mg protein. Succinate–cytochrome *c* reductase and NADPH–cytochrome *c* reductase activities are given in μmoles cytochrome *c* reduced per min/mg protein. In calculating percentage total protein contamination the mitochondrial contribution represents an average of the succinate oxidase and succinate–cytochrome *c* reductase values.

Prep.	*Fraction*	*Succinoxidase activity value*	*Mitochondrial protein* (%)	*Succinate–cytochrome c reductase activity*	*Mitochondrial protein* (%)	*NADPH–cytochrome c reductase activity*	*Microsomal protein* (%)	*Total protein contamination* (%)
1	Mitochondria	0.147		0.179				
	Microsomes					0.0782		
	Nuclear membrane	0.00518	3.52	0.00513	2.87	0.00513	6.56	9.76
2	Mitochondria	0.175		0.122				
	Microsomes					0.0842		
	Nuclear membrane	0.0032	1.83	0.00431	3.53	0.00862	10.2	12.88
3	Mitochondria	0.120		0.165				
	Microsomes					0.0725		
	Nuclear membrane	0.00412	3.40	0.00372	2.25	0.00454	6.26	9.09

taminating membranes as well as intact nucleoli and nucleoplasm (Fig. 5). Both outer and inner membranes are present. As can be seen in Fig. 6, the outer nuclear membrane is recognized by the presence of ribosomes on its outer surface while the inner nuclear membrane is to a large extent devoid of ribosomes. At the nuclear pores the two membranes appear to fuse together.

Besides nuclear membrane, the fraction contains a small amount of microsomal and mitochondrial membrane contamination. We have estimated this contamination to be less than 10 %. Fragments of nucleoli are also present, and only very rarely, fragments of nuclei.

Enzymatic evaluation of mitochondria and microsomal contamination in the nuclear membrane fraction

Mitochondrial and microsomal contamination were evaluated, as in the nuclear fraction, using succinoxidase and succinate–cytochrome *c* reductase as markers for mitochondria and NADPH–cytochrome *c* reductase as an indicator of microsomes. In this case, however, the mitochondrial and microsomal fractions were treated with deoxyribonuclease and $MgCl_2$ under the same conditions as were the nuclei for nuclear membrane isolation. The results are presented in Table V. Mitochondrial contamination averaged 2.92 and 2.88 % using succinoxidase and succinate–cytochrome *c* reductase, respectively, as indicators while microsomal protein averaged 7.67 %. Total percentage protein contamination due to mitochondria and microsomes averaged 10.5 % in three different preparations.

DISCUSSION

In order to obtain reasonably pure nuclear membrane fractions nuclei of the highest purity must be used. Small amounts of contamination in the nuclear fraction could lead to substantial amounts in the membrane fraction. A 5 % contamination in the nuclei increases to 33 % in the membrane fraction assuming 100 % recovery for the contamination and 10 % protein recovery for nuclear membrane. We therefore emphasize purifying the nuclei as much as possible and carefully evaluating the remaining slight degree of contamination. In our investigations we have carefully analyzed nuclear and nuclear membrane fractions both ultrastructurally and biochemically. In estimating microsomal and mitochondrial contamination, these two methods of analysis correlated well.

An attractive feature of using deoxyribonuclease–$MgCl_2$ treatment to isolate nuclear membrane is that it also serves to get rid of mitochondrial contamination. Thus the typical 1 % mitochondrial protein in the nuclei preparation becomes only 2–5 % in the nuclear membrane fraction rather than the expected 10 %. Another advantage of this method is that the treatments do not appear to result in loss of outer membrane ribosomes. This provides a convenient ultrastructural handle for distinguishing outer membrane from inner membrane (which lacks attached ribosomes). Work on the separation of these two membranes is currently in progress. In the past year three different groups of investigators have presented work on the isolation of nuclear membranes[6–8]. We realize, however, that in order to do extensive biochemical fractionation studies of membranes, substantial amounts of membrane fractions are desired. In the procedure presented here 200–400 mg of membrane protein can be isolated in a single preparation.

NOTE ADDED IN PROOF (Received May 19th, 1970)

It was recently called to our attention that procedures for isolation of nuclear membrane were reported by W. W. FRANKE[27,28] and by M. M. BORNENS[29].

ACKNOWLEDGMENTS

The research has been supported by the National Institute of General Medical Science under Grant GM10741. F. L. Crane is supported by Career Grant K6-21839 and Ronald Berezney by training grant 5T1 GM1195. We would also like to gratefully acknowledge the help of Mrs. Doris Andres, Mr. Graham Richardson, and Mr. Gary Wright in the large scale preparation of nuclei.

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