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ISOLATION AND LIPID COMPOSITION OF NUCLEAR MEMBRANES FROM MACRONUCLEI OF *TETRAHYMENA PYRIFORMIS*

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SUMMARY

A procedure was developed for isolation of macronuclei and nuclear membranes from the ciliated protozoan *Tetrahymena pyriformis* E, and the lipid composition of the isolated nuclear membranes was determined.

This method involves cell lysis with octanol, separation of the nuclear membrane with 0.2 M phosphate–1 M NaCl and purification on a discontinuous sucrose gradient. By phase-contrast and electron microscopic examination, our preparations were pure and preserved the typical nuclear membrane morphology: inner and outer nuclear membranes, and nuclear pore complexes. As for lipid distribution, the three major phospholipids in the membranes were phosphatidylcholine (31.0%), phosphatidylethanolamine (26.1%) and 2-aminoethylphosphonolipids (23.3%) and the molar ratio of a sterol-like lipid, tetrahymanol to phospholipid phosphorus was 0.036. These results were compared to other membrane fractions of *Tetrahymena*.

INTRODUCTION

The ciliated protozoan *Tetrahymena pyriformis* is an ideal model system for the study of membrane biosynthesis because of several advantages; rapid bacteria-like growth, highly defined subcellular organelles as in typical eukaryotes and the presence of specific lipids (2-aminoethylphosphonolipids and a triterpenoid, tetrahymanol). Therefore, we have performed a number of experiments, attempting to obtain useful information about membrane synthesis. Procedures for isolating various membrane fractions *e.g.* cilia, pellicles, mitochondria, microsomes from *T. pyriformis* were developed by Nozawa and co-workers[1–5] and striking differences in lipid distribution among the diverse membranes were discovered. Of particular interest is the enrichment of phosphonolipids and tetrahymanol in surface membranes: cilia and pellicles[1,3].

However, our method using a high phosphate buffer was not successful for isolation of nuclear membranes; addition of phosphate buffer induces immediate disintegration of nuclear membranes while other membrane fractions remain intact. In contrast, a number of procedures have been developed for separating nuclear membranes from mammalian cells but only few for *Tetrahymena* cells[6,7].

For isolation of *Tetrahymena* nuclei, prior to separation of nuclear membranes, several detergents, *e.g.* Triton X-100 (refs 8, 9) or Nonidet P-40 (ref. 10) have been

employed to lyse the cells. Electron microscopy reveals that nuclei prepared in this way may have lost outer membrane. A better procedure therefore has been sought and Gorovsky's octanol method[11] which is originally based on the method of Kuehl[12] for plant nuclei, proved to be suitable for isolation of nuclei for separating intact nuclear membranes.

No information has been reported regarding lipid compositions of the isolated nuclear membrane from *Tetrahymena*, although some morphological studies of nuclear pore complexes have appeared[6,7,13,14].

In this paper we present a method for the isolation and purification of nuclear membranes from *Tetrahymena pyriformis* in logarithmic growth phase, and a report on lipid composition of the isolated nuclear membrane.

MATERIALS AND METHODS

Growth of T. pyriformis cells

T. pyriformis, strain E was grown at 28 °C in an enriched proteose peptone medium as previously discussed[1]. 200-ml cultures were harvested after 38–40 h, when cells were in logarithmic growth phase (approx. $5 \cdot 10^5$–$6 \cdot 10^5$ cells/ml). Cells used in this experiment grew exponentially with a doubling time of about 4 h. Log-phase cells were quickly chilled to 4 °C in acetone–solid CO_2 and centrifuged at $164 \times g$ for 5 min in a Sorvall RC-2B refrigerated centrifuge (GSA rotor).

Isolation of macronuclei from T. pyriformis

All procedures were carried out under 4 °C unless otherwise stated. Macronuclei were isolated by a slight modification of the method by Gorovsky[11] and the outline of isolation procedure is presented in Fig. 1. The harvested cells were suspended in a solution of 0.1 M sucrose, 1.5 mM in $MgCl_2$ and 4% in "Gum arabic" (Koso chemical, Tokyo), pH 6.7 and spun down at $164 \times g$ for 5 min. The washed cells were then resuspended in the same solution now containing 24 mM *n*-octanol–0.01% supermidine, and homogenized gently with several strokes by hand in a loosely fitting Teflon homogenizer. The degree of cell lysis was followed with a phase contrast microscope (Olympus Model EHT, Tokyo). To the cell lysate suspension was immediately added 3 vol. of the $MgCl_2$–sucrose solution. During this procedure, care was taken not to expose isolated nuclei too long to octanol solution. Then, the diluted suspension was centrifuged at $365 \times g$ for 5 min in order to sediment crude macronuclei which were still heavily contaminated with small particles. Subsequently, the macronuclear pellet was resuspended in $MgCl_2$–sucrose solution and centrifuged as above. This washing step and centrifugation was repeated twice. For further purification, the nuclear suspension was layered on a discontinuous sucrose gradient of 1.0 M and 1.5 M and centrifuged at $10\,400 \times g$ for 5 min in the Sorvall HB-4 swinging bucket rotor. Thus, the highly purified macronuclei were sedimented to the bottom of the tube.

Separation of nuclear membranes from isolated macronuclei

For separation of nuclear membranes (Fig. 2) from nuclei isolated by the above described procedure, we used a high salt medium to disrupt the nuclei: to nuclear pellet was added a small amount of 0.2 M phosphate buffer in 0.25 M sucrose con-

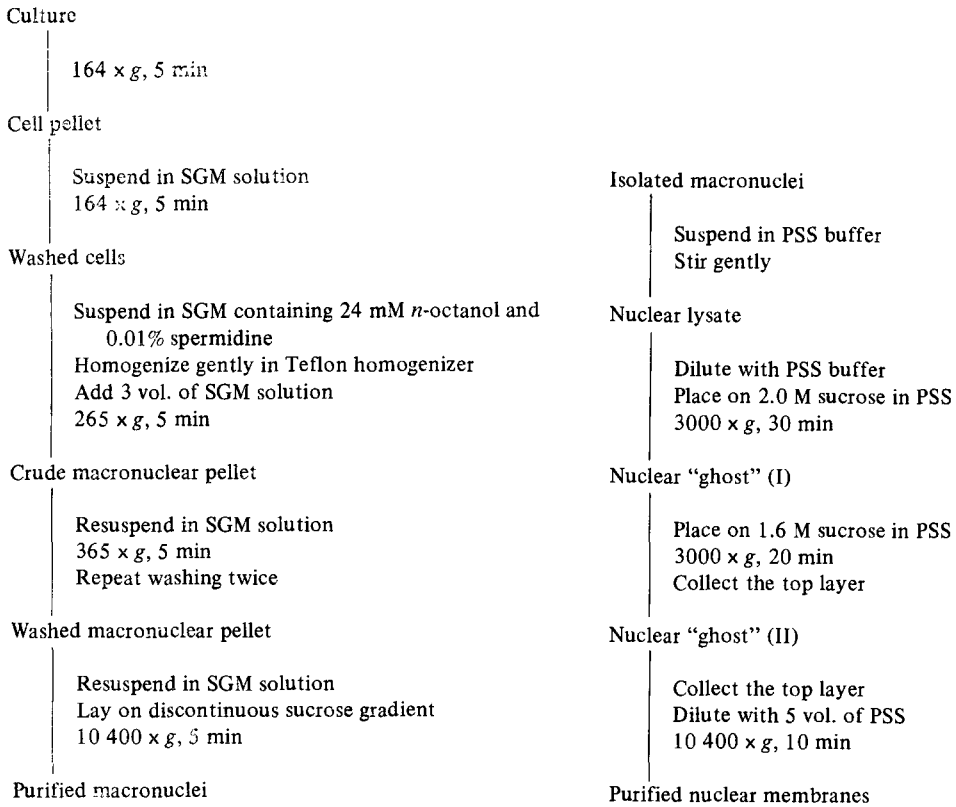

Fig. 1. Preparation of *T pyriformis* E macronuclei. SGM solution: 0.1 M sucrose, 15 mM $MgCl_2$ and 4% gum arabic.

Fig. 2. Separation of nuclear membranes from isolated macronuclei. PSS buffer: 0.2 M phosphate buffer, containing 1 M NaCl and 0.25 M sucrose.

taining 1 M NaCl (pH 7.2). By this hypertonic shock almost all of the naked nuclei were immediately ruptured leaving the intact nuclear ghosts. Then the suspension was placed on 2.0 M sucrose–phosphate buffer and centrifuged at 3000 × *g* for 30 min. The top layer containing the nuclear membrane was carefully removed with a syringe, subsequently overlayered on 1.6 M sucrose–phosphate buffer and spun for 20 min at 3000 × *g*. The membrane fractions were diluted with 5 vol. of 0.2 M phosphate buffer–1 M NaCl and centrifuged at 10 400 × *g* for 10 min. Thus the nuclear membranes were pelleted at the bottom.

Lipid extraction and analysis

Lipids were extracted by the method of Bligh and Dyer[15]. Silica gel G thin-layer chromatography for neutral lipids utilized the following solvent system: light petroleum–diethyl ether–acetic acid (70:30:1, by vol.). Phospholipids were separated by using the solvents chloroform–acetic acid–methanol–water (75:25:5:2.2, by vol.).

Phosphorus content of individual phospholipids was determined by the method of Bartlett[16] modified by digestion with $HClO_4$ according to Marinetti[17].

Tetrahymanol content was determined by gas chromatography following the method described previously[3]. An aliquot of total lipids containing about 30 μg of tetrahymanol was mixed with β-amyrin (Koch-Light Laboratories, U.S.A.) as an internal standard and then hydrolysed in 2 M KOH in 50% ethanol–water for 2 h on a boiling water bath. The combined benzene extracts from the hydrolysate were subjected to gas chromatographic analysis. For this purpose samples in benzene (10 μl) were applied to a 100-cm column packed with 3% OV-17 on 80–100 mesh Chromosorb (Gaschrokogyo, Tokyo) and analysed isothermally at 271 °C in a Hitachi gas chromatograph (Model K-53). The conditions for operation are the following: injection temperature, 310 °C; column temperature, 271 °C; pressures for N_2, H_2 and air, 1.0 kg/cm^2, 1.1 kg/cm^2 and 2.2 kg/cm^2, respectively.

Phase-contrast and electron microscopy

For monitoring the degree of purity of isolated nuclei and nuclear membranes, a phase-contrast microscope (Model EHT) was used. Photographs of unfixed samples were taken with a Zeiss Ultraphot-3 microscope.

For transmission electron microscopy samples of isolated nuclear membranes were examined principally as described previously[18], except for the fixation procedure. In the present study the membrane suspension was fixed in freshly prepared 2% glutaraldehyde in 50 mM cacodylate buffer (pH 7.4) for 1 h and subsequently in 2% OsO_4 in the same buffer for 1 h. Then, the fixed specimens were dehydrated in a graded series of acetone solutions, and embedded in epoxy resin. Ultra-thin sections were cut with glass knives using Ultrome (LKB). Thin-sectioned specimens were examined with the Hitachi HS-8 electron microscope.

For negative staining the membrane samples were air-dried on formvar-coated grid and stained with 2% phosphotungstic acid (pH 7.2).

RESULTS

Preparation of macronuclei and nuclear membranes

It is a prerequisite to isolate pure intact nuclei prior to preparation of nuclear membranes. In general, the main difficulty in isolating nuclei is to break the cells without damaging the nucleus. For this reason, several different methods including homogenization, sonification and detergents (Triton X-100, Tween-80 and Nonidet P-40) were tried, that would break the cells but leave the nuclei intact: the entire nuclear surface should be well preserved. However, it appeared that nuclei prepared by these methods have lost some parts of the outer nuclear membrane (Nozawa, Y., unpublished observation). Instead, use of *n*-octanol proved to be the most satisfactory so that nuclei were isolated from *Tetrahymena* by a modification of the method of Gorovsky[11] (Fig. 1). Nevertheless, particular care must be taken to keep the cells in octanol medium only for as short a period as possible.

Phase-contrast microscopic examination of nuclei thus isolated demonstrated that they are highly purified and show no breakage (Fig. 3).

Separation of nuclear membranes was achieved by disruption of the isolated nuclei with 0.2 M phosphate buffer in 0.25 M sucrose containing 1 M NaCl (pH 7.2) and centrifugation on high density sucrose solutions (1.6 and 2.0 M) for purification. The degree of purity of the nuclear membranes thus prepared proved to be satisfactory,

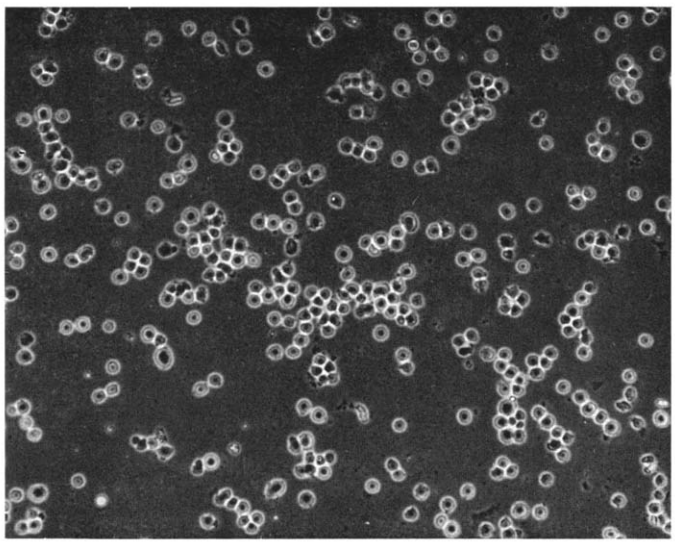

Fig. 3. Phase-contrast photomicrograph of isolated macronuclei from *T. pyriformis* E, demonstrating high degree of purity and intactness. × 480

yet, in some cases, we had difficulty in eliminating the small fragments of nucleoplasm from the nuclear membranes. Fig. 4 shows isolated nuclear membranes observed by phase-contrast microscopy using a Zeiss Ultraphot-3 Microscope, revealing very little contamination by other membranous components. The nuclear membranes are largely uniform in both shape and size. In addition, examination of thin-sectioned samples of the nuclear membrane under the electron microscope revealed almost

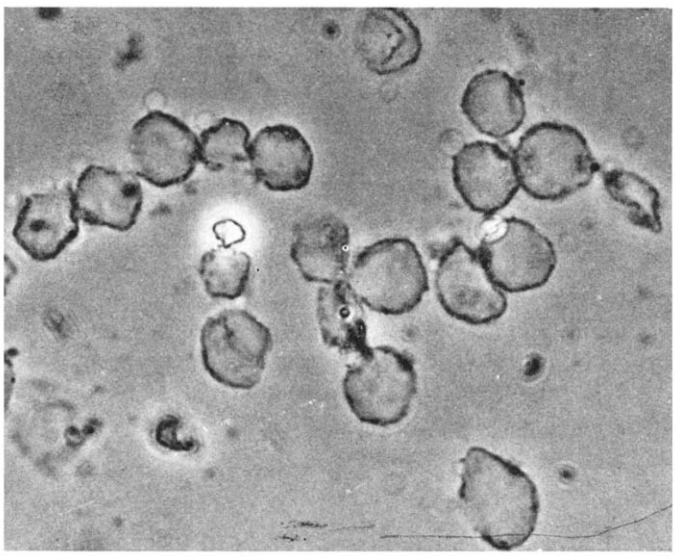

Fig. 4. Isolated macronuclear membranes observed by phase-contrast microscopy, revealing little contamination by other membrane components. × 1500

complete release of nuclear content, but in some preparations small amounts of amorphous material, possibly chromatin were observed. The membrane integrity characteristic of the nuclear membrane was well preserved, with a bilaminar structure of the inner and outer membranes. It is also noteworthy that nuclear pores can be seen at many sites on the membrane (Fig. 5a). The presence of these nuclear pore complexes, containing central granules, was confirmed by negative staining with 2% phosphotungstic acid (Fig. 5b).

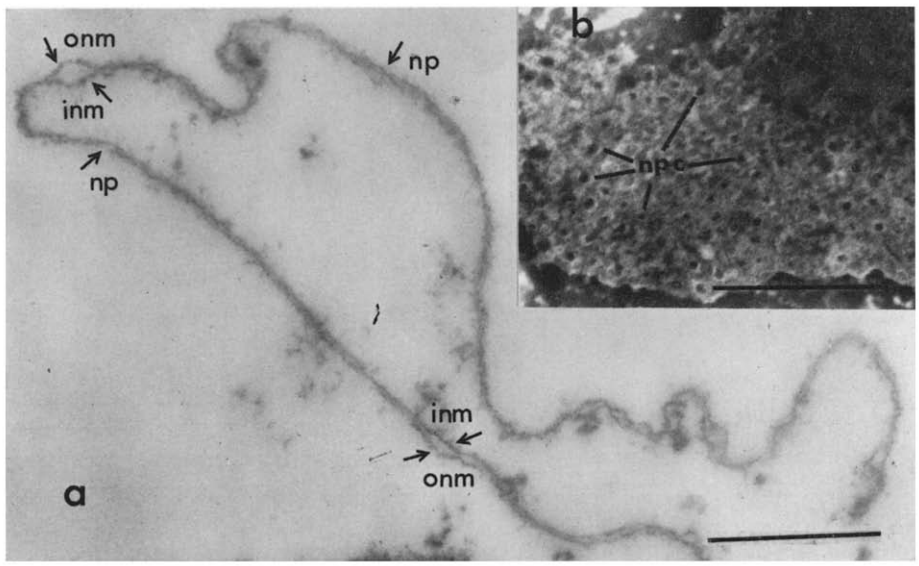

Fig. 5a. Electron micrograph of an isolated nuclear membrane showing the inner and outer nuclear membranes. Nuclear pore complexes are also observed. np, nuclear pores; inm, inner nuclear membrane; onm, outer nuclear membrane. The scale indicates 1 μm. b. A surface view of isolated nuclear membranes, negatively stained with 2% phosphotungstic acid, showing the presence of typical nuclear pore complexes. The scale indicates 1 μm.

Lipid compostion of isolated nuclear membranes

The nuclear membranes contain the same major phospholipids as other *Tetrahymena* membrane fractions: phosphatidylcholine, phosphatidylethanolamine and 2-aminoethylphosphonolipids which are shown on thin-layer chromatogram in Fig. 6, and, in addition, one principal neutral lipid, sterol-like compound tetrahymanol. As can be seen from Table I, which shows phospholipid distribution, a striking difference appears between the nuclear membranes and surface membrane fractions (pellicular and ciliary membranes). The nuclear membrane shows a higher content of phosphatidylcholine than phosphatidylethanolamine which is in agreement with results for nuclear membranes from mammalian sources[19–24], while the pellicular membrane shows the reverse; phosphatidylethanolamine > phosphatidylcholine. Smaller amounts of lysophosphatidylcholine lysophosphatidylethanolamine, *plus* lyso-2-aminoethylphosphonolipids and cardiolipin were also present.

Contrary to our expectation of close similarity in lipid composition between nuclear membrane and microsomes which has been found for mammalian liver[20,23],

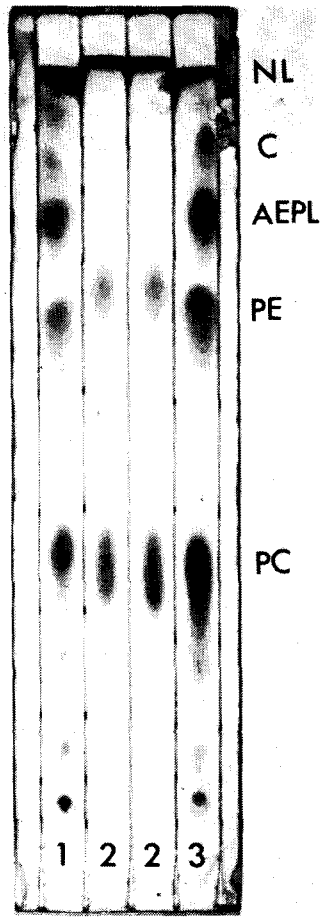

Fig. 6. A thin-layer chromatogram of the phospholipids of *Tetrahymena* nuclear membranes. AEPL, 2-aminoethylphosphonolipids; PE, phosphatidylethanolamine; PC, phosphatidylcholine; NL, neutral lipids; C, cardiolipin; 1, nuclear membrane; 2, egg yolk phospholipids; 3, whole cells.

TABLE I

PHOSPHOLIPID COMPOSITION OF ISOLATED NUCLEAR MEMBRANE FROM *T. PYRIFORMIS*

Figures are expressed as percent of total phospholipid phosphorus. The plate for thin-layer chromatography was developed with chloroform–acetic acid–methanol–water (75:25:5:2.2, by vol.). Phosphorus was determined by the method of Barlett[16] modified with $HClO_4$ according to Marinetti[17]. More details are given in Materials and Methods.

Phospholipids	*Nuclear membrane**	*Pellicles***	*Cilia***	*Mitochondria***	*Microsomes**
Phosphatidylcholine	31.0 ± 1.15	25	28	35	35
Phosphatidylethanolamine	26.1 ± 0.30	34	11	35	34
2-Aminoethylphosphonolipid	23.3 ± 0.43	30	47	18	23
Lysophosphatidylcholine	6.0 ± 2.50	5	1	2	1
Lysophosphatidylethanolamine + + lyso-2-aminoethylphosphonolipid	5.6 ± 1.28	3	9	0	3
Cardiolipin	3.2 ± 0.35	2	1	10	1

* Mean values ± S.D. of six individual nuclear membrane preparations.

** From data of Nozawa and Thompson[1].

the present data showed some difference in content of phosphatidylethanolamine: 26.1% in nuclear membrane and 34% in microsomes. However, phosphatidylcholine and 2-aminoethylphosphonolipids are present in identical amounts in both of the membranes. The molar ratio of sterol-like lipid, tetrahymanol to phospholipid phosphorus which is shown in Table II, is in nice agreement between nuclear membranes (0.036) and microsomes (0.041). Thus it is not unreasonable to suggest that there is some resemblance in lipid distribution between these two membrane fractions, unlike other subcellular membrane fractions of this cell.

TABLE II

TETRAHYMANOL CONTENT OF NUCLEAR MEMBRANES AND OTHER MEMBRANE FRACTIONS FROM *T. PYRIFORMIS*

Figures are expressed as averages from three individual nuclear membrane preparations. Tetrahymanol content was determined by gas chromatography using β-amyrin as internal standard. Phospholipid phosphorus was analysed by the same method as in Table I. See Materials and Methods for details.

	Nuclear membrane	*Pellicles**	*Cilia**	*Mitochondria**	*Microsomes**
Tetrahymanol (μg/μg of phospholipid phosphorus)	0.466	1.175	4.164	0.671	0.481
Molar ratio (tetrahymanol/ phospholipid phosporus)	0.036	0.084	0.30	0.048	0.041

* From data of Thompson *et al.*[3].

DISCUSSION

The ciliate *T pyriformis* has proved to be a particularly useful model system for membrane studies[1,25]. Thompson and co-workers[1–4] have conducted a number of experiments with this system, in which we have shown the striking differences in the lipid composition among the various membrane fractions.

In addition to these studies on lipid, we carried out labeling experiments with radioactive lipid precursors, which showed interesting results concerning the biosynthesis and movement of lipids between membranes in the cell[2]: the labeling pattern was specific for each membrane fraction and different from one membrane to another. These results seem fairly consistent with the work by Chlapowsky and Band[26] on *Acanthamoeba palestinensis*.

However, our procedure for membrane isolation from *Tetrahymena* using high phosphate buffer was not suitable for isolating nuclear membranes, since by this method the nuclei were found to fragment readily and disappear during the preparative steps. Therefore, the lipids of the nuclear membrane have not been characterized until now.

A satisfactory method has been developed for isolation of intact nuclear mem-

branes from *Tetrahymena pyriformis*. The major difficulties encountered in separation of intact nuclear envelopes are (1) quick disruption of the isolated nuclei without fragmentation and (2) solubilization of nucleoplasm which otherwise causes contamination in the nuclear membrane preparation.

The use of high concentration of salt, as has been reported for rat liver nuclei[27–29], was applied to *Tetrahymena* and proved to be effective. Since in some cases loss of outer nuclear membranes can occur (Nozawa, Y., unpublished observation), we did not use sonication and detergents (Triton X-100, Tween 80 and Nonidet P-40). Franke[6] and Wunderlich[7,14] isolated nuclear membranes from *T. pyriformis*, strain GL by hypotonic shock with water or dilute sucrose solution and obtained interesting information relating to nuclear pore complexes by extensive morphological studies. But we felt that a disadvantage of hypotonic stress is the difficulty in separating a satisfactory nuclear membrane fraction which preserves the entire intact membrane integrity. Our nuclear membrane preparations isolated by the procedure presented here were shown to be intact and morphologically pure. Both inner and outer membranes and nuclear pores were well preserved (Figs 5a and 5b).

Lipid distribution of the isolated nuclear membranes prepared as above was examined and compared with that of other membrane fractions previously reported[13]. Based on lipid phosphorus distribution, the major phospholipids were phosphatidylcholine (31.0%), phosphatidylethanolamine (26.1%) and 2-aminoethylphosphonolipids (23.3%). The principal neutral, sterol-like lipid, tetrahymanol was also found: the molar ratio of tetrahymanol to phospholipid phosphorus was 0.036. It is of interest to note that the distribution pattern of lipid of the nuclear membrane is like to that of the microsomal fraction, except for the presence of significant cardiolipid (see Table I). This might reflect the structural similarity of both of these membranes and, in fact, electron microscopy showed direct continuity between the outer nuclear membrane and the endoplasmic reticulum. In comparison with data of phospholipid composition of nuclear membranes from mammalian livers[19,20,22,23], the marked differences are the lower content of phosphatidylcholine and the lack of sphingomyelin, and the inclusion of an appreciable amount of specific structural lipids, 2-aminoethylphosphonolipids in *Tetrahymena* nuclear membrane. Labeling experiments to follow biosynthetic features of the nuclear membrane are now in progress, and the results will appear elsewhere.

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