CHARACTERIZATION OF NUCLEAR MEMBRANES AND ENDOPLASMIC RETICULUM ISOLATED FROM PLANT TISSUE

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ABSTRACT

Nuclei, nuclear membranes and rough endoplasmic reticulum (rER) were isolated from onion root tips and stems. Structural preservation and purity of the fractions was determined by electron microscopic and biochemical methods. Gross compositional data (protein, phospholipid, nonpolar lipids, sterols, RNA, DNA), phospholipid and fatty acid patterns, enzyme activities (ATPases, ADPase, IDPase, glucose-6-phosphatase, 5′-nucleotidase, acid phosphatase, and NADH- and NADPH-cytochrome *c* reductases), and cytochrome contents were determined. A stable, high salt-resistant attachment of some DNA with the nuclear membrane was observed as well as the association of some RNA with high salt-treated nuclear and rER membranes. The phospholipid pattern was identical for both nuclear and rER membranes and showed a predominance of lecithin (about 60%) and phosphatidyl ethanolamine (20–24%). Special care was necessary to minimize lipid degradation by phospholipases during isolations, Nonpolar lipids, mostly sterols and triglycerides, accounted for 35–45% of the membrane lipids. Sterol contents were relatively high in both membrane fractions (molar ratios of sterols to phospholipids ranged from 0.12 to 0.43). Sitosterol accounted for about 80% of the total sterols. Palmitic, oleic, and linoleic acids were the most prevalent acids in membrane-bound lipids as well as in storage lipids and occurred in similar proportions in phospholipids, triglycerides and free fatty acids of the membrane. About 80% of the fatty acids in membrane phospholipids and triglycerides were unsaturated. A cytochrome of the b_5 type was characterized in these membranes, but P-450-like cytochromes could not be detected. Both NADH and NADPH-cytochrome *c* reductases were found in nuclear and rER membranes and appeared to be enriched in rER membranes. Among the phosphatases, Mg^{2+}-ATPase and, to lesser extents, ADPase, IDPase and acid phosphatase activities occurred in the fractions, but significant amounts of monovalent ion-stimulated ATPase, 5′-nucleotidase and glucose-6-phosphatase activities did not. The results obtained emphasize that the close biochemical similarities noted between rER and nuclear membranes of animal cells extend to these fractions from plant cells.

The progress in research on cellular membranes that has been achieved in the past two decades has come, to a large part, from studies of subcellular fractions. Such studies are more frequent and much more advanced with animal cells than with plant material. This lag in current knowledge of composition and function of plant membranes is especially conspicuous for the plasma membrane and for the endomembranes such as nuclear envelope, endoplasmic reticulum (ER), Golgi apparatus (GA), and the various vesicles and vacuoles. Only a few studies have dealt with the preparation and characterization of plant endomembranes of the ribosome-associated category, i.e. the nuclear envelope, the rough endoplasmic reticulum (rER), the annulate lamellae (AL; 30), and perhaps, at least in some cell types, also the outer membrane of mitochondria and plastids (for references see 13, 26, 27, 77). This article presents a characterization of membranes of nuclear envelopes and rER from two tissues of the onion, *Allium cepa*.

MATERIALS AND METHODS

Materials

All fractions were obtained from either bulb stem plates or tips of water-grown adventitious roots of the onion, ***Allium cepa*** **L.** All chemicals used were of reagent grade and were purchased from Merck (Darmstadt, West Germany), Serva (Heidelberg, Germany), or Boehringer (Mannheim, Germany), if not otherwise indicated.

Isolation Procedures

TOTAL HOMOGENATE: The tissue was incubated for 1–6 h in cold medium (see below) or was homogenized immediately, especially in experiments intended to minimize hydrolysis of phospholipids by phospholipases A and D, at the desired concentration (0.3 M sucrose, 50 mM Tris-HCl, pH 7.2, 3% purified gum arabic, 3 mM octanol; see 21, 25, 55, 82, 97) in a rotating knife device (E. Bühler, Tübingen, Germany) four times at high speed for 2–4 s each, with a cooling interval. In some experiments, 5 mM or 10 mM EDTA and/or 10 mM NaF was included in all media in order to reduce degradation of phospholipids by endogenous phospholipase activities. The homogenate was used after two filtrations through fine-meshed nylon cloth and flannel.

NUCLEI: Nuclei were isolated from the homogenate according to the method previously described (21, 25, 82), with the modification that, in the case of the stem material, 5 or 10 mM $MgCl_2$ was added to all the isolation media. In preparations from onion root tissue, divalent cations were present at lower concentrations (1–3 mM) or were totally omitted.

NUCLEAR MEMBRANES: Nuclear membranes were prepared either as previously described (21, 25) or, in particular for large scale preparations, with the use of the following modification. The nuclear pellet was resuspended in 0.1 mM Tris-HCl (pH 7.5) and sonicated 10 times, for 2 s each, with cooling intervals (Bransom Sonic Power, Danbury, Conn.). This suspension was centrifuged at 150 *g* for 5 min. The supernate was layered directly on top of a 66% (wt/vol) sucrose solution and centrifuged at 75,000 *g* for 90 min in a WKF P50K ultracentrifuge (Weinkauf, Brandau, Germany). The material accumulated at the interface was collected, diluted with 10 mM Tris-HCl (pH 7.2), and centrifuged at 110,000 *g* for 120 min. For preparation of high salt-extracted nuclear membranes, the sonicated nuclear fraction was dialyzed against a large vol of 1.5 M KCl (0.25 M sucrose, 0.01 M Tris-HCl, pH 7.2) for 90 min and then further extracted for 60 min in a two-to-threefold volume of this medium. The pellet obtained after centrifugation at 110,000 *g* for 120 min contained the nuclear membrane material. It was then resuspended in 1 mM Tris-HCl (pH 7.5) and processed further by sucrose gradient centrifugation as previously described (see above). For studies of nucleic acid attachment to membrane material by centrifugation in cesium chloride solutions (cf. 6,29), pellets of nuclear membranes or nuclei were resuspended in 1 mM Tris-HCl (pH 7.5), intensely treated with 20–30 strokes in tightly fitting homogenizers of the Potter-Elvehjem or Dounce types and extracted with the 20-fold volume of high salt medium for 4 h under stirring. The membranous material was then recovered by centrifugation at 110,000 *g* for 2 h. The pellet obtained was thoroughly resuspended, usually with the aid of sonication, in 3 ml of 3.0 M or 4.0 M CsCl (10 mM Tris-HCl, pH 7.5), put on the bottom of a centrifuge tube and overlayered with 3 or 4 M CsCl (same buffer). After 10-16 h centrifugation at 75,000 *g* at room temperature a flat density gradient had established. The floated "skin" containing the lipoproteinaceous membrane was recovered from the top of the gradient. In addition, we separately collected the upper half and the bottom half, including the pellet. The fractions were dialyzed against 10 mM Tris-HCl (pH 7.4). The dialyzed material was precipitated with cold trichloroacetic acid (TCA, 10% final concentration) and collected by centrifugation at 2,000 *g* for 15 min.

TOTAL AND ROUGH MICROSOMES: Microsomal fractions were prepared from the supernate of the first centrifugation step of the nuclear isolation or were prepared directly after homogenization. The "postmitochondrial supernates" obtained after two centrifugations at 12,000 *g* for 10 min were centrifuged at 100,000 *g* for 120 min. The resulting pellet was designated "crude microsomes". For further fractionation, this material was suspended through intensive homogenization in 0.3 M sucrose (10 mM Tris-HCl, pH 7.2), which in some experiments contained 2 mM $MgCl_2$, and was loaded on top of a linear 30–65% (wt/vol) sucrose gradient (10 mM with respect to Tris-HCl, pH 7.2). After 180 min of

centrifugation the "rough microsomes" were banded at a mean peak buoyant density of 1.184 ± 0.007 g/cm³ (mean from 30 different preparations). This material was collected by conventional methods, diluted with 0.3 M buffered sucrose and centrifuged at 110,000 *g* for 120 min. For preparation of high salt-extracted rough microsomes the microsomal fractions were dialyzed and extracted in the same way as described for the nuclear membranes. The extracted microsomal membranes were purified by centrifugation in linear sucrose gradients (mean peak density: 1.166 g/cm³) and collected as described for the nuclear membranes.

"TOTAL PARTICULATE" FRACTIONS": This fraction was prepared as the sediment obtained from the homogenate after high-speed centrifugation (2 h at 110,000 *g*).

CHEMICAL ANALYSES: The gross chemical composition of the cellular fractions was determined as follows. Total lipids, total nonpolar lipids, phospholipids and sterols were estimated in the chloroform-methanol extract that had been washed with salt solution (20). Phosphate was determined by the procedure of Gerlach and Deuticke (36), and the corresponding amount of phospholipids was calculated by assuming a mean mol wt of 770 daltons. Sterols were determined either directly or after hydrolysis of the esterified and glycosylated compounds by the Liebermann-Burchard reaction as described by Abell et al. (2) and Skipski and Barclay (91), or by gas chromatography (see below). DNA was measured by the diphenylamine reaction (9) and RNA by the orcinol method (72), or both nucleic acids were determined by their phosphorus contents according to Schmidt and Thannhauser (89), with slight modifications (82). A modified Nessler reaction (96) and the method of Lowry et al. (63), using bovine serum albumin as standard, were used for protein estimations.

For examination of the phospholipid pattern the fractions were suspended in distilled water, and lipids were extracted by adding a 20-fold volume of chloroform-methanol (2:1, vol/vol) and stirring several hours to overnight. The solution was filtered, washed according to Folch et al. (20), and evaporated to near dryness *in vacuo* without heating. Lipids were dissolved in chloroform. Total lipid was determined gravimetrically and lipid phosphorus by colorimetry (see above). Phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on 20 × 20-cm plates coated with silica gel HR Merck, and the amounts of the individual components were determined by phosphorus analysis (86). Lipids, separated by TLC into triglycerides, diglycerides, free fatty acids, sterols, sterol esters, and phospholipids were identified by co-chromatography with authentic references and with the use of selective spray reagents (51, 91).

Free sterols were isolated from the lipid extract by TLC and were determined quantitatively as their acetates (11) by gas-liquid chromatography (GLC, on a glass column, packed with 1.3% OV1, 5 ft × 0.4 cm, 230°C, isothermal). Cholesterol, β-sitosterol, campesterol, and stigmasterol were routinely used as reference substances. Campesterol was added as internal standard (40 μg/mg PL) for the determinations of free sterols. In addition, sterols were determined colorimetrically (37).

Fatty acids were determined by GLC (93). The amount of free fatty acids and triglycerides relative to the phospholipids was calculated by the ratio of the saponified fatty acids of each lipid fraction, separated by thin-layer chromatography, containing an equal amount of tricosanic acid as internal standard.

Enzyme Assays

ATPASE ACTIVITIES: ATPase activities were essentially determined as described previously (28). The reaction was initiated by addition of the suspended material and carried out at 30°C for 15 min. Enzyme was measured as the release of inorganic phosphate (19). In addition, the enhanced activity in the presence of Na^+ and K^+ and its inhibition by ouabain was examined (28). Moreover, the activity was determined with [γ-^{32}P]ATP (from The Radiochemical Centre, Amersham, England) by the isotope distribution method of Lindberg and Ernster (60).

NUCLEOSIDE DIPHOSPHATASE: Nucleoside diphosphatase activities were assayed according to Novikoff and Heus (79) with the substrates IDP and ADP (both as trisodium salts). The released inorganic phosphate was determined by the procedure of Chen et al. (10).

5′-AMPASE: 5′-AMPase activity was determined according to Heppel and Hilmoe (42). Phosphate release was determined as mentioned above.

ACID PHOSPHATASE: Nonspecific phosphatase activity at low pH was assayed by the method of Schmidt (88), with some minor modifications. The reaction misture contained, in 1 ml, 50 μmol sodium acetate-acetic acid buffer (pH 5.2) and 40 μmol β-glycerophosphate (disodium salt). The released inorganic phosphate was determined according to Fiske and SubbaRow (19).

NADH-CYTOCHROME C REDUCTASE: NADH-cytochrome *c* reductase was determined according to the method of Mahler (65) which was slightly modified. The reaction vessel contained, in a total vol of 3 ml, 50 μmol potassium phosphate buffer (pH 7.8), 240 nmol cytochrome *c* (Fe^{3+}), 3 μmol KCN, 0.6 μmol NADH, and a NADH regenerating system consisting of 100 μmol alcohol and 300 mU of alcohol dehydrogenase (EC, 1.1.1.1., Boehringer, Mannheim, Germany), and enzyme suspension. The reaction was started by addition of NADH after preincubation for 5 min at 30°C. In inhibition experiments, rotenone (Sigma Chemical Co., St. Louis, Mo.) was dissolved in ethanol and was added to the sample cuvette while ethanol was added to the reference cuvette. Rotenone had been postpurified by recrystallization from trichloroethylene before use. The

absorbancy increase at 550 nm was measured with a Beckmann DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) equipped with an ultrathermostat for 3–5 min vs. a reference cuvette containing the same reaction mixture except for the enzyme protein. An extinction coefficient difference of 20.5 mM^{-1} cm^{-1} for reduced minus oxidized cytochrome *c* was used for the calculation of the enzyme activity (66).

NADPH-CYTOCHROME C REDUCTASE: NADPH-cytochrome *c* reductase was assayed according to Masters et al. (71). The sample cuvette contained, in a total of 3 ml, 50 μmol potassium phosphate buffer pH 7.8, 240 nmol cytochrome *c* (Fe^{3+}), 3 μmol KCN, 0.3 μmol EDTA, 6 μmol NADPH, enzyme protein and a NADPH regenerating system consisting of 0.4 μmol trisodium isocitrate, and about 200 mV of isocitrate dehydrogenase (EC, 1.1.1.42). The determination of enzyme activity was carried out as described above for the NADH-cytochrome *c* reductase.

CYTOCHROME OXIDASE. Cytochrome oxidase activity was measured spectrophotometrically according to Smith (92; see also 47). Cytochrome *c* was reduced as described by Wharton and Tzagoloff (99).

Cytochrome Determinations

Cytochromes were analyzed spectrophotometrically either at room temperature in 3-ml cuvettes with a 1-cm optical path or at 77°K in 2-ml cuvettes with a 2-mm optical path. Membrane fractions were dispersed in 0.05 M potassium phosphate buffer (pH 7.5). For low temperature spectra, glycerol was added to a final concentration of 25%. The sample cuvette was made anaerobic with N_2, a few grains of dithionite were added, and the difference spectrum of the reduced versus the oxidized sample was recorded. Cytochrome P-450 was determined from the difference spectrum of the dithionite-reduced and CO-treated sample vs. the dithionite-reduced sample (80). The concentrations of the cytochromes were calculated from the extinction coefficients given by Vanneste (98). The intensification of the low temperature spectrum when compared to the room temperature spectrum was estimated from the absorbancy increase of the Soret band of the *b*-type cytochromes.

Light and Electron Microscopy

The fractions were either studied directly by phase- and interference-contrast light microscopy or were fixed as pellets or in suspension. They were processed for electron microscopic observations of ultrathin sections or negatively stained preparations as described (21, 22, 25, 28). Micrographs were made with Siemens electron microscopes IA and 101 or the Zeiss EM10. For determinations of cross contaminations, membrane profile lengths were traced in randomly chosen sections and were classified as nuclear envelope, rough microsomes, total smooth-surface membranes, and mitochondrial and plastidal membranes (32, footnote 1).

RESULTS AND DISCUSSION

Morphology and Purity of Fractions

The structural preservation and purity of nuclei and nuclear membrane fractions from onion tissue as we prepare them has been described (21–25, 27). Fig. 1 gives light and electron micrographs of the nuclear fraction from onion root tips. The purity of this fraction was comparable or superior to that achieved by others with plant material (cf. 25, 56, 85, 97). Morphometric analysis by membrane profile tracing of three onion root tip nuclear fractions revealed (as percentage of total membrane profile length) 81% nuclear membrane, 11% rough-surfaced membranes not associated with nuclei, 4% smooth-surfaced membranes (not specified), 1.5% mitochondrial membranes and 0.5% plastidal membranes. Nuclear fractions from root tips were generally more homogeneous than corresponding fractions from stem material.

Nuclear envelopes were preserved over an average of about three-fourths of the nuclear surface. Polyribosomes were associated with the outer nuclear membrane, but at reduced density compared to the situation *in situ*. Most of the chromatin appeared to be condensed, leaving the nucleolus and the interchromatinic strands. The nuclear membranes and pore complexes showed normal ultrastructural organization, as described in detail previously (21–24, 41). The outermost layer of chromatin appeared to be in direct contact with the inner nuclear membrane in some areas, but was separated from this membrane by electron-translucent particles, about 200 Å wide, in other regions (Fig. 2). Distinct, fine threadlike connections between the chromatin and the nuclear envelope were regularly observed (Fig. 2; for reviews see 23–25, 27, 29, 30). Occasionally, osmiophilic knobs with diameters up to 200 nm were recognized at or within the nuclear envelope. These knobs sometimes displayed a typical myelinization pattern (Fig. 3 and reference 82). The frequency of these knobs, which probably consist of lipids, was varia-

[1] Franke, W. W., T. W. Keenan, J. Stadler, E.D. Jarasch, R. Genz, and J. Kartenbeck. 1975. Nuclear membranes from mammalian liver. VII. Characteristics of highly purified nuclear membranes and comparison with other membranes. Manuscript submitted for publication.

ble between different nuclei as well as within different regions of the same nucleus. The formation of such lipid droplets or myelin figures may result from rearrangement of membrane lipids following degradation of phospholipids (see following section). That this rearrangement occurred was suggested by the reduced frequency of such bodies in preparations isolated more rapidly than normal or when phospholipase inhibitors were included in isolation media.

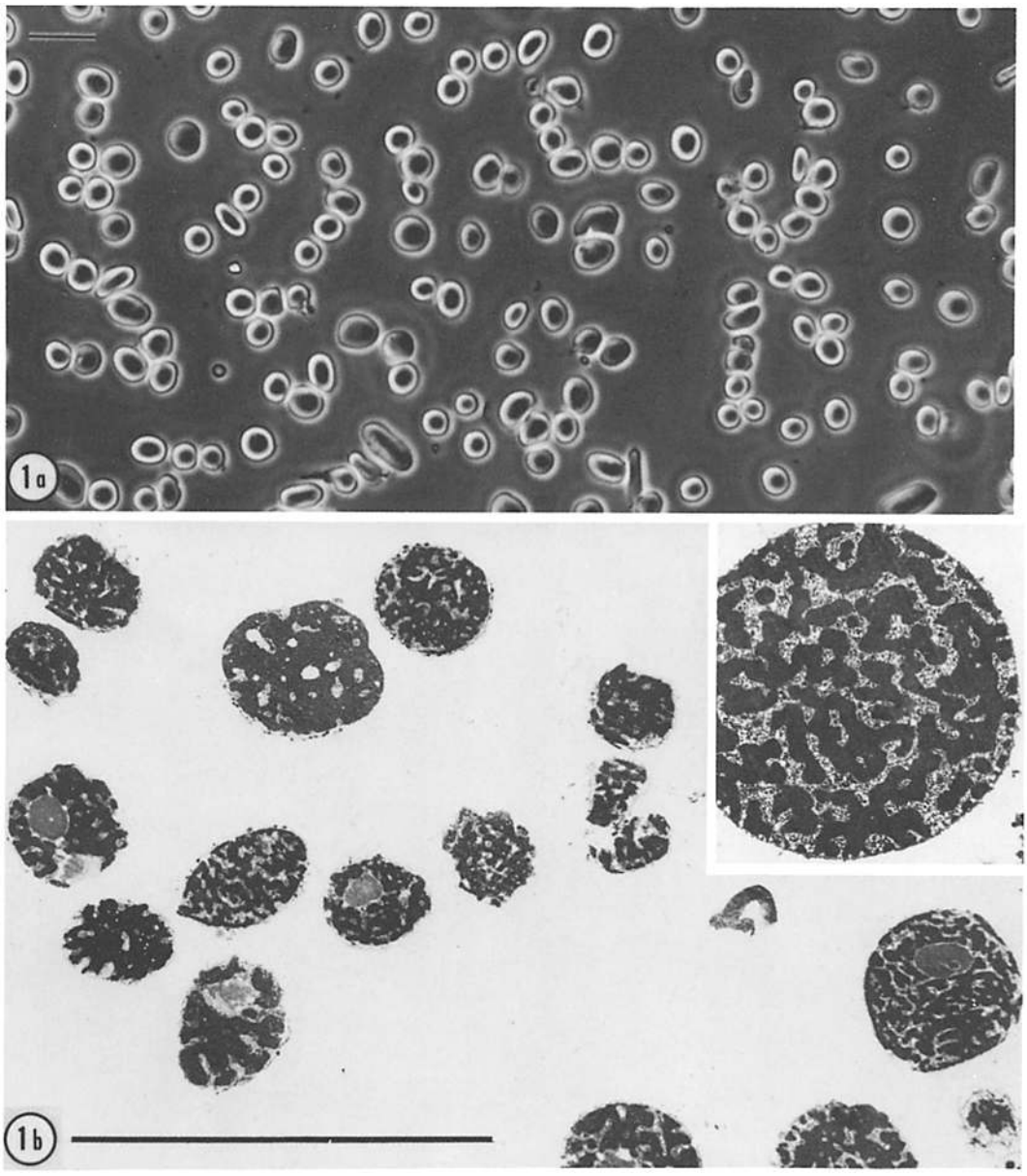
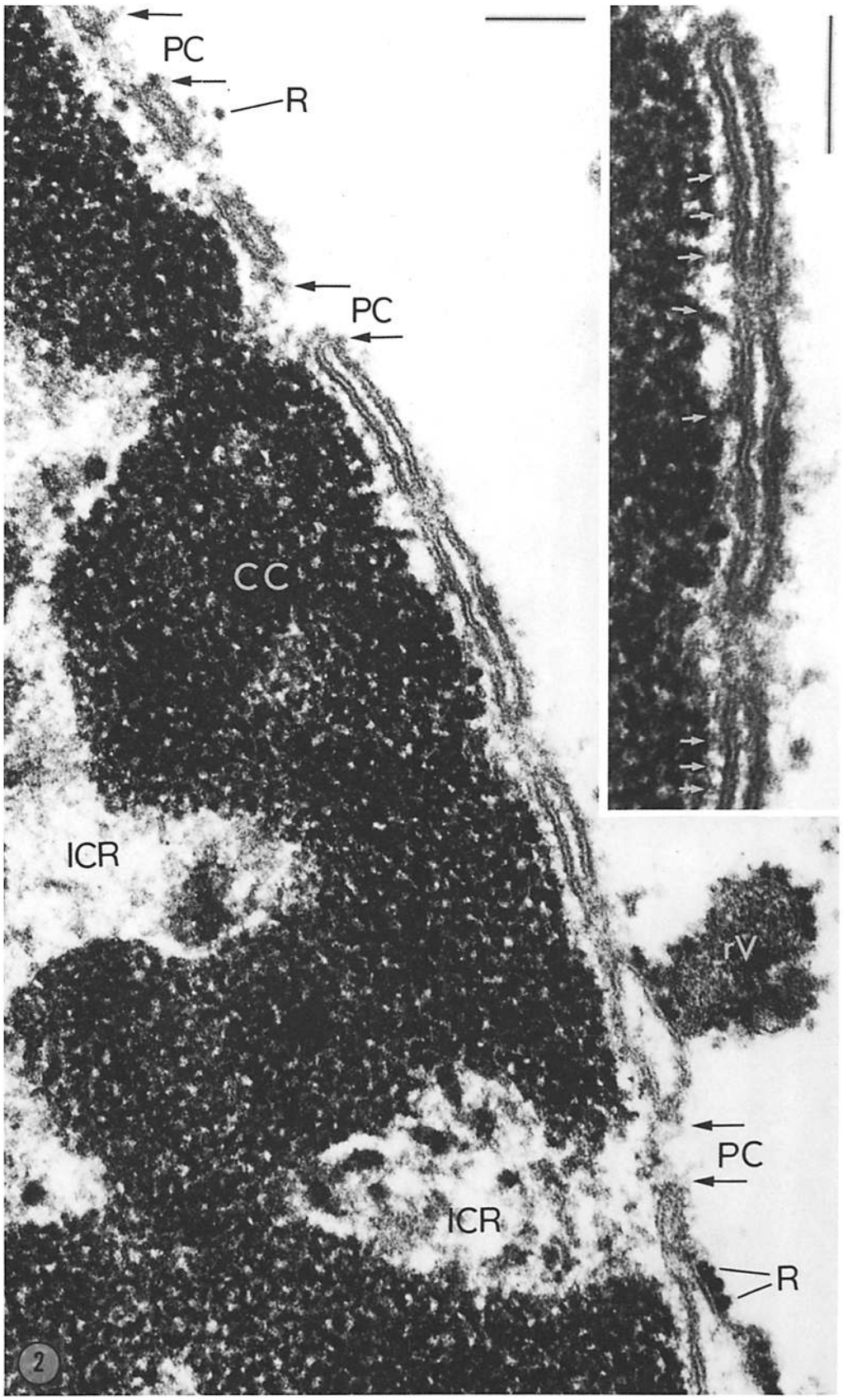


FIGURE 1 Survey micrographs of the nuclear fractions isolated from onion root tip as revealed in the light microscope (*a*, phase contrast) and in the electron microscope (*b*). Note the heterogeneity in nuclear sizes and shapes. In most nuclei the chromatin appears rather condensed (insert in *b*). Some broken nuclei are observed but cytoplasmic contamination is very low. (*a*) × 450; (*b*) × 2,700; insert, × 6,900. Scales indicate 20 μm.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The yield of nuclear membranes was poor, compared to that attained in various animal cells (for references see 23, 24, 28, 49, 53, 101, footnote 1), primarily as a result of low recovery of nuclei from the tissue (10–20% on the basis of DNA determinations). The relative recovery of nuclear membranes from isolated nuclei, however, was as good as with animal material (note, e.g., the 59% phospholipid recovery; see below). Maximally, we obtained 1-mg nuclear membrane protein from a total of ca. 4,000 root tips.

The crude microsomal fraction consisted mainly of membranous vesicles and sheets but also contained aggregates of free ribosomes and lipid

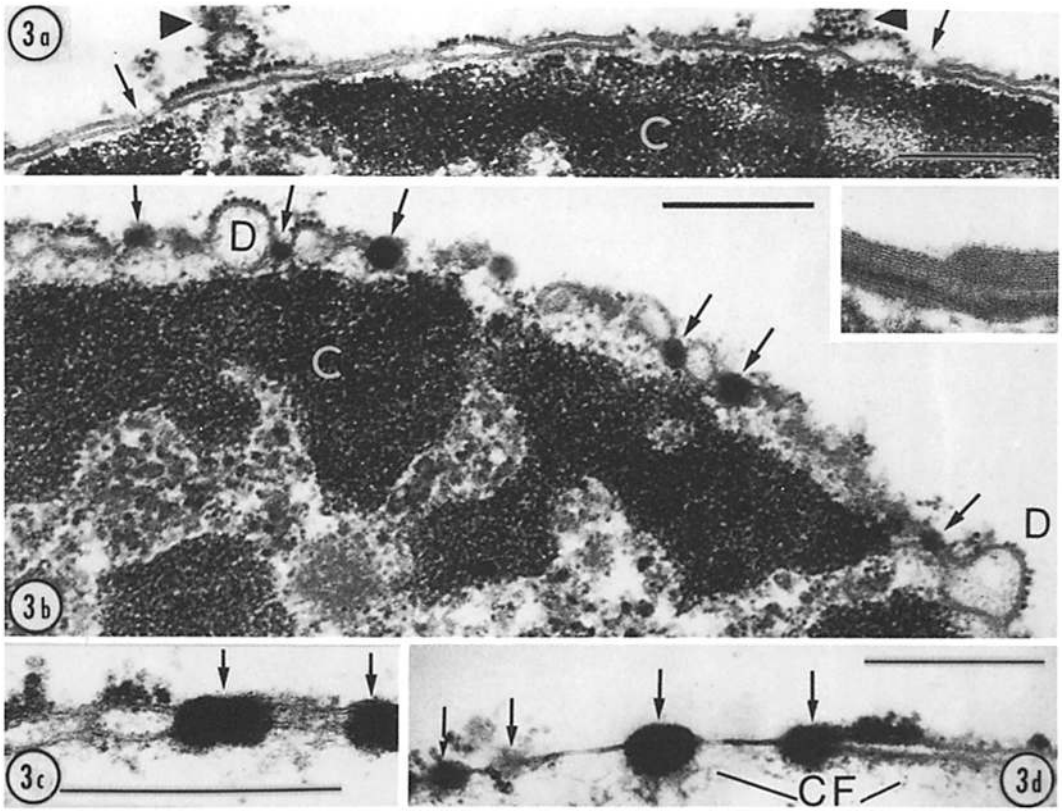

FIGURE 3 Details of nuclear envelope fine structure in isolated nuclei from onion root tips. While the majority of nuclei showed the normal, well preserved double membrane appearance of the envelope (*a*, arrows denote pore complexes) a certain proportion of the nuclei revealed localized lipid formations that occurred either as osmiophilic nodules associated with one of the two nuclear membranes (*b* and *d*) or spanning the whole perinuclear cisterna (*c*) or appeared in typical lamellar myelin configurations (insert in *b*; periodicity ca. 40 Å). Probably, such structures reflect rearrangements of lipid material liberated during phospholipid breakdown in the course of the isolation. (*a* and *b*) × 60,000; (*c*) × 10,000; (*d*) × 72,000; (*b*, insert) × 70,000. Scales indicate 0.3 μm.

FIGURE 2 Periphery of a nucleus isolated from an onion root tip cell with its nuclear envelope as revealed in the electron microscope at higher magnification. Note the condensed appearance of the chromatin and the well preserved ultrastructure of the nuclear envelope with its two membranes, ribosomes (R) associated with the outer membrane, threadlike connections (some are indicated by the white arrowheads in the insert) to the peripheral, condensed chromatin (CC), and pore complexes (PC; the outer annuli are denoted by short horizontal arrows). ICR, interchromatinic regions; rV, rough-surfaced vesicle. × 160,000; insert, × 220,000. Scales indicate 0.1 μm.

bodies. Morphometry of such fractions from root tips, for example, showed that 72% (mean from three different preparations) of the identified membrane profiles were from rER as judged by ribosome attachment. This fraction of rER-derived vesicles and sheets increased to more than 83% in the "purified rough microsomes" prepared therefrom (Fig. 4 *a*). Only 2.5% of the membrane profiles could be identified as mitochondrial. The purified rER fraction (Fig. 4; for plant microsomal fractions cf. 4, 15, 43, 44, 61, 62) also contained fewer lipid nodules and free ribosomes but still some smooth surfaced membrane sheets and a conspicuous type of vesicle that was "smooth" on its outer surface but showed ribosomes in its interior (Fig. 4 *a–e*). The latter might well have been derived from vesiculated plasmalemma (Fig. 4). Loosely and irregularly packed fibrillar contents were noted in some of the rER vesicles, possibly representing intracisternally located products.

After sonication and high salt extraction, nuclear and microsomal membrane fractions revealed an almost identical appearance characterized by smaller vesicle sizes and a marked increase in small sheetlike fragments. While ribosomes were absent on outer vesicle surfaces, some ribosome-like granules were still recognized within some of the vesicles (e.g., Fig. 4 *e*).

Gross Chemical Composition and Buoyant Density

The composition of fractions from the two onion tissues is given in Table I. The DNA, RNA, protein and phospholipid contents of nuclear fractions are in basic agreement with data reported for nuclei from other plant materials (for reviews see 25, 30, 85, 94, 97). The composition of microsomal fractions is close to that reported for rER-derived fractions from a variety of plant materials (4, 61; for further references see 32), but this fraction is distinguished by its RNA content from "smooth vesicle" fractions from plants (32, 61). Our onion nuclear membrane composition is similar to that reported for pea plumule nuclear membranes (94), except for a higher phospholipid content in our fractions. This may be indicative of more efficient removal of nonmembranous materials from our fractions. Extraction with high salt combined with sonication resulted in a great reduction of the RNA content of both membrane fractions, which probably reflects the removal of a considerable proportion of the ribosomal material (3), in agreement with the noted absence of identifiable ribosomes in fractions after treatment. It is noteworthy that an appreciable amount of RNA is still found in fractions after such treatment with salt. This RNA persists in fractions even after incubation in the presence of puromycin (3). This is in contrast to observations with animal cell fractions (3, 28) and bean hypocotyls (15), but may be explained by the occurrence of smooth-surfaced but ribosome-enclosing vesicles in our fractions. The retention of significant amounts of DNA in extracted nuclear membranes correlates with similar observations with animal cells (23–25, 27–30, 49 53, 101) and, as in animal cells (29), this DNA was not completely removed by digestion with pancreatic deoxyribonuclease (82).

The stable association of both DNA and RNA with nuclear membranes was also observed after flotation in 3 and 4 M CsCl solutions upon which 2.3% of nuclear DNA, 15% of nuclear RNA and 59% of nuclear phospholipids were recovered in the floated membrane material (details given in 82). The recovery of nearly 60% of the phospholipid illustrates the relatively good recovery of membrane material, in correspondence with recoveries from hepatocyte nuclei (e.g., 74). We believe that this nuclear membrane-associated DNA represents the remainder of the most peripheral chromatin stably connected with the inner nuclear membrane. This probably contains blocks of constitutive heterochromatin (for reviews see 24, 27, 29, 33). The relatively high protein and nucleic acid content of the isolated nuclear membrane probably explains the high mean peak buoyant density ($\rho_4^{20} = 1.212 \pm 0.008$ g/cm^3 as determined by isopycnic centrifugation in sucrose gradients) which is in the same range as the figures determined for nuclear membranes isolated from animal sources by the techniques (24, 28, 49, 53, 101). This mean peak density is, however, lower than that reported by Stavy et al. (94) for nuclear membranes from pea apices in sorbitol gradients. The density of our rER membranes (1.184 g/cm^3) is similar to that for rER fractions from animal and plant cells (see references given above). After sonication and high salt treatment, the mean peak density for both nuclear membranes and rER shifted by about 0.01 g/cm^3.

Lipid Composition of Fractions

Lipid contents and patterns for the fractions are given in Tables I and II. Considerable degradation

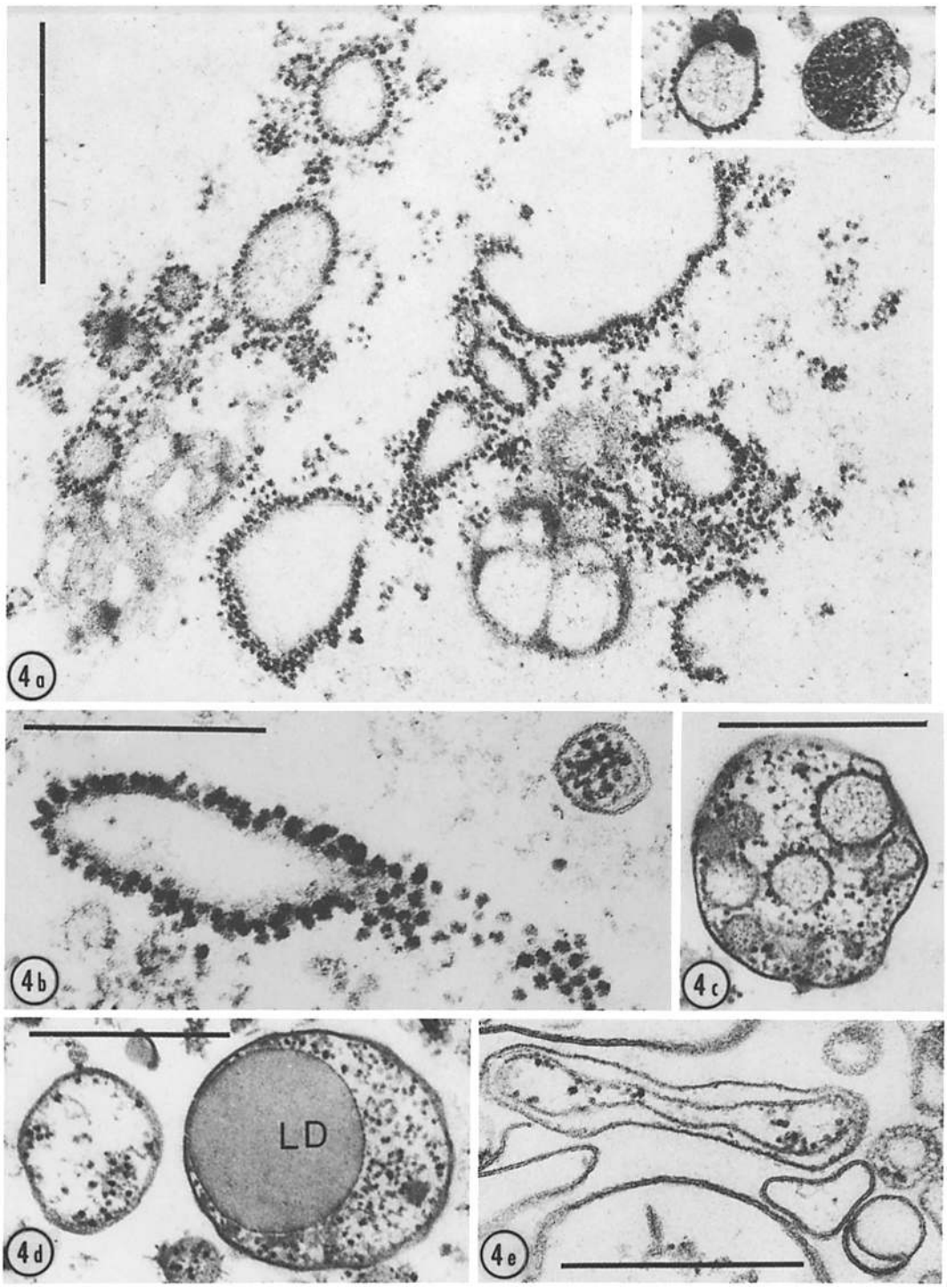

FIGURE 4 Electron micrographs of ultrathin sections through pellets of purified fractions of rough microsomes isolated from onion root tips. The predominance of ribosome-covered vesicles and membrane fragments is obvious in the survey micrograph (*a*). Elongated cisternal fragments were also seen (e.g. *b*). Occasionally, one notes localized formations of small osmiophilic nodules (insert in *a*), indicative of artificial lipid rearrangements. The fractions also contained minor amounts of smooth-surface vesicles with about the same sedimentation rate and buoyant density as the rough microsomes; such vesicles contained variable amounts of ribosomes (e.g., in the insert in *a*; the vesicle in the upper right of *b*; and *c-e*) and probably reflect vesiculations from plasmalemma or tonoplast. Some of these "heavy" smooth-surfaced vesicles contained rER-derived vesicular elements (*c*) and cytoplasmic lipid droplets (LD in *d*). Fig. 4 e illustrates that some ribosome-like structures can still be visualized within such vesicles after treatment of the fractions with high salt concentrations. (*a*) × 80,000; insert, × 60,000; (*b*) × 145,000; (*c*) × 62,000; (*d*) × 60,000; (*e*) × 72,000. Scales indicate 0.5 μm.

TABLE I

Gross Composition Data (%Weight of Total Sum) in Fractions from Onion Tissues

Tissue	Fraction	DNA	RNA	Protein	Phospholipids	Nonpolar lipids*	RNA / DNA	RNA / Protein	Phospholipids / Protein	Nonpolar lipids / Phospholipids
Stem	Nuclei	30.5	9.0	55.8	2.6	2.2	0.30	0.16	0.05	0.85
	Nuclear membranes, extracted	11.3	7.2	68.4	7.7	5.4	0.64	0.11	0.11	0.70
	Crude microsomes	0.3	38.0	35.4	14.2	12.0	127	1.07	0.40	0.85
	Rough microsomes	<0.1	16.4	52.6	16.6	14.4		0.31	0.32	0.87
	Rough microsomes, extracted	<0.1	12.1	57.9	17.2	12.8		0.21	0.30	0.74
Root tip	Nuclei	30.2	15.5	52.2	1.2	0.7	0.51	0.30	0.02	0.58
	Nuclear membranes, not exposed to high ionic strength	18.7	14.1	56.6	6.9	4.0	0.75	0.25	0.12	0.58
	Nuclear membranes, extracted	1.7	5.5	67.7	14.9	10.0	3.24	0.08	0.22	0.66
	Crude microsomes	3.2	41.3	39.4	9.2	7.0	12.9	1.05	0.23	0.76
	Purified rough microsomes	<0.1	23.7	52.3	13.7	9.7		0.45	0.26	0.70
	Purified rough microsomes, extracted	<0.1	12.6	63.3	13.9	10.1		0.20	0.22	0.73

* Triglycerides, diglycerides, sterols, sterol esters, and glycolipids.

of phospholipids was noted in all fractions during isolation unless precautions (especially working fast and addition of lipase inhibitors) were taken. Such lipid-degrading enzymes, especially phospholipase D, are common in plant tissues (35, 50). Lecithin was the most abundant phospholipid, constituting 60% or more of the phospholipids in both membrane fractions (Table II). These data, taken together with analyses of various endomembranes as well as mitochondrial, plastidal, and plasma membranes from other plants (e.g., 1, 5, 7, 16, 51, 73, 76, 83), suggest that in plants, as in various animal cells, the content of lecithin is higher in nuclear and ER membranes than in smooth endomembranes, plasma membrane, and the inner membranes of mitochondria and plastids. Phosphatidyl ethanolamine was the second most abundant phospholipid in our fractions (Table II). The low level of cardiolipin observed corresponds well with the low levels of mitochondrial contamination observed by electron microscopy and by cytochrome oxidase determination (cf. a following section).

Nonpolar lipids constituted a relatively high but constant proportion of the membrane lipids (Table I). Sterol contents were particularly high in fractions from stems when compared with the cholesterol contents in such membranes from animal cells (e.g., 24, 78). Sitosterol and campesterol were the major constituents of the sterol fraction (for related sterol patterns cf. references 8, 11, 31, 39, 52, 81); a chromatographic peak corresponding to cholesterol represented only a trace of the total sterols (Table II; assays using the method of Clark et al., reference 12, were negative; for a report of high cholesterol levels in microsomes from other plants and in nuclear membranes from pea plumules, see references 8, 94). Sterol esters were present in levels of less than 3% of the amounts of total free sterols. Others have likewise reported relatively high sterol contents in plant nuclei and nuclear membranes (94), microsomes (17, 45) and plasma membranes (7, 45, 51). In certain animal cells sterols are enriched in Golgi apparatus and plasma membrane relative to nuclear envelope and ER (for references see 24, 78), but presently available data on this point are lacking for plant cells. We did not observe specific enrichment of sterols or sterol esters in nuclear membranes relative to microsomes as has been reported by Kemp and Mercer (52) in corn shoots.

Fatty acid patterns for the phospholipids, triglycerides and free fatty acids were rather uniform (Table II) and revealed the dominance of unsaturated acids in these lipids. Palmitic acid was the only saturated acyl group in appreciable amounts. In addition to the normal (cis) palmitoleic acid which occurred only in trace amounts, we noted a related fatty acid that might be an isomer of palmitoleic acid (Table II; for the common occurrence of such a fatty acid in plant material see also 34). Significant amounts of linolenic acid occurred in all fractions from root tips but in stem material only in triglyceride components (cf. 5, 73; see, however, also 76). The fatty acid patterns of the membrane fractions exhibited a strong similarity to those determined in homogenates and total particulate material which contains considerable amounts of storage fat droplets (82) and also resemble the patterns described in defined fractions of storage oil bodies and lipid-containing vesicles (40).

When fractions were prepared in the presence of lipase inhibitors, the amount of free fatty acids was not altered in the membrane fractions (Table II) but was markedly increased in the homogenates and total particulate fractions, a result which is unexpected and yet unexplained. The data suggest that lipid bodies, which were sometimes noted in association with the membranes (see above and Figs. 3 and 4), are not aggregates of free fatty acids but, in our opinion, might rather represent rearrangements of membrane-derived sterols (note also the ca. 40 Å periodicity in Fig. 3 *b*).

Enzyme Acitivities and Cytochrome Determinations

Enzyme activities measured in the fractions are listed in Table III. Mg^{2+}-activated ATPase was present in the same order of magnitude as found in a variety of other membrane fractions from various plants (e.g., 45, 59, 70, 94; for further references, see 31). Its enrichment in purified microsomes and in nuclear membranes was only moderate, which might be explained by the occurrence of such activities in various nonmembranous structures. Stimulation of ATPase activity by monovalent cations, which has been described in various membranes and membrane fractions from other plants and has been discussed by some authors as being specific for plasma membrane (e.g., 45, 59, 100), was not significant in fractions from root tips and was only weakly expressed in

TABLE II

*Lipid Composition Data of Fractions from Onion Root Tip and Stem**

	Total particulate fraction	Nuclei	Nuclear membranes	Crude microsomes	Purified rough microsomes
Identified phospholipids (*% of total lipid phosphorus*)					
(1) Phosphatidylcholine	24.8 (54.0)	27.1	25.2 (52.2)	19.2	30.3 (50.3)
(2) Lysophosphatidylcholine	6.6 (0.8)	5.2	3.2 (1.4)	5.8	4.4 (1.5)
(3) Phosphatidic acid	23.9 (2.5)	24.6	34.3 (4.5)	31.6	24.6 (4.6)
(4) Sum of 1 and 2	31.4 (54.8)	32.3	28.4 (53.6)	25.0	34.7 (51.8)
(5) Sum of 1 to 3	55.3 (57.3)	56.9	62.7 (58.1)	56.6	59.3 (56.4)
(6) Phosphatidylethanolamine	20.3 (21.6)	20.4	17.1 (25.8)	19.3	21.4 (26.6)
(7) Lysophosphatidylethanolamine	2.4 (0.2)	4.9	3.5 (1.9)	5.3	2.5 (2.1)
(8) Sum of 6 and 7	22.7 (21.8)	25.3	20.6 (27.7)	24.6	23.9 (28.7)
(9) Phosphatidylinositol	9.5 (7.1)	8.0	7.4 (7.3)	7.9	7.4 (5.9)
(10) Phosphatidylserine	2.5 (2.4)	3.1	2.0 (ND)	4.3	2.1 (2.4)
(11) Phosphatidylglycerol	5.6 (6.1)	5.6	5.4 (5.2)	5.2	6.0 (5.2)
(12) Cardiolipin (plus one partially separated unidentified minor component)	4.3 (5.2)	1.2	2.1 (1.8)	1.6	1.3 (1.2)
Molar ratio of total sterols to total phospholipids‡	0.24 [0.18] 0.29 [0.29]	0.13 0.36	0.18 [0.12] 0.36 [0.22]	0.20 [0.17] 0.52 [0.38]	0.17 [0.14] 0.43 [0.39]
Major sterols (*%wt of sterols*)					
β-Sitosterol	74.5	80.7	85.5	82.2	81.0
Campesterol	9.7	7.1	8.0	7.5	6.6
Triglycerides (*mg/mg phospholipids*)	0.89 (1.13)	0.38	0.44	0.54	0.47 (0.59)
Free fatty acids (*μg/mg phospholipids*)	95 (256)	35	30	39	35 (30)
Fatty acid composition (% wt)§					
Phospholipids					
16:0	32(30);21(17)		33;22		32;26(22)
16:1	tr(tr) ;tr(tr)		tr ;tr		tr ;tr(tr)
16:1‖	2(1) ; 2(5)		tr ;tr		tr ; 3(5)
18:0	2(2) ;tr(tr)		3; 2		2; 1(tr)
18:1	7(8) ;32(34)		8;20		6;16(19)
18:2	49(51);44(43)		51;52		53;52(53)
18:3	6(7) ; 1(1)		5;tr		7;tr(tr)
Triglycerides					
16:0	28(25);14(10)		nd		26;13(12)
16:1	tr(tr) ;tr(tr)				tr ;tr(tr)
16:1‖	tr(tr) ;tr(tr)				tr ;tr(tr)
18:0	2(2) ; 2(1)				2; 1(tr)
18:1	7(8) ;25(27)				7;15(15)
18:2	54(54);54(58)				57;65(67)
18:3	9(10); 4(4)				7; 6(5.5)
Free fatty acids					
16:0	38(36);32(27)		nd		36;36(34)
16:1	tr(tr) ;tr(tr)				tr ;tr(tr)
16:1‖	tr(tr) ;tr(tr)				tr ;tr(tr)
18:0	2(2) ;11(19)				5; 5(3)
18:1	10(9) ;29(27)				9;18(19)
18:2	43(46);27(25)				45;40(41)
18:3	7(7) ; 1(2.5)				5; 1(1)

ND, not detected (i.e. not separated from lecithin) in this series of experiments; nd, not determined; tr, traces. Brackets give values from isolations carried out in the presence of 10 mM EDTA and 10 mM NaF as phospholipase inhibitors.

* Data from stem material are listed here only for determinations which showed considerable tissue differences, i.e. contents of total sterols and the fatty acid compositions.

‡ Values determined by colorimetry; values determined by gas chromatography are given in the squared parentheses; mol wt of 415 D and 770 D have been used; values below the line are from stem material.

§ Values to the right of the semicolons are for stem material.

‖ Unidentified acid which is possibly an isomer of the normally occurring 16:1 (see text).

homogenates and crude microsomes from stem tissue (see also 94). Inhibition with ouabain was never observed (for references on ouabain effects in plant cells see 31, 70). The membrane-bound Mg^{2+}-ATPase activities were only slightly reduced after treatment of the fractions with high salt concentrations, indicative of its constitutive membranous character. ADPase and IDPase activities were present at similar levels in all fractions, which emphasizes their widespread, nonspecific occurrence among endomembranes (for references see 14, 31, 38, 84). Significant enzymatic hydrolysis of 5'-AMPase was not found (for references as to the dubious occurrence of this enzyme in plant membranes, see 31, 56, 59, 94). Acid phosphatase was not enriched in any of the membranous fractions and occurred in the isolated membranes as well as in a form not sedimentable at 100,000 *g* (for references see 14, 31, 43, 56). Some authors have reported the occurrence of glucose-6-phosphatase in plant nuclear and microsomal membranes (56, 94). However, experiments performed to characterize the G-6-P hydrolysis activity in fractions from onion tissues (82) rather favour the notion that the activity found in plants reflects the presence of a nonspecific acid phosphatase (58; see also 59).

Both NADH- and NADPH-cytochrome *c* reductase were present in nuclear and rER membranes at similar levels (Table III). Their true enrichment in rER might even by higher than indicated since some degradation and inactivation during the preparation might be expected. The activities were in the same range as those described in (partly smooth) microsomal fractions from various plant cells (for references see 16, 31, 43, 59, 62, 67-69, 82). The NADH-cytochrome *c* reductase is not inhibited by rotenone; therefore a significant contribution of the mitochondrial NADH dehydrogenase in our microsomal and nuclear membrane preparations can be excluded. The presence of NADPH-cytochrome *c* reductase activity in nuclear membranes is interesting in view of the continuing debate as to its significance in this structure in animal cells (for reviews see 23, 24, 49). Both reductases had the same pH and temperature optimum (pH 8.0, 30°C) and reduced endogenous cytochrome b_5 (see below) to the same extent. Maximal enzyme activity for both electron donors was obtained in the presence of low concentration of detergents (e.g., 0.1% Triton X-100). Concentrations higher than 0.5% Triton X-100 inhibited the enzyme activity. On the other hand, the NADPH-dependent activity was much more resistant to extraction of phospholipids (for details see 82). Further, the NADH-cytochrome *c* reductase activity followed Michaelis-Menten kinetics with respect to NADH whereas the NADPH-cytochrome *c* reductase showed more complex kinetics, suggesting the presence of two components with

TABLE III

Specific Enzyme Activities in the Fractions from Onion Stem and Onion Root Tip

Tissue	Fraction	Mg^{2+}— ATPase	$(Na^+ + K^+)$— Mg^{2+}— ATPase*	ADPase	IDPase	Acidic phosphatase	NADH-cytochrome *c* reductase	NADPH-cytochrome *c* reductase
Stem	Homogenate	0.92	<0.01	0.66	2.39	12.60	15.5	26.6
	Nuclei	0.37	<0.01	0.68	2.30	<0.02	19.4	23.7
	Nuclear membranes	1.01	<0.01	0.37	3.06	0.03		
	Crude microsomes	2.80	<0.01	0.61	1.27	1.30	19.4	26.8
	Rough microsomes	7.50	<0.01	0.80	1.52	0.33	25.6	38.4
Root tip	Homogenate	1.31	0.02	0.50	2.75	1.90	87.0	128
	Nuclei	1.96	<0.01	0.14	1.75	<0.02	25.6	48.2
	Nuclear membranes	2.63	<0.01	0.40	2.12	<0.02	61.7	78.3
	Crude microsomes	2.26	0.06	0.50	4.62	2.45	142	156
	Purified rough microsomes	3.03	<0.01	0.68	4.83	0.46	264	258

* These values are taken from colorimetric determinations using the Fiske und SubbaRow method; values of determinations using γ^{32}P-ATP as substrate were basically identical.

Activities are given as μmoles inorganic phosphate released per hour per mg protein or as nmoles cytochrome *c* reduced per min per mg protein.

different affinities for NADPH. Our routine measurements were made at 2 mM NADPH concentration, i.e. 10-fold higher than the substrate concentration used in the NADH-cytochrome *c* reductase assay. No significant increase of activity was observed at higher NADPH concentrations.

In the difference spectrum of the dithionite-reduced vs. oxidized microsomes and nuclear membranes, a major peak at 427 nm and two minor peaks at approximately 531 and 560 were detected which reflect the presence of a *b*-type cytochrome. Better resolution of the cytochrome constituents was achieved with difference spectra at 77°K (Fig. 5). The Soret band of the *b*-type cytochrome had shifted to 425 nm, and a shoulder at about 445 nm could be recognized. The β-band at about 531 nm was resolved into two peaks at 526 and 533 nm, and the α-band at 560 nm had split into two symmetrical peaks at 552 and 558 nm, respectively. In addition, a band at 601 nm was noted. This band and the shoulder at 445 nm are indicative of the presence of some cytochrome aa_3, most probably contained in the small amounts of mitochondrial contaminants demonstrated in the microsomal fraction (see above). Using an extinction coefficient of 13.1 mM^{-1} cm^{-1} for the wavelength pair 605–630 nm in the dithionite-reduced vs. oxidized difference spectrum (98) and assuming that the low temperature spectrum was intensified by a factor of 13 relative to the room temperature spectrum, we estimated a concentration of 0.018 nmol cytochrome aa_3 per mg protein in the microsomal fraction, corresponding to a mitochondrial contamination of less than 5%. In the nuclear membrane fraction the cytochrome aa_3 content was too low to be determined. It was not possible to calculate mitochondrial contaminations from measurements of cytochrome oxidase activity by the polarographic assay. Using the ascorbate-*N*,*N*,*N'*,*N'*-tetramethylphenylenediamine-cytochrome *c* system (46) as electron donor, we found relatively high apparent respiratory rates which, however, were due to the presence of some ascorbate-oxidizing activity (for details see 31, 82). NADH oxidase activity could not be demonstrated in the membrane fractions, not even in the presence of an excess of cytochrome *c*. With the spectrophotometric assay (92), very low cytochrome *c* oxidase activity was recorded in the microsomal fractions (about 25 nmol of cytochrome *c* reduced per min per mg protein) and even less was found in the nuclear membranes.

The *b*-type cytochrome which was firmly attached to the rER and nuclear membranes, even after treatment at high ionic strength, could be reduced by either NADH or NADPH, and this

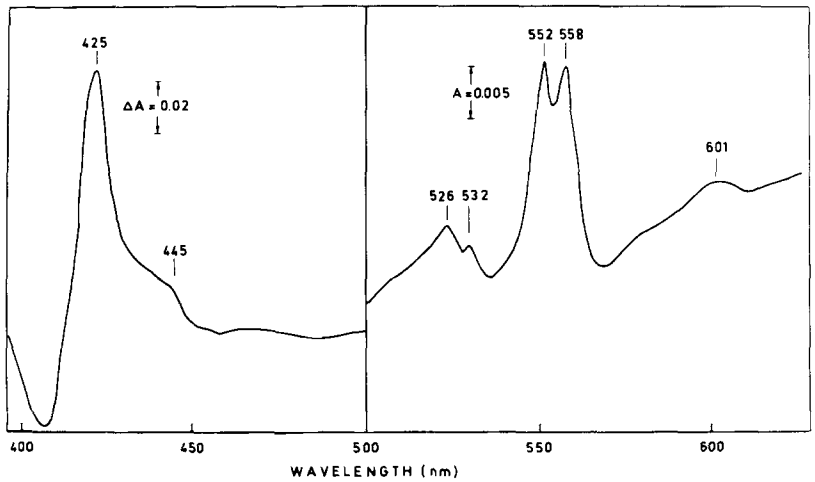

FIGURE 5 Dithionite-reduced versus oxidized difference spectrum of onion root tip microsomes at 77°K. Samples containing 3.3 mg protein per ml in 0.1 M potassium phosphate buffer, pH 7.4, and 50% glycerol were treated with a stream of oxygen for 5 min in 1-ml cuvettes with 2-mm optical path, then frozen in liquid nitrogen (see Methods), and a base line was recorded. Thereafter, both samples were thawed, the reference treated with oxygen again, and to the sample a few grains of dithionite were added and the whole sample was flushed with nitrogen for another 5 min. Then both the sample and reference were frozen again, and the difference spectrum was recorded. The curve shown has been corrected for the base line.

reduction was not inhibited by rotenone or antimycin. However, the peaks of the difference spectra recorded at room temperature using these electron donors were shifted to shorter wavelengths, compared with the dithionite-reduced spectrum (at 554, 526, and 424 nm). About 26% of the total cytochrome detected in the dithionite-reduced spectrum was reduced by 0.3 mM of the pyridine nucleotides under aerobic conditions. Ascorbate at concentrations of 2 mM was as effective a reducing agent as NADH or NADPH. The peaks at 552 and 558 nm (Fig. 3) most probably do not indicate the existence of two different *b*-type cytochromes but result from the splitting of one α-band at low temperatures (cf. 18). These properties of the onion membrane *b*-cytochrome suggest a close relationship to the microsomal cytochrome b_5 of animal cells (54; cf. 31, 75), to the cytochrome *b*-555 described in mung bean microsomes (48, 90) and to the plant b_3 cytochrome described by Martin and Morton (67–69). It differs, however, from the mitochondrial cytochrome *b*-555 of some plants (57, 95) and the "soluble" cytochromes *b*-555 and b_3 of some plants (64, 90; for a detailed discussion of the confusing literature on plant *b*-cytochromes and for further references see 31). A probably related microsomal cytochrome b_6 has also been described in castor bean endosperm (62) and in turnips (87). Using an $\varepsilon_\lambda$tinction coefficient of 160 $mM^{-1}\ cm^{-1}$ for the wavelength pair 426 to 410 nm as reported for the animal cytochrome b_5 (54), we calculated a content of 0.10 nmol per mg protein in the microsomes and about 0.025 nmol per mg protein in the nuclei. This figure approaches the values reported for microsomes and nuclear membranes from animal tissues (see the reviews quoted above).

Cytochrome P-450 could not be detected in the membrane fractions studied, either at room temperature or at 77°K. Carbon monoxide had no effect on the difference spectra of the reduced cytochromes, except for a small peak at 434 nm of cytochrome a_3 in the microsomal fraction. In the total homogenate from onion root tips, however, a small peak at 421–422 nm appeared in the carbon monoxide difference spectrum which possibly might be attributed to P-420, the denatured product of cytochrome P-450.

Concluding Remarks

The results of the present study demonstrate that nuclear membranes can be isolated and purified from plant tissue in yields sufficient for biochemical work, although much more preparative effort is required in working on a large scale than with many animal tissues. The data obtained emphasize the close biochemical similarity of the ER and the nuclear envelope, in accord with the morphological demonstrations of the continuity of these two membrane systems in most cells (see the reviews quoted), though not in all (e.g., 32). Many properties of the nuclear and rER membranes from the onion tissues studied closely resemble those found in the corresponding membranes of animal cells (e.g., references in 23, 24, 27, 49, 53, 101). This holds, for example, for the phospholipid pattern, the cytochromes present, various enzyme activities, and the tight associations with nucleic acids. There are, however, basic differences in other membrane properties, the most striking differences being the sterol patterns and the contents of oleic, linoleic, and linolenic acids in the onion endomembranes. It may be that these latter differences in lipid composition are mutually compensatory in order to establish a specific degree of membrane fluidity. In animal cells it has been suggested, on the basis of ultrastructural and biochemical criteria, that subclasses of the endomembrane-plasma membrane group can be distinguished, such as nuclear membrane and rER membrane, smooth surfaced ER, Golgi apparatus, secretory vesicles, and plasma membrane. The few analytical data published as to the composition of plant membrane fractions could be brought into superficial agreement with this concept as far as differences in lipid pattern, enzyme activities and cytochrome contents are concerned but conclusions would be premature. Definitely, much more analytical work on sufficiently pure plant membrane fractions has to be done before concepts of membrane homology and differentiation can be established for plant cells.

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