

ENDOPLASMIC RETICULUM AS THE SITE OF LECITHIN FORMATION IN CASTOR BEAN ENDOSPERM

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ABSTRACT

The properties of a discrete membranous fraction isolated on sucrose gradients from castor bean endosperm have been examined. This fraction was previously shown to be the exclusive site of phosphorylcholine-glyceride transferase. The distribution of NADPH-cytochrome c reductase and antimycin insensitive NADH-cytochrome c reductase across the gradient followed closely that of the phosphorylcholine-glyceride transferase. This fraction also had NADH diaphorase activity and contained cytochromes b_5 and P 450. On sucrose gradients containing 1 mM EDTA this fraction had a mean isopycnic density of 1.12 g/cm³ and sedimented separately from the ribosomes; electron micrographs showed that it was comprised of smooth membranes. When magnesium was included in the gradients to prevent the dissociation of membrane-bound ribosomes, the isopycnic density of the membrane fraction with its associated enzymes was increased to 1.16 g/cm³ and under these conditions the electron micrographs showed that the membranes had the typical appearance of rough endoplasmic reticulum. Together these data show that the endoplasmic reticulum is the exclusive site of lecithin formation in the castor bean endosperm and establish a central role for this cytoplasmic component in the biogenesis of cell membranes.

INTRODUCTION

Radioactive choline supplied to castor bean endosperm tissue is readily incorporated into organelle membranes in the form of lecithin (23). Three major organelle components containing [¹⁴C]lecithin were obtained when homogenates prepared from [¹⁴C]choline-treated tissue were fractionated on sucrose gradients: mitochondria, glyoxysomes, and a third heterogeneous fraction, recovered at a lower density in the sucrose gradients, designated the light membranous fraction (23). The kinetics of [¹⁴C]choline incorporation into these fractions showed that labeled lecithin is first formed in the light membranous fraction, and subsequently appears in mitochondria and glyoxysomes, indicating that lecithin formed by

components of the light membranous fraction serves as a precursor for the biogenesis of the membranes of these other organelles. In further studies, using modified sucrose gradients, the light membranous fraction was separated into two clearly defined bands of particulate material (25). The lecithin synthesizing enzyme, phosphorylcholine-glyceride transferase, was confined to one of these bands, at a density of 1.12 g/cm³, and was not detected in mitochondria and glyoxysomes.

The distinctive localization of the enzyme forming lecithin in a single separated cell fraction establishes the role of this fraction in the biosynthesis of other lecithin-containing or-

ganelle membranes. In this paper we present data which show that the cytoplasmic origin of this fraction is the endoplasmic reticulum.

MATERIALS AND METHODS

Seeds of castor bean (*Ricinus communis* var. Hale) were soaked in running tap water for 1 day and germinated in moist vermiculite in darkness at 30°C.

Homogenization

20 endosperm halves, removed from 4-day old seedlings, were homogenized by chopping for 15 min with a single razor blade in 8 ml of grinding medium contained in a Petri dish on ice. The grinding medium contained 150 mM tricine (pH 7.5), 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA (pH 7.5), and 13% (wt/wt) sucrose.

The crude homogenate was filtered through two layers of nylon cloth, and the volume of the filtered homogenate was adjusted to 10 ml with grinding medium. Cell debris was removed by centrifuging at 270 g for 10 min.

Fractionation of Cellular Components by Sucrose Density Gradient Centrifugation

5 ml of the 270 g supernatant was applied to gradients prepared as follows.

NONLINEAR GRADIENTS: The gradient consisted of (a) a 6 ml cushion of 60% (wt/wt) sucrose, (b) 20 ml of sucrose solution increasing linearly in concentration from 32 to 60% (wt/wt) sucrose, and (c) a 5 ml layer of 20% sucrose (gradient A). All sucrose solutions were prepared in 1 mM EDTA (pH 7.5).

LINEAR GRADIENTS: A gradient of 20 ml of sucrose solution increasing linearly in concentration from 16 to 60% sucrose was constructed over a 10 ml cushion of 60% sucrose (gradient B). Sucrose solutions contained 150 mM tricine (pH 7.5), 10 mM KCl, and 1 mM EDTA (pH 7.5). When appropriate, 3 mM MgCl₂ was included to prevent the dissociation of membrane-bound ribosomes (38).

Gradient C was prepared as for gradient B, except that 8 ml of 16% sucrose were placed on top of the linear gradient. All gradients were contained in 38.5-ml polyallomer tubes and centrifuged for 4 h at 20,000 rpm using a SW 27 rotor in a Beckman L2-65B ultracentrifuge at 4°C (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). After centrifugation, 0.6 ml fractions were collected using an ISCO density gradient fractionator, model 640 (Instrumentation Specialties Co., Lincoln, Nebr.).

Enzyme Assays

PHOSPHORYLCHOLINE - GLYCERIDE TRANSFERASE: This enzyme was assayed as described previously (25), except that 1,2-dipalmitin and Tween-20 were omitted from the reaction mixture.

CYTOCHROME C REDUCTASES: The reaction mixture contained, in a final volume of 1.0 ml, 20 mM potassium phosphate (pH 7.2), 0.2 mM NADH or NADPH, 0.02 mM cytochrome c, and 10 mM KCN. The reaction was started by adding enzyme, and the reduction of cytochrome c was followed at 550 nm in a Hitachi Perkin-Elmer recording spectrophotometer (Perkin-Elmer Corp., Ultek Div., Palo Alto, Calif.). Antimycin A dissolved in 10 μ l of ethanol was added to certain reaction mixtures to give a final concentration of 1 μ M. Controls with boiled enzyme or with reduced pyridine nucleotide omitted showed no activity.

NADH DIAPHORASE: The reaction mixture contained, in a final volume of 1.0 ml, 50 mM potassium phosphate (pH 7.5), 0.1 mM NADH, and 0.04 mM 2,6-dichlorophenolindophenol (DCPIP). The reaction was started by the addition of enzyme, and the reduction of DCPIP was followed at 600 nm.

GLUCOSE-6-PHOSPHATASE: This enzyme was assayed as described by Castelfranco et al. (3) using an incubation time of 60 min at 30°C. Enzymically released inorganic phosphate was determined by the Fiske and Subbarow method (13) as modified by Anderson and Tolbert (1).

DIFFERENCE SPECTRA: Difference spectra were determined on particulate material banding on nonlinear sucrose density gradients at 1.12 g/cm³; a Cary model 14 spectrophotometer was used (Varian Associates, Instrument Div., Palo Alto, Calif.). The suspended particles were added to each of two 1 ml cuvettes (1 cm light path) and a wavelength scan was made to give a base line. Cytochrome b₅ was demonstrated by the difference spectrum between the oxidized reference and a sample which had been reduced by the addition of a few crystals of sodium dithionite, 0.2 mM NADH, or 0.2 mM NADPH.

The presence of cytochrome P 450 was demonstrated by recording spectral changes resulting from carbon monoxide binding to the dithionite-reduced pigment (33). Carbon monoxide was bubbled for 30 s through the sample cuvette, and the spectrum was recorded against a dithionite-reduced reference cuvette.

Electron Microscopy

After sucrose density gradient centrifugation, fractions containing particulate components to be

examined were pooled and mixed with an equal volume of 5% glutaraldehyde in 0.1 M potassium phosphate (pH 7.2) containing sucrose at a concentration equal to that in the pooled sample.

After standing overnight at 4°C, preparations were diluted with one-third their volume of 0.05 M potassium phosphate (pH 7.2) and centrifuged at 150,000 *g* for 1 h.

Pellets were washed with 0.05 M potassium phosphate (pH 7.2) containing the appropriate sucrose concentration and then treated for 4 h with 2% OsO₄ in the same buffer-sucrose solution, washed, and treated overnight with 2% OsO₄ in 0.05 M potassium phosphate (pH 7.2). Samples were finally washed, dehydrated in ethanol series, and embedded in Spurr's low viscosity medium (39). Silver sections were cut, poststained in uranyl magnesium acetate (15) and lead citrate (41), and examined on a RCA-EMU 34 electron microscope.

Other Methods

The distribution of RNA was determined by the orcinol method (4). Purified yeast RNA (Sigma Chemical Co., St. Louis, Mo.) was used as a standard. Aliquots of 0.4 ml from each gradient fraction were removed and added to 5 ml of 5% (wt/vol) trichloroacetic acid. The precipitated material was collected by centrifugation, washed several times to insure the complete removal of sucrose, and assayed for RNA. Sucrose concentrations were determined refractometrically.

RESULTS

Distribution of Enzymes After Nonlinear Sucrose Density Gradient Centrifugation

PHOSPHORYLCHOLINE-GLYCERIDE TRANSFERASE: As reported previously (25), this enzyme was exclusively localized in a narrow band of particulate material recovered at a sucrose density of 1.12 g/cm³ (Fig. 1 *a*).

CYTOCHROME C REDUCTASES: Both the NADH- and NADPH-cytochrome *c* reductase activity showed a single distinctive peak in the 1.12 g/cm³ fraction, coinciding exactly with the distribution of phosphorylcholine-glyceride transferase activity in the gradient (Figs. 1 *b, c*). Appreciable NADPH-cytochrome *c* reductase was also present in the soluble fraction at the top of the gradient (Fig. 1 *b*), and some NADH-cytochrome *c* reductase was also present in the mitochondria (Fig. 1 *c*). The mitochondrial NADH-cytochrome *c* reductase activity was severely inhibited by 1 μM antimycin A (94% inhibition), while both

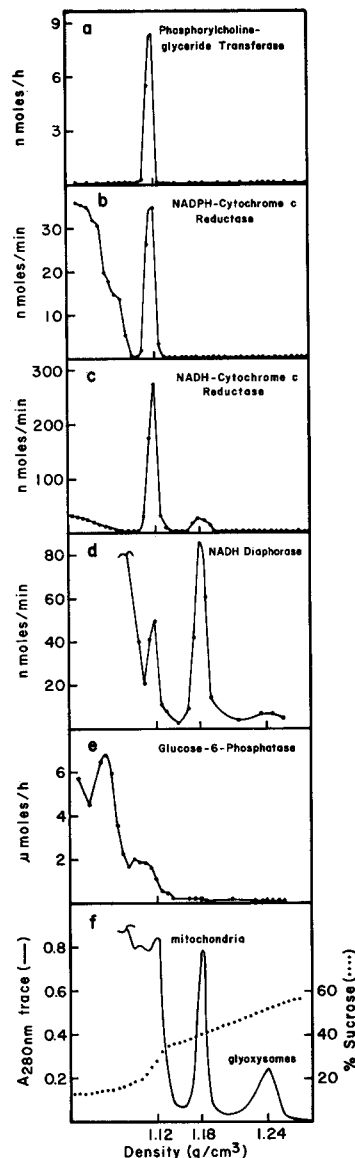


FIGURE 1 The distribution of (*a*) phosphorylcholine-glyceride transferase, (*b*) NADPH-cytochrome *c* reductase, (*c*) NADH-cytochrome *c* reductase, (*d*) NADH diaphorase, (*e*) glucose-6-phosphatase, and (*f*) protein ($A_{280\text{nm}}$) when 5 ml of extract was centrifuged on gradient A. Mitochondria and glyoxysomes were located by marker enzymes, fumarase and isocitrate lyase, as described previously (23, 25).

the NADH- and NADPH-cytochrome *c* reductases of the 1.12 g/cm³ band were unaffected by this concentration of the antibiotic.

NADH DIAPHORASE: This enzyme was found

to be largely soluble, with 85% of the total activity remaining at the top of the gradient (Fig. 1 *d*). In particulate fractions, activity was present in the 1.12 g/cm³ band and, to a greater extent, the mitochondria (Fig. 1 *d*). Low levels of activity were also detected in the glyoxysomes, probably representing contamination of this organelle fraction (24).

GLUCOSE-6-PHOSPHATASE: This enzyme, which in some animal tissues is associated with the endoplasmic reticulum (34), did not appear to be associated specifically with any separated particulate component (Fig. 1 *e*).

Cytochromes b₅ and P 450

The oxidized vs. dithionite-reduced spectrum obtained with the 1.12 g/cm³ density fraction, recovered from nonlinear sucrose gradients, is shown in Fig. 2 *a*. The peaks at 555, 427, and 410 nm are typical of cytochrome b₅ (17). Reduction of the cytochrome b₅ component also occurred upon addition of NADH and, to a lesser extent, NADPH (Figs. 2 *b*, *c*). The dithionite-reduced spectrum had a shoulder at 450 nm which was not observed in the pyridine nucleotide-reduced spectra. This 450 nm peak, emphasized in the dithionite-reduced minus NADH-reduced difference spectrum (Fig. 2 *d*), suggests that cytochrome P 450 is present in the preparation. This was confirmed by the response of dithionite-reduced preparations to carbon monoxide. A typical difference spectrum, with a clear peak at 450 nm, was obtained (Fig. 2 *e*).

Effect of Mg⁺⁺ on Sedimentation Behavior

The location of marker enzymes and distinctive cytochromes in the band recovered at 1.12 g/cm³ provided evidence that the membranes in this fraction were derived from endoplasmic reticulum and thus that this cellular component is the site of lecithin synthesis *in vivo*. Electron micrographs of cells of castor bean endosperm have shown that the endoplasmic reticulum is richly endowed with ribosomes and is predominantly "rough" in character (29, 42). Studies on cell-free preparations from rat liver have shown that the controlled use of EDTA results in dissociation of the ribosomes from the membranes and further that the attachment can be maintained by appropriate concentrations of Mg⁺⁺ (38).

The following considerations show that under

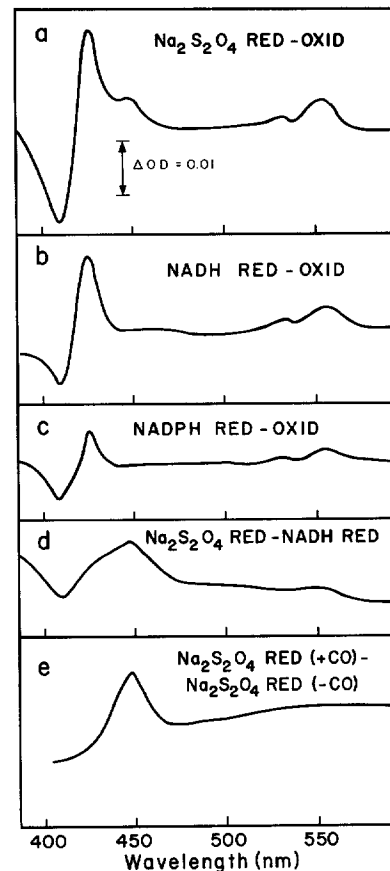


FIGURE 2. Difference spectra obtained with material banding at density 1.12 g/cm³. The presence of cytochrome b₅ is revealed after reduction (RED) with dithionite (*a*), NADH (*b*), and NADPH (*c*). Components reduced by dithionite but not by NADH include cytochrome P 450, (*d*). The typical P 450 spectrum in the presence of CO is shown in (*e*). OXID, oxidation.

the conditions of separation described above, i.e., on gradients containing EDTA, the membranes of the endoplasmic reticulum are separated from ribosomes:

(*a*) Assay for RNA over the gradient showed that the major peak was at a slightly lower density than that of the marker enzymes, with a minor peak in the mitochondrial region (Fig. 3 *c*). On a slightly modified gradient in which 8 ml of 16% sucrose was interposed between the 16–60% sucrose and the sample (gradient C) a clear resolution of the ribosome and membrane peaks was obtained, as shown by the distribution of RNA and NADH-cytochrome c reductase (Fig. 4).

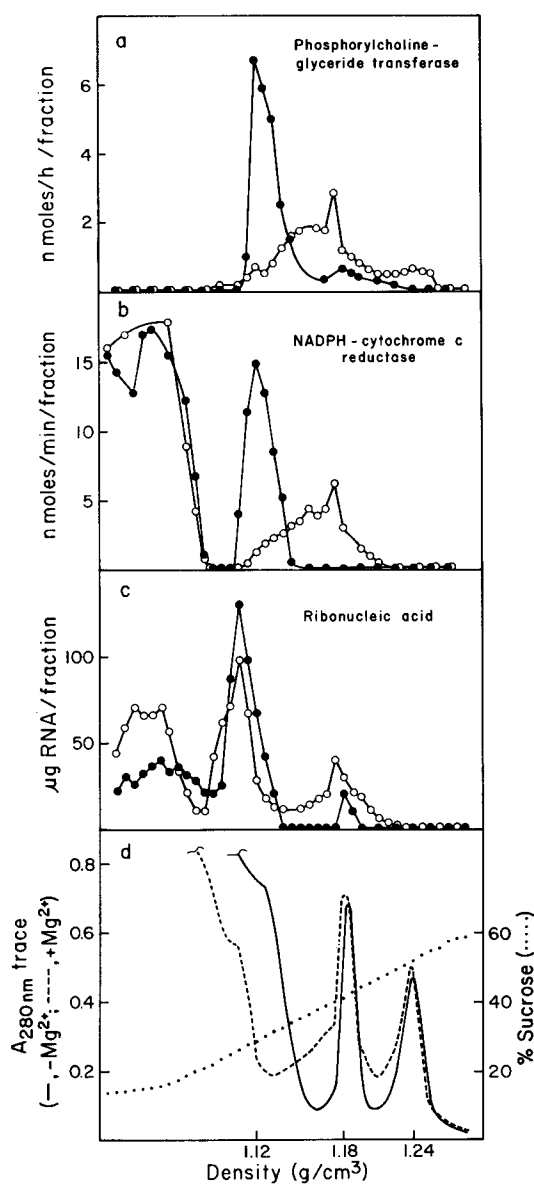


FIGURE 3 The effect of inclusion of magnesium chloride (3 mM) in the sucrose gradient on the sedimentation behavior of (a) phosphorylcholine-glyceride transferase, (b) NADPH-cytochrome c reductase, (c) RNA, and (d) protein (A_{280} nm). (—●—) centrifugation on gradient B without magnesium chloride, (—○—) centrifugation on gradient B with magnesium chloride. In (d) the continuous line shows the protein distribution in the absence of magnesium chloride and the dashed line the distribution in the presence of magnesium chloride. The dotted line shows sucrose concentration.

The RNA peak presumably includes ribosomes originally free in the cytoplasm and those dissociated from the rough endoplasmic reticulum.

(b) Electron micrographs of material at density 1.12 g/cm³ from standard gradients (Fig. 5 a) showed typical segments of endoplasmic reticulum with smooth surfaces, i.e., without attached ribosomes.

On the other hand, when Mg²⁺ was included in the gradient, the sedimentation behavior of the membranes and RNA was drastically modified and in a way showing that ribosome attachment has been maintained.

As shown in Fig. 3, there was no effect of Mg²⁺ on the equilibrium position of the protein peaks representing mitochondria and glyoxysomes, but that corresponding to the membranes of the endoplasmic reticulum at density 1.12 was largely lost and was spread broadly around density 1.16 g/cm³. Correspondingly, the peaks of phosphorylcholine-glyceride transferase (Fig. 3 a) and that of NADPH-cytochrome c reductase (Fig. 3 b) had moved to the same region of the gradient.

Fig. 3 c shows the effect of Mg²⁺ on the RNA distribution. In its absence most of the RNA (ribosomal) was found in a sharp peak at density 1.11 g/cm³ with a small peak in the mitochondrial region. In the presence of Mg²⁺ a greater portion of the RNA moved lower into the gradient and was spread broadly around density 1.16 g/cm³, the region occupied by the enzymes in Figs. 3 a and 3 b. We presume that the increased RNA recovered in this region in the presence of Mg²⁺ is in ribosomes associated with the membranes bearing the two enzymes (see below). The bulk of the RNA which was not affected by the inclusion of Mg²⁺ presumably represents free ribosomes; that portion recovered higher in the gradient in the presence of Mg²⁺ was not characterized.

The relatively broad spread of each of the components on gradients containing Mg²⁺ has been observed previously (43) and is attributed to the range of equilibrium densities resulting from differences in the proportion of ribosomes to membranes in the separated segments of endoplasmic reticulum.

When material at density 1.16 g/cm³ from the gradient including Mg²⁺ was examined under the electron microscope the structures shown in Fig. 5 b were observed. This fraction shows

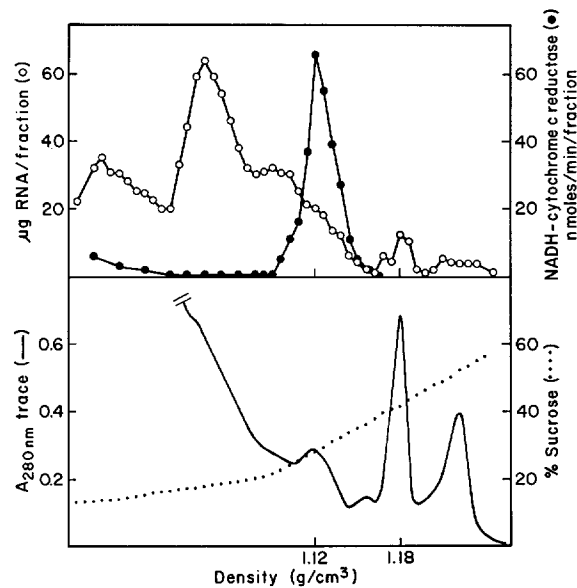


FIGURE 4 The distribution of RNA (—○—○—), NADH-cytochrome c reductase (—●—●—), and protein ($A_{280\text{ nm}}$) (—), when 5 ml extract was centrifuged on gradient C.

clearly the rough-surfaced membranes typical of rough endoplasmic reticulum. The elongated vesicular nature of the membranes and dense association of ribosomes is quite similar to that seen in the electron micrographs prepared from intact endosperm tissue (29, 42).

DISCUSSION

By all the criteria examined in the present work, the particulate fraction isolated from castor bean endosperm cells which contains phosphorylcholine-glyceride transferase (25) is derived from the endoplasmic reticulum. This conclusion is based on the presence in this fraction of NADH- and NADPH-cytochrome c reductase (7, 11, 34), NADH diaphorase (9, 11), cytochromes b_5 (6, 17, 40) and P 450 (10, 33), the effect of Mg^{++} on the centrifugal behavior of this fraction, and the electron microscope evidence (11, 43).

The membranes bearing the phosphorylcholine-glyceride transferase are free from ribosomes and are isolated as a sharp, discrete peak in the standard gradients (25). The fact that inclusion of Mg^{++} in the gradients yielded enzymatically active fractions of higher density comprised of rough endoplasmic reticulum, and the prevalence of similar structures in sections of living cells (29, 42) strongly suggest that this is the location of the enzyme *in vivo*.

Membranes of mitochondria and glyoxysomes from castor bean endosperm contain lecithin as a major structural phospholipid (23, 25). Insofar as the pathway of synthesis from choline is the source of lecithin, and since the organelles themselves do not contain the phosphorylcholine-glyceride transferase, the endoplasmic reticulum must have a functional role in the biogenesis of these organelles. Such a role is consistent with kinetic data obtained when $[^{14}C]$ choline was supplied to castor bean endosperm (23). Labeled lecithin was initially formed in a membranous fraction which included the endoplasmic reticulum-derived material, and subsequently appeared in the membranes of mitochondria and glyoxysomes (23).

Extensive studies on rat liver by Dawson and co-workers (21, 22, 28) have provided biochemical evidence for the synthesis of mitochondrial lecithin on the endoplasmic reticulum, and a recent suggestion has been made whereby the lecithin might be transferred (45). Electron micrographs have shown direct continuities between the endoplasmic reticulum and the microbody membrane in animal and plant tissues (see reviews in 18, 19, 42). These observations have prompted the suggestion that microbodies are formed directly from the endoplasmic reticulum by vesiculation (19). Continuities between the endoplasmic

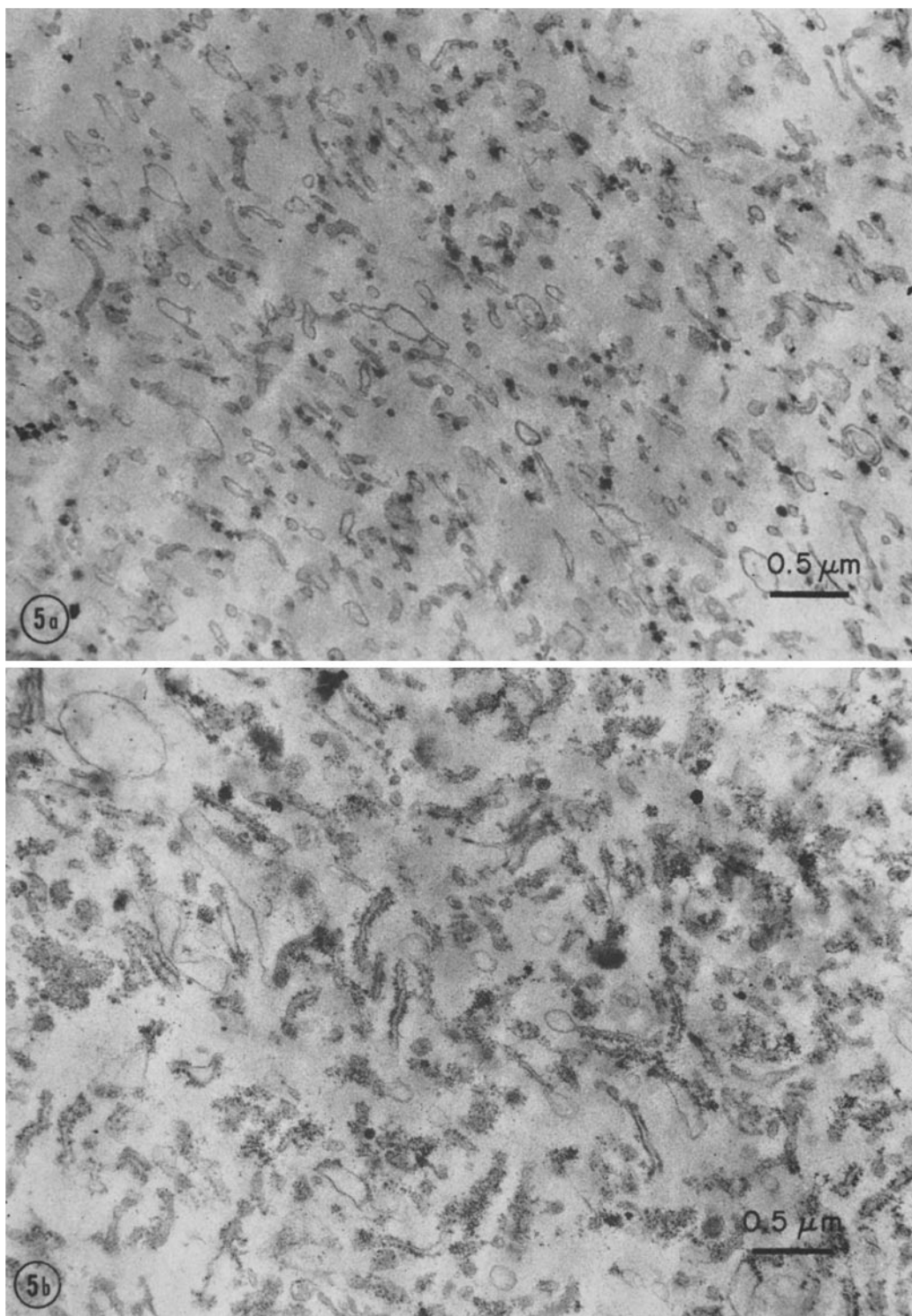


FIGURE 5 Electron micrographs of material (a) from the fraction recovered at density 1.12 g/cm^3 in standard gradients without magnesium, as in Fig. 1 ($\times 23,000$) and (b) from the fraction at density 1.16 g/cm^3 in gradients including magnesium chloride as in dashed lines in Fig. 3 ($\times 24,000$).

reticulum and the outer mitochondrial membrane have also been reported (2, 14, 30, 36).

The present demonstration that phosphorylcholine-glyceride transferase is specifically associated with the membranes of the endoplasmic reticulum refines and confirms earlier conclusions based on the fractionation of cell extracts by differential centrifugation (25). Although those fractionation techniques do not achieve a clean separation of organelle fractions, phosphorylcholine-glyceride transferase was predominantly recovered in microsomal pellets in studies involving both animal (28, 44) and plant (8, 20, 31) tissues. Such crude microsomal pellets from plants were previously shown to contain NADH- and NADPH-cytochrome c reductases and NADH diaphorase (16, 26, 27, 35) as well as cytochromes b_5 and P 450 (5, 16, 32, 37). The present work shows that all of these components are specifically associated with the membranes of the endoplasmic reticulum from castor bean endosperm. Little is known about the function of these constituents in plants; their association in a fraction which can readily be isolated offers a useful system for investigation of their possible roles (12) in plant metabolism.

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