Effect of Various Hepatic Membrane Fractions on Microtubule Assembly—with Special Emphasis on the Role of Membrane Phospholipids

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ABSTRACT This report describes an interaction between rat brain microtubule protein and various hepatic fractions in vitro. Purified preparations of Golgi membranes, plasma membranes, rough and smooth endoplasmic reticulum, nuclear membranes, and mitochondria were obtained from the livers of 200-g rats. Several concentrations of fresh or sonicated frozen membranes were incubated with twice-cycled rat brain microtubule protein in a microtubule assembly buffer for 60 min at 30°C. Changes in microtubule assembly were assessed either by quantitative electron microscopy on negatively stained samples or by spectrophotometric methods. The results show that all the tested membranes "bound" microtubule protein, preventing assembly: Golgi and plasma membranes, as well as mitochondria, were especially potent in this regard. To identify the membrane-associated components responsible for microtubule protein binding, the membranes were extracted with methanol-chloroform, and liposomes were prepared from the resulting lipids. Microtubule protein incubated with these liposomes showed a differential ability to assemble that was similar to the effect obtained with intact membranes. Membrane-extracted phospholipids were identified as the lipid component responsible for these changes, with the negatively charged phospholipids (cardiolipin and phosphatidylserine) being uniquely active. These findings indicate that hepatic membranes differentially interact with brain microtubule protein; this interaction may be dependent on membrane phospholipids.

In a previous in vivo study (32), we noted a unique association between microtubules and Golgi complexes in rat hepatocytes following colchicine treatment. This led to the suggestion that microtubules may normally aid in maintaining the structural integrity of the cisternal membranes in Golgi complexes and, in so doing, play a crucial, if permissive, role in hepatic lipoprotein secretion. In the present study, we wish to see whether isolated Golgi complexes show any special interactions with microtubule protein in vitro. Accordingly, Golgi complexes and various other hepatic membranes were isolated and incubated with twice-cycled brain microtubule protein. The results show that all liver membranes "bind" microtubule protein, but that some membranes, such as those derived from the Golgi complex and cell surface, are particularly effective in this regard. Subsequent incubation with the lipids of the various membrane preparations show an effect similar to that obtained with the intact membranes themselves and suggests that membrane phospholipids may play an important role in microtubule protein-membrane interactions.

MATERIALS AND METHODS

Incubation of Microtubule Protein with Liver Membranes

Various concentrations of fresh, intact, or frozen-sonicated hepatic membranes were preincubated with twice-cycled rat brain microtubule protein (120 μg per incubation vial or 460 μg/ml) for 30 min at 30°C, pH 6.4, in the following buffer: 100 mM 2-[N-morpholino]ethane sulfonic acid [MES]; 0.5 mM $MgCl_2$; and 1 mM EGTA. No microtubules were assembled under these conditions. Following the preincubation period, GTP (final concentration, 0.5 mM) and glycerol (final concentration, 2 M) were added and the incubation was allowed to proceed for 60 min at 30°C. When intact organelles, such as mitochondria and nuclei, were used for incubation with microtubule protein, buffer solutions for control and experimental samples were made isotonic with additional sucrose. This enrichment with sucrose did not affect the membrane-induced changes in microtubule

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assembly. In most cases, the incubated samples were negatively stained and microtubule assembly was quantified by electron microscopy. To validate this method for quantitation of microtubule assembly, certain samples were also assayed by spectrophotometric methods.

PREPARATION OF RAT BRAIN MICROTUBULE PROTEIN: Microtubule protein was prepared by the technique of Borisy et al. (8), as modified by Asnes and Wilson (4). In brief, brains removed from 140–160-g rats were homogenized with two to three strokes in glutamate-phosphate buffer (20 mM PO_4 + 100 mM sodium glutamate, pH 6.75) in a motor-driven, glass-teflon homogenizer, and centrifuged at 33,000 *g* for 40 min at 4°C. Assembly of the supernatant protein took place in phosphate-glutamate buffer (containing 2.5 mM GTP + 0.5 mM $MgCl_2$ + 1 mM EGTA) at 37°C. A second cycle was carried out as described above, after which the assembled protein was aliquoted and frozen. Before use, the microtubule protein was thawed, depolymerized at 4°C, centrifuged at 33,000 *g* for 40 min, and used in the disassembled state. The protein concentration of each aliquot was measured by the technique of Lowry et al. (22), as modified by Markwell et al. (23). Polyacrylamide gel electrophoresis indicated the presence of high molecular weight proteins ($\sim M_r$ 300,000) and tubulin ($\sim M_r$ 55,000) as well as small, variable amounts of an intermediate molecular weight protein ($\sim M_r$ 70,000) in the cycled protein.

PREPARATION OF RAT LIVER MEMBRANES: All membranes were isolated from young rats (200–250 g) obtained from Simonsen Laboratories (Gilroy, Calif.). Intact Golgi membranes were obtained by the method of Morré (24). In addition, light (GF_1) and medium (GF_2) Golgi membrane fractions were isolated from animals (without alcohol priming) as described by Ehrenreich et al. (15) and Bergeron et al. (7). Rough- (RER) and smooth-surfaced endoplasmic membranes (SER) were obtained from heavy fractions of the same preparations from which the different Golgi preparations had been derived (7). Plasma membranes were prepared as described by Neville (25), and modified by Ray (31). Intact nuclei and nuclear membranes were isolated by the procedure of Berezney et al. (5), or sonicated to include nuclear organelles. Intact mitochondria were isolated and purified according to the procedure of Bustamente et al. (10). Isolated membranes were stored overnight in 0.25 M sucrose either at 4°C or frozen.

The purity of the various isolated fractions was determined by electron microscopy (see Materials and Methods) and by enrichment of key enzymes in specific samples. GF_1 and GF_2 Golgi fractions were examined for galactosyltransferases by the method of Bretz and Staubli (9), using fetuin free from N-acetylneuramic acid and galactose (DSG-fetuin) and mucin free from N-acetylneuramic acid (DS-mucin) as acceptor proteins: galactosyltransferase was expressed as nanomoles of galactose incorporated per hour per milligram protein. Plasma membrane fractions were assayed for 5′ nucleotidase (3); activity was expressed as micromole Pi released per minute per milligram protein. Mitochondrial fractions were examined for succinic-2 (p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltrazolium-reductase activity (29) which was expressed as micromoles 2 (p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltrazolium-reduced per minute per milligram protein. The purity of the nuclei could be determined at the light microscope level and no specific enzyme activities were measured. SER and RER were assayed for glucose-6-phosphatase (3) activities expressed as micromole Pi released per minute per milligram protein as well as by NADPH-ferricyanide reductase activity (35) expressed as micromoles ferricyanide reduced per minute per milligram.

ELECTRON MICROSCOPIC PROCEDURES: Immediately following the various incubations, the microtubule protein-membrane suspensions were diluted 1:2 with assembly buffer, and representative drops of the sample were placed on Formvar- and carbon-coated 400-mesh grids. The grids were subsequently covered with a layer of 0.02% cytochrome *c* and stained with 0.5% uranyl acetate. Random pictures were obtained from each sample by photographing the centers of five predetermined grid openings at × 12,000. Microtubule assembly was quantified by determining the total microtubule length per area photographed (21).

To judge the purity of the various membrane preparations, 0.17-ml samples of each preparation were fixed for 10 min in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.0, mOS 420) and spun for 2 min in an airfuge centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 100,000 *g*. The pelleted material formed a thin shell on the sides and bottom of the centrifuge tubes, and the orientation of these membrane shells was maintained throughout the processing procedures. As a consequence, all the layers formed during the pelleting of a sample could be viewed in a single section.

TURBIDIMETRIC METHOD: To validate the electron microscopic assay for microtubule assembly, hepatic membranes were incubated with microtubule protein as before, and microtubule assembly was monitored by changes in turbidity (19). Specifically, Golgi or SER membranes (1.8 mg protein/ml) were mixed with microtubule protein (1.8 mg/ml) in assembly buffer (100 mM MES; 0.5 mM GTP; 2 M glycerol, 0.5 mM $MgCl_2$, 1 mM EGTA, and absorbtion at 350 nm was measured in 4-min intervals at 30°C (Gilford Spectrophotometer, model 250; Gilford Instrument Laboratories Inc., Oberlin, Ohio). Because the membrane preparations tended to settle during the 40-min experimental period, it was necessary to mix the samples gently just before each measurement.

MISCELLANEOUS TECHNIQUES: (*a*) Colchicine binding. On several occasions, incubated samples were spun (100,000 *g* for 1 h); and the supernatant fluid was checked for colchicine binding activity using the method of Ostlund et al. (28).

(*b*) Binding experiments. To check for specific binding between microtubule protein and membranes, standard binding competition experiments were carried out with iodinated (20) microtubule protein (19 μCi/μg protein sp act). Membrane fractions (120 μg protein) were incubated with increasing concentrations of ^{125}I-microtubule protein (4.5, 9, 18, 45, and 90 μg) in a final volume of 0.27 ml containing 20 mM sodium phosphate–100 mM sodium glutamate buffer, pH 6.75. After incubation for 1 h at 30°C, the bound tubulin was separated from free tubulin by centrifugation at 50,000 *g* for 30 min in the cold (4°C). The pellet in each case was washed once in buffer and counted for radioactivity in a gamma spectrometer. The amount of ^{125}I-microtubule protein that specifically bound to membranes was computed from the difference between total binding and that observed in the presence of excess unlabeled microtubule protein (2.5 mg).

Incubation of Microtubule Protein with Membrane Lipids

LIPOSOME FROM MEMBRANE-EXTRACTED LIPIDS: Total lipids were extracted from two preparations each of Golgi membranes (24), plasma membranes, nuclei, RER, SER, and mitochondria, using chloroform/methanol according to the method of Folch et al. (18), as modified by Radin (30). The dried lipids were reconstituted in buffer and sonicated to form liposomes. Incubations with liposomes and microtubule protein were carried out as described for the intact membranes, with the phospholipid content of the liposomes determining the amount of liposomes added per incubation tube.

LIPOSOMES OF MEMBRANE-EXTRACTED PHOSPHOLIPIDS: Phospholipids were separated from the neutral membrane lipids of the GF_1 and GF_2 fractions of Golgi membranes, using activated silicic acid (Unisil; Clarkson Chemical Co., Williamsport, Pa.) column chromatography, as described by Dittmer and Wells (14). The various dried lipid fractions were reconstituted with buffer, sonicated, and incubated with microtubule protein as described for intact membranes.

ANALYSIS OF MEMBRANE-EXTRACTED PHOSPHOLIPIDS: Extracted lipids from each membrane preparation were applied as a single spot (~20 μg phosphorus) to thin-layer chromatography plates and developed in the first dimension with chloroform/methanol/28% aqueous ammonia (65:25:5 vol/vol) and in the second dimension with chloroform/acetone/methanol/acetic acid/water (3:4:1:1:0.5 vol/vol). Identification of the phospholipids was made possible with authentic reference lipids (Sigma Chemical Co., St. Louis, Mo.) or group-specific reagents. Quantitative estimation of individual phospholipids was done by exposing the thin-layer plates to iodine vapor; then the stained areas were marked, the iodine was allowed to evaporate, and the appropriate areas were removed. The phospholipids were eluted from the silica gel using chloroform/methanol (2:1; 10 ml), chloroform/methanol/acetic acid/water (25:15:4:2; 5 ml), methanol (5 ml), and finally chloroform/methanol (2:1; 5 ml). The eluates were evaporated to dryness under nitrogen at 40°C. Phosphate content was measured according to the method of Ames (2), and multiplied by 25 to determine phospholipid levels.

LIPOSOMES FROM COMMERCIALLY OBTAINED PHOSPHOLIPIDS: Phosphatidylcholine (lecithin), phosphatidylserine, phosphatidylethanolamine, and diphosphatidyl glycerol (cardiolipin) from nonliver sources were obtained commercially (Sigma Chemical Co.). Liposomes were prepared either from the pure phospholipids or from specific combinations of the different phospholipids. The liposomes were incubated with microtubule protein, and microtubule assembly was quantified electron microscopically or spectrophotometrically, as described above.

RESULTS

Microtubule Assembly after Incubation of Brain Microtubule Protein with Intact Liver Membranes

PURITY OF RAT LIVER MEMBRANES: Pelleted membranes prepared in the airfuge centrifuge permitted all stratified layers of a pellet to be examined in the same section. Golgi pellets contained profiles of sacs, vesicles, and tubules characteristic of this fraction (Fig. 1*A*, [6]). The plasma membrane

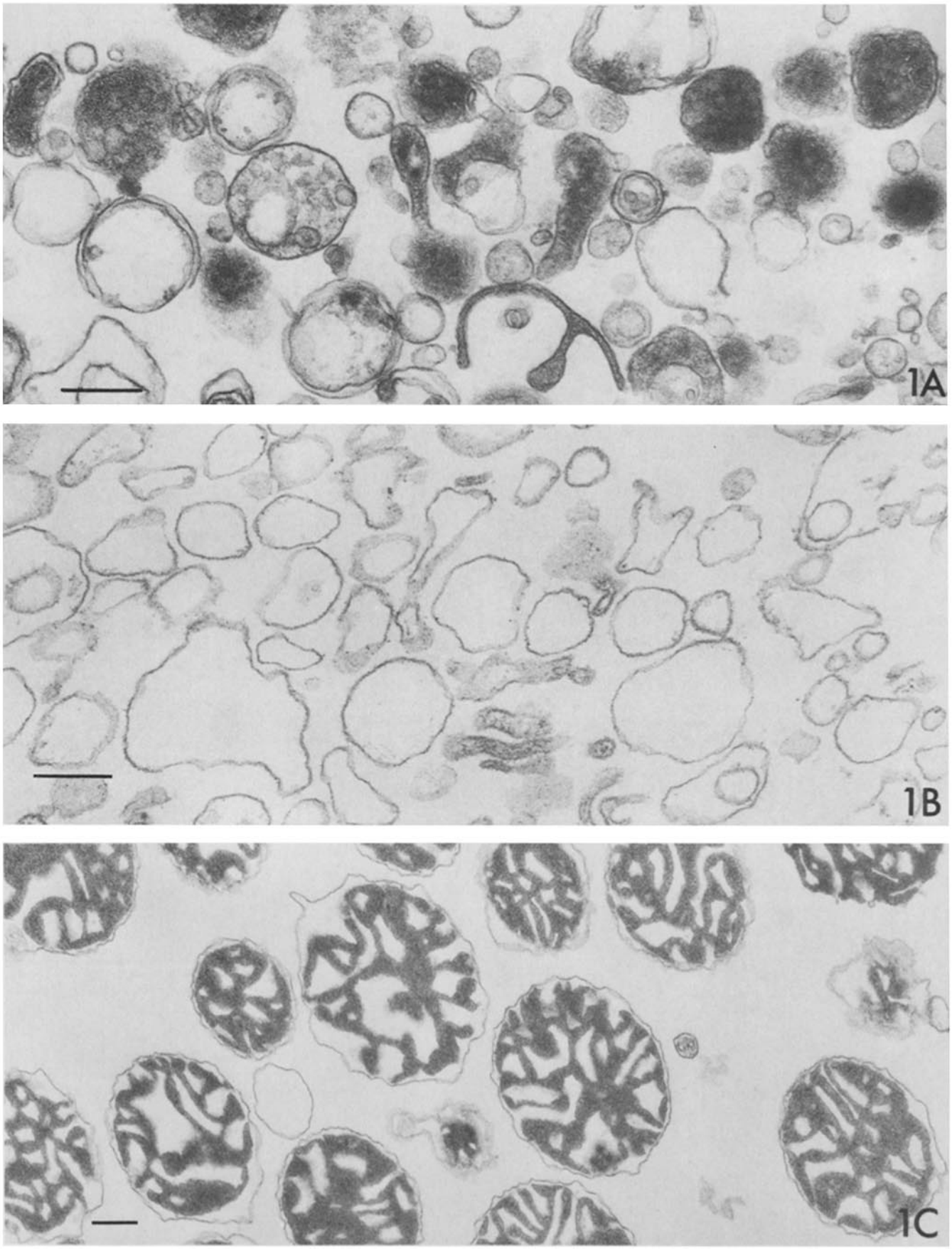
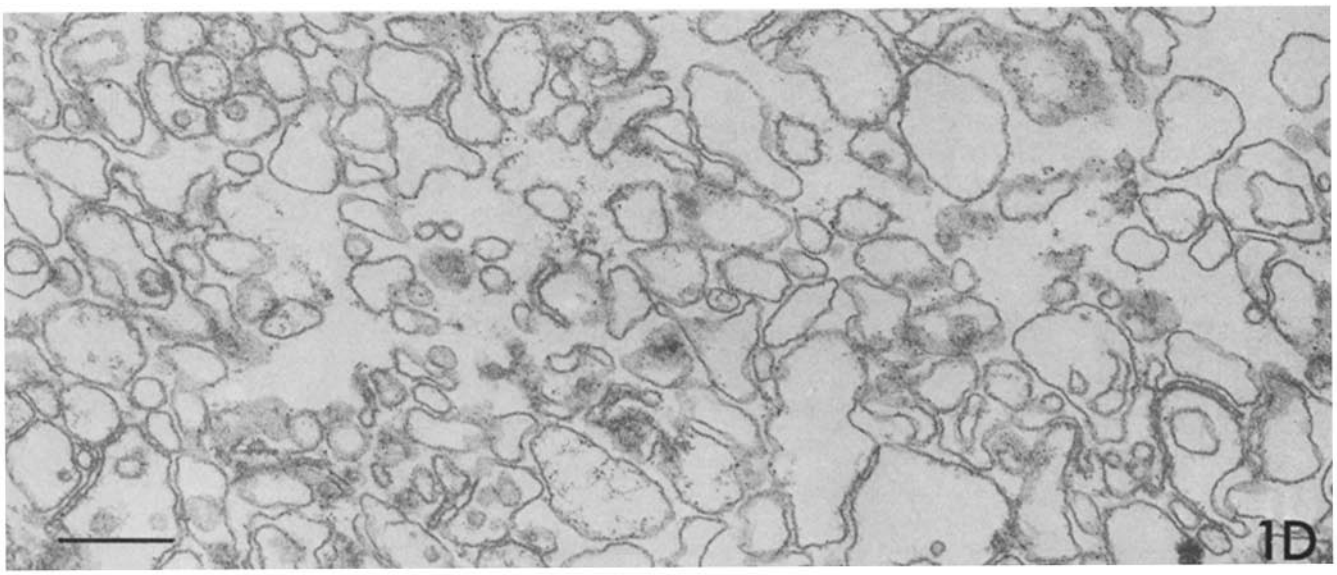


FIGURE 1 Representative micrographs of isolated rat liver fractions used in the various incubations of this study. Bars, 0.25 μm. (1A) Mixed GF_1 and GF_2 fractions of Golgi samples. × 60,000. (1B) Plasma membrane fraction. × 60,000. (1C) Intact mitochondria. × 35,000. (1D) Nuclear membranes. × 60,000. (1E) Smooth endoplasmic reticulum. × 60,000. (1F) Rough endoplasmic reticulum. × 60,000.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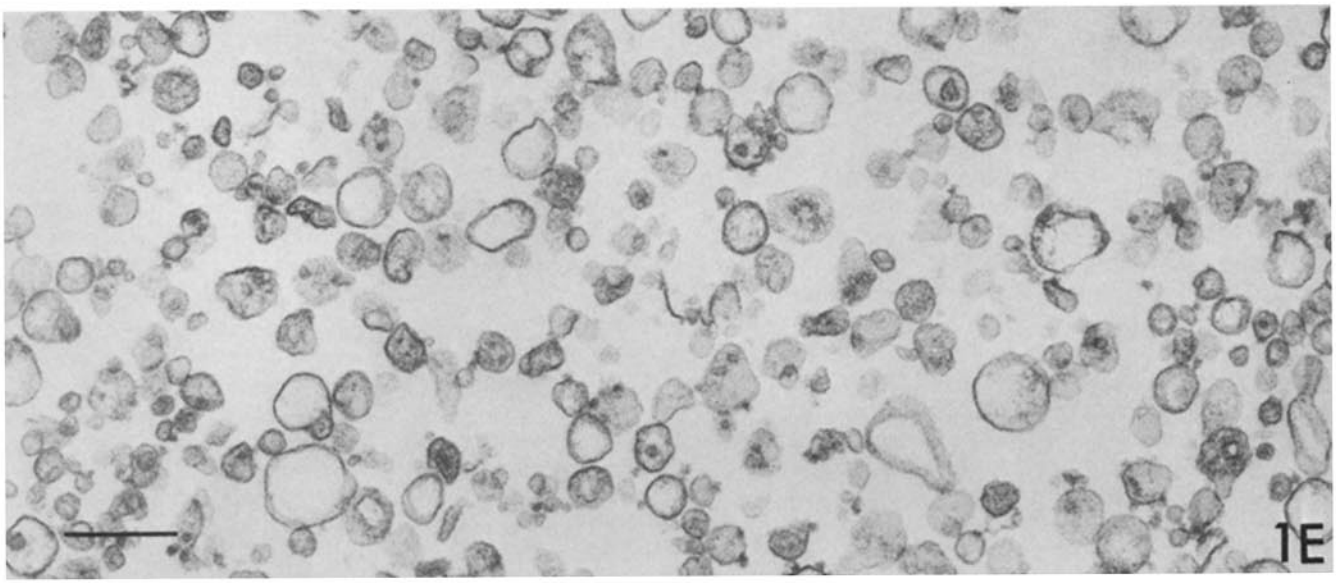
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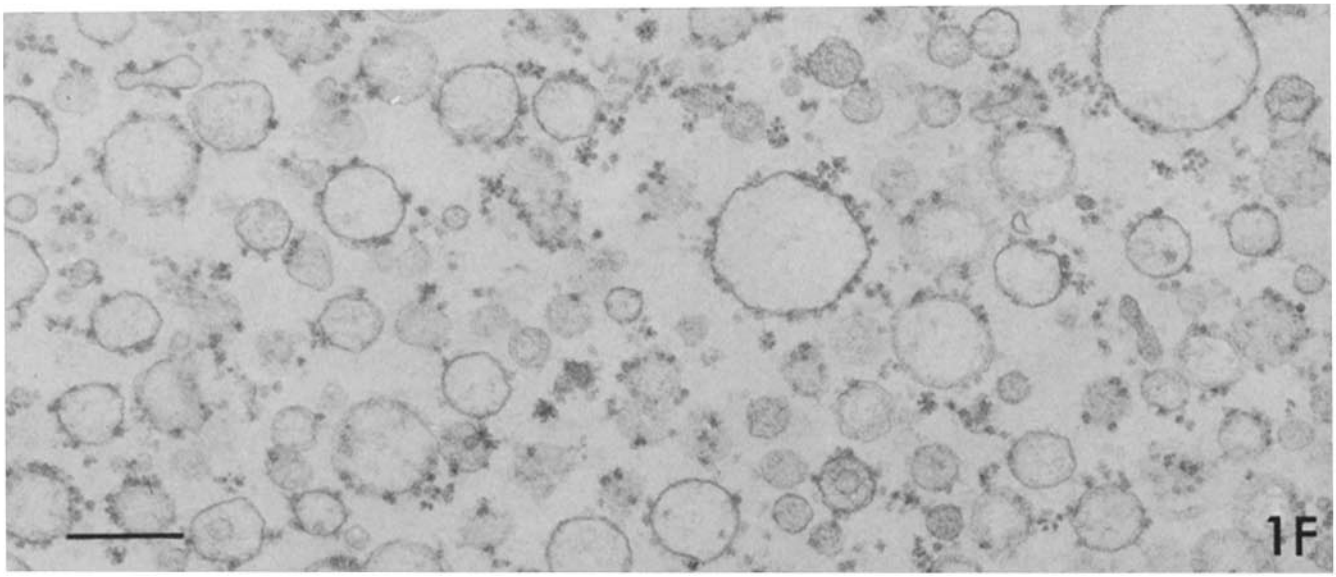
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preparation contained large and small, flattened, empty vesicles, and occasional clusters of ribosome-studded vesicles (Fig. 1 *B*). Preparations of isolated mitochondria were homogeneous, containing only a small fraction (<10%) of peroxisomes, lysosomes, or broken or damaged organelles (Fig. 1 *C*). Pellets of nuclear membranes contained primarily profiles of large, flattened vesicles with some contaminating (<5%) chromatin material (Fig. 1 *D*). The SER pellet contained basically small (1,000 Å Diam) round vesicles with smooth surfaces: a small proportion (~10%) of rough-surfaced vesicles were also present (Fig. 1 *E*). The RER was exceptionally homogeneous, containing only ribosome associated vesicles (Fig. 1 *F*).

Enrichment of specific enzymes in the various fractions is shown in Table I and indicates that the purity of the hepatic subcellular fractions of this study is comparable to that reported by others (3, 6, 9, 17, 26).

EFFECT OF LIVER MEMBRANES ON MICROTUBULE ASSEMBLY: Fig. 2 is representative of the microtubule assembly obtained when microtubule protein (120 μg/incubation vial) is assembled under the conditions described in Materials and Methods. (For quantitation, such preparations are diluted 1:2 with assembly buffer before the grids are prepared).

Fig. 3 compares the effects of various concentrations of different hepatic membrane preparations on microtubule assembly. The bar on the far left shows the extent of microtubule assembly when no membrane protein is added to the incubation medium. As can be seen, Golgi and plasma membranes, as well as mitochondria, totally interfere with microtubule assembly in all membrane protein concentrations assayed. On the other hand, SER and RER membranes (even when obtained from the same preparations that provided Golgi membranes) do not interfere with microtubule assembly when 60 μg of membrane protein is incubated with 120 μg of microtubule protein; however, as the membrane/microtubule protein ratio increases, these membranes show significant inhibitory effects on microtubule assembly. On the other hand, nuclear membranes appear to have a minor effect on reducing microtubule assembly at all membrane concentrations tested.

TABLE I

Characterization of Membrane Fractions

Fraction	Enzyme	Activity*		Relative specific activity‡
		Homogenate	Purified fraction	
Golgi	Galactosyltransferase (DSG-fetuin)	7.7	365	47.4
	Galactosyltransferase (DS-mucin)	5.0	591	118.0
Plasma membrane	5′ Nucleotidase	9.9	291	29.4
Mitochondria	Succinic-INT-reductase	0.032	0.174	5.4
SER[1]	Glucose-6-phosphatase	0.140	0.380	2.7
SER[2]	NADPH-ferricyanide reductase	0.074	0.407	5.5
RER[1]	Glucose-6-phosphatase	0.140	0.490	3.5
RER[2]	NADPH-ferricyanide reductase	0.074	0.458	6.2

* Enzyme activities were expressed as follows: Golgi, nmol galactose incorporated/h/mg protein; plasma membrane, μmol Pi released/min/mg protein; mitochondria, μmol INT-reduced/min/mg protein; SER[1] and RER[1], μmol Pi released/min/mg protein; SER[2] and RER[2], μmol ferricyanide reduced/min/mg protein.

‡ Relative specific activity: specific activity of enzyme in purified fraction per specific activity of enzyme in homogenate.

Fig. 4 confirms the findings of Fig. 3, using different quantitative methodology. In this case, microtubule protein was incubated with equal concentrations of Golgi or SER protein under assembly conditions, and measurements of turbidity at 350 nm indicated microtubule assembly. Compared with the buffer control, the addition of SER inhibited microtubule assembly by 50% at 30 min. In contrast, the addition of equal amounts of Golgi membrane protein produced total inhibition of microtubule assembly.

Although not shown, hepatic cytosol (100,000-*g* supernatant fluids obtained after homogenization of fresh rat liver) does not by itself interfere with microtubule assembly. However, if the liver membranes are incubated with microtubule protein for the full 90 min (30 min preincubation plus 60 min incubation) in nonassembly buffer, and then spun (100,000 *g* for 1 h), the resulting supernatant fluid contains only a fraction of the microtubule protein (defined here as colchicine-binding activity) present in control tubes not incubated with membranes. Thus, when 120 μg of Golgi membrane protein is incubated with 120 μg of microtubule protein, only 15% of the colchicine binding (present in incubated control tube) remains in the supernatant fluid after 90 min of incubation. However, when 120 μg of SER membrane protein is incubated with 120 μg of microtubule protein, 48% of the control colchicine binding protein remains in the supernate. This remaining colchicine binding activity correlates well with the percent reduction in assembled microtubules seen after incubation with the respective membranes in Figs. 3 and 4, and suggests that microtubule protein associates with the membranes during the incubation period and is removed from solution. As a result, the microtubule protein left in solution is below the critical concentration for assembly. Increasing twofold the concentration of cofactors (GTP, $MgCl_2$, and EGTA) does not alter the results. On the other hand, the addition of a basic protein, such as histone (0.4 mg/ml), to the supernatant fluid of Golgi-incubated samples will induce assembly where none was apparent before. However, the microtubules induced by the addition of histone show a horizontal repetitive pattern not seen in the standard assembled microtubules of Fig. 2.

Microtubule Assembly after Incubation of Brain Microtubule Protein with Liver Membrane Lipids

Preliminary tests showed that the association between microtubule protein and hepatic membranes did not involve high-affinity binding. Thus, when iodinated microtubule protein was used as a marker, binding to 120 μg of sonicated Golgi membranes was not saturable, nor was the binding displaceable by unlabeled microtubule protein of increasing concentrations.

These tests led us to examine other, nonprotein constituents of biological membranes to see which components might be responsible for the observed membrane association with microtubule protein. The work of Caron and Berlin (11) and Daleo et al. (13) gave us reason to believe that membrane phospholipids may be involved. Accordingly, the lipids of the various membrane preparations were extracted, reconstituted, and sonicated to provide liposomes for incubation with microtubule protein. Although the chloroform/methanol-extracted mem-

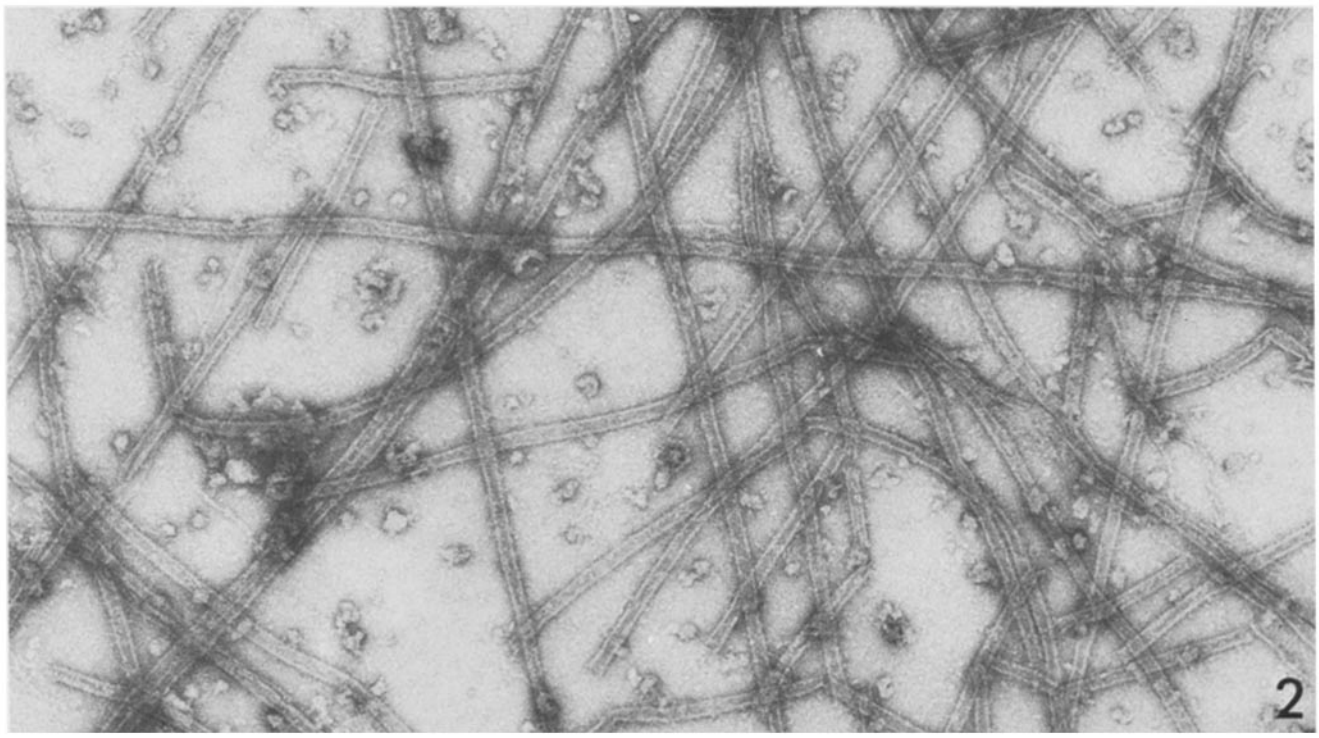

FIGURE 2 Representative assembly of microtubules under the conditions of this study (120 μg protein/incubation). For this micrograph, no dilution was made before staining with cytochrome c and uranyl acetate. × 60,000.

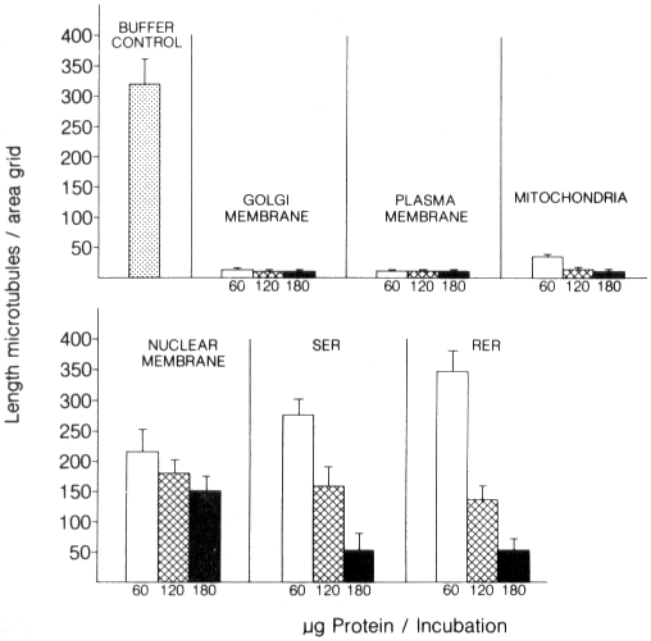

FIGURE 3 Bar graph showing the effect of different concentrations of sonicated hepatic membranes on microtubule assembly quantitated by electron microscopy. The results were obtained from incubations of each type of membrane fraction from three separate experiments. Mitochondria were incubated as intact organelles, though the protein concentration used was for a sonicated mitochondrial preparation. The control contained no addition of membranes. Microtubule protein (120 μg protein) was added to all tubes, and incubations were carried out as described under Methods.

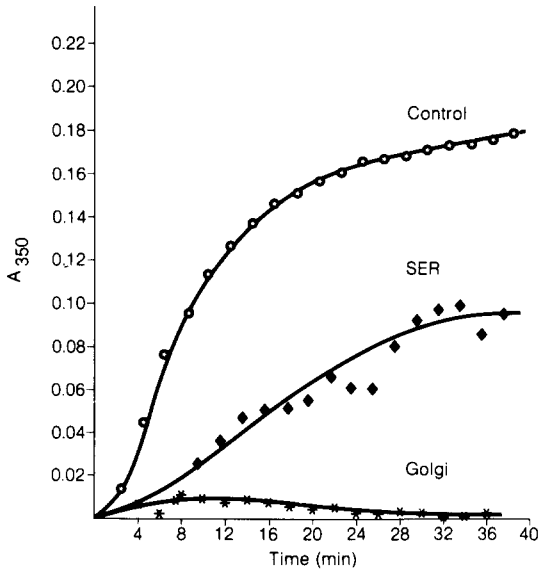

FIGURE 4 Effect of Golgi membrane and SER fractions on microtubule assembly quantitated spectrophotometrically. All tubes contained 1.8 mg/ml microtubule protein and 1.8 mg/ml membrane protein. ○, control; ●, SER; *, Golgi.

branes (protein) themselves did not affect microtubule assembly, liposomes prepared from the extracted lipids did, as shown in Fig. 5. Most of the liposomes from the membranes behaved as did the intact (nonextracted) membranes themselves; e.g., Golgi, cell surface, and mitochondrial lipids interfered with microtubule assembly, as did the intact membranes from which the lipids were extracted. SER and RER liposomes did not interfere with assembly when used at the same concentrations as the other membranes, and this was in keeping with the effect of intact SER and RER on microtubule assembly. On the other hand, it was found that lipids from nuclear membranes were as effective as plasma membranes in interfering with assembly and, in this case, the situation differed from that found with the nonextracted nuclear membranes.

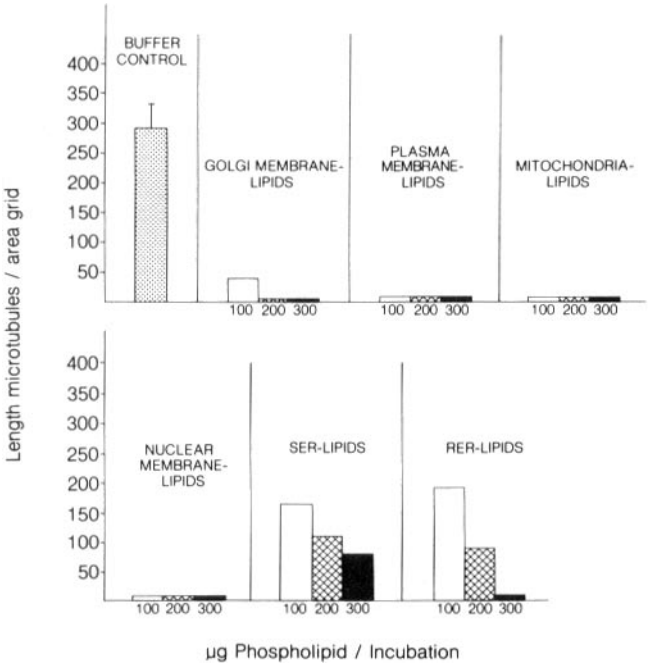

FIGURE 5 Bar graph showing effect of membrane-derived lipids on microtubule assembly quantitated by electron microscopy. Incubation details are identical to those in Fig. 3. The results were obtained from two different preparations of each type of membrane. The lipid concentrations were determined by the phospholipid content of the extracted material.

That the inhibitory effect of the membrane lipids was due to the phospholipid (rather than the neutral lipid) content of the membranes was shown when Golgi-extracted phospholipids were incubated with microtubule protein. Table II indicates that the phospholipid liposomes from Golgi membranes were as effective as intact Golgi membranes (Fig. 2) or extracted Golgi lipids (Fig. 5) in inhibiting microtubule assembly. On the other hand, the Golgi-extracted neutral lipids (cholesterol, fatty acids, cholesterol esters, and triglycerides) did not interfere with microtubule assembly.

Table III shows the phospholipid composition of the various membrane preparations used. It appears that similar types of phospholipids exist within the different membranes, though the content of individual phospholipids varies. For example, Golgi membranes and mitochondria are rich in the negatively charged phospholipid cardiolipin, but relatively poor in another negatively charged phospholipid, phosphatidylserine. In contrast, plasma membranes have a large amount of phosphatidylserine and very little cardiolipin.

Microtubule Assembly after Incubation of Brain Microtubule Protein with Commercially Obtained Phospholipids

Various neutral and negatively charged phospholipids (100–300 μg) were incubated with 120 μg of microtubule protein under assembly conditions. The neutral phospholipids, phosphatidylcholine (lecithin), and phosphatidylethanolamine, had no effect on microtubule assembly, although some liposome aggregation occurred. On the other hand, the negatively charged phospholipids (phosphatidylserine and cardiolipin) were extremely effective in inhibiting microtubule assembly. Fig. 6*A* shows the striking effect of incubating microtubule protein with liposomes constructed from different ratios of lecithin and cardiolipin. When the liposome concentration was comprised of >50% neutral phospholipids,no effect was seen on microtubule assembly. However, when the liposome concentration included >50% negatively charged phospholipids, such as cardiolipin, no microtubules were assembled.

This effect of negatively charged liposomes on microtubule assembly was confirmed by turbidimetric measurements as shown in Fig. 6*B*. That is, phosphatidylcholine (lecithin) incubation with microtubule protein had little or no effect on microtubule assembly, whereas equal amounts of cardiolipin totally prevented assembly.

DISCUSSION

Data from this study indicate that certain isolated and purified membranes of rat liver interfere with the assembly of brain

TABLE II

Effect of Various Lipid Components of Golgi Membranes on Microtubule Assembly

Golgi membrane components*	Microtubule assembly
	length microtubules/area grid
Membranes (nonextracted)	10
Membrane total lipids	35
Membrane neutral lipids	330
Membrane phospholipids	0
Membrane neutral lipids + phospholipids	0
Buffer control	308

* The membrane fractions (7, 15) and derived lipids from the same preparation were sonicated and incubated with 120 μg microtubule protein as described under Methods. The buffer control contained no membrane components. Nonextracted membranes were added in an equal amount (120 μg protein) to microtubule protein. The lipid concentration used for the remaining tubes was equivalent to 100 μg phospholipids.

TABLE III

Phospholipid Composition of Hepatic Subcellular Fractions

	Percent of phospholipid (phosphate)					
Phospholipid class	Nuclear membrane	Plasma membrane	Golgi fraction*	RER	SER	Mitochondria
Phosphatidylcholine (lecithin)	53.3	34.3	39.2	48.6	43.2	42.3
Phosphatidylethanolamine	20.1	18.3	22.7	24.3	28.8	34.6
Phosphatidylinositol	6.9	7.8	11.7	10.5	11.2	2.6
Phosphatidylserine	10.7	11.6	5.9	8.1	6.9	0.9
Diphosphatidylglycerol (cardiolipin)	0.8	0.8	3.8	1.5	0.9	18.4
Phosphatidic acid	0.3	0.6	1.3	1.0	0.8	0.1
Lysophosphatidylcholine	1.9	3.3	3.2	1.8	2.0	0.2
Lysophosphatidylethanolamine	‡	0.8	0.6	0.4	‡	‡
Lysophosphatidylserine	‡	0.3	0.3	‡	‡	‡
Sphingomyelins	2.5	19.6	7.2	2.7	4.5	0.6
Unidentified	3.5	2.5	4.1	1.1	1.7	0.3

* Golgi fraction prepared by Morré procedure (19).
‡ Not detected.

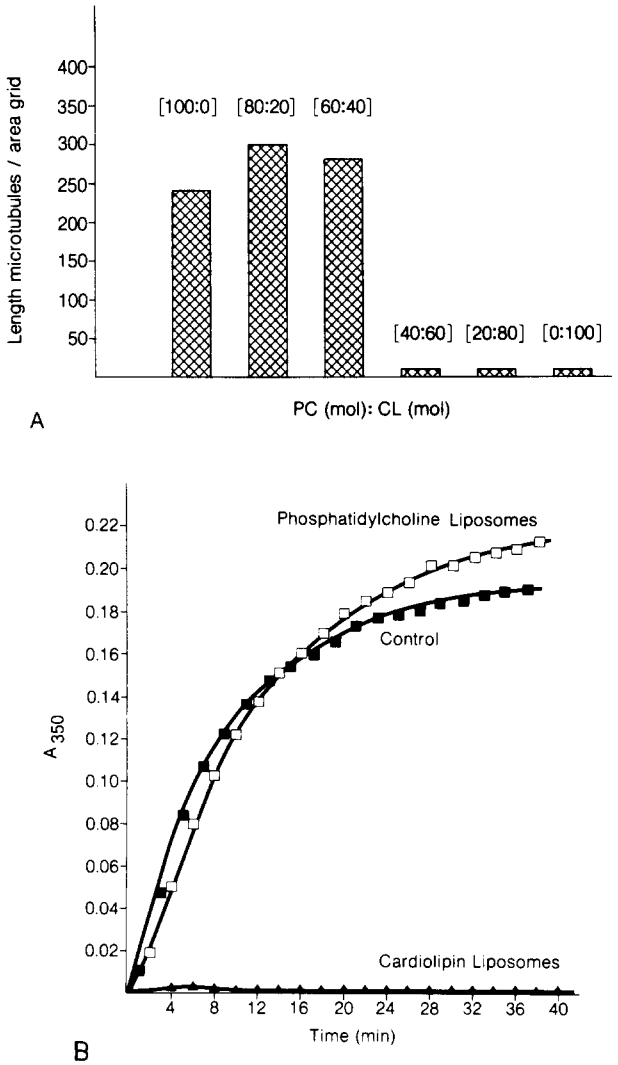

FIGURE 6 Effect of liposomes from commercially obtained phospholipids on microtubule assembly. (*A*) Phosphatidylcholine (PC) and cardiolipin (CL) were combined in various ratios and incubated with microtubule protein (100 μg phospholipid + 120 μg microtubule protein). Quantitation of microtubule assembly was carried out microscopically. (*B*) Phosphatidylcholine or cardiolipin liposomes were incubated with microtubule protein (400 μg phospholipid + 1.9 mg microtubule protein/ml). Quantitation of microtubule assembly was carried out spectrophotometrically. ■, control; □, phosphatidylcholine liposomes; ▲, cardiolipin liposomes.

microtubule protein under in vitro conditions. We believe that membranes have this effect on microtubule assembly because they "bind" the unassembled microtubule protein, removing it from solution. As a result, the protein concentration of the microtubule protein is lowered below its critical assembly concentration and microtubules fail to form. Evidence for this comes from several observations. First, electron microscopy indicates that membrane vesicles become sticky when incubated with microtubule protein, and aggregate with one another and with newly formed microtubules. Second, this aggregated material is removable by centrifugation; the resulting supernatant fluid has reduced colchicine-binding activity in keeping with the level of inhibition of microtubule assembly noted with the membrane preparation used. Finally, the microtubule protein that remains in solution following this postincubation spin has been found to be assembly-competent, since it forms microtubules when an independent basic protein such as histone is added to lower the critical assembly concentration (16). In certain respects, the results of these experiments resemble those of Sherline et al. (34), who found that microsomal fractions from rat brain bind tubulin directly and inhibit microtubule assembly in vitro.

The question that arises is why do liver membranes bind to microtubule protein? Clearly, this is not a specific protein-protein interaction. Our attempts to show specific protein binding have been unsuccessful. ^{125}I-microtubule protein binding to Golgi membranes was not saturable, even when large concentrations of microtubule protein were used: in addition, the observed binding was not displaceable with unlabeled microtubule protein. In a recent paper, Caron and Berlin (11) showed, however, that microtubule protein can be selectively adsorbed onto liposomes constructed from lecithin. This adsorption induces stacking and/or fusion of liposomes into multilamellar structures, indicating strong protein-lipid interactions. Using a similar approach, we have been able to show that liposomes constructed entirely of liver membrane-extracted lipids form aggregates when incubated with microtubule protein. In addition, some of these liposome preparations interfere with microtubule assembly and, as such, duplicate the in vitro behavior of the intact (unextracted) membranes from which they were derived. For example, intact Golgi and plasma membranes, and liposomes derived from these membranes, effectively inhibit microtubule assembly over a wide concentration range. On the other hand, intact SER and RER membranes are much less potent in inhibiting microtubule assembly, as are the lipids derived from these membranes. However, when one specifically examines the phospholipid composition of these membranes, it is not immediately apparent that the content or the type of phospholipids in the different membranes can account for the differential behavior noted. On the other hand, phospholipids derived from Golgi membranes (and separated from neutral lipids) interfere with microtubule assembly, as do the intact Golgi membranes or the combined extracted lipids from the Golgi fraction. In addition, liposomes made from pure, commercially obtained phospholipids show differences in their ability to influence microtubule assembly: neutral phospholipids such as phosphatidylcholine (lecithin) and phosphatidylethanolamine have no effect on assembly over a wide concentration range, whereas negatively charged phospholipids such as phosphatidylserine and cardiolipin (diphosphatidyl glycerol) are very effective in this regard. In fact, in liposomes constructed with different ratios of neutral (lecithin) and negatively charged (cardiolipin) phospholipids, no decrease in microtubule assembly is found until >50% of the liposomes contain the negatively charged lipid. These effects on microtubule assembly with cardiolipin and lecithin were observed also by Daleo et al. (13). Taken together, the observations suggest that phospholipid "binding" to microtubule protein is in some way responsible for the observed membrane aggregation. Precisely how this occurs, and whether phospholipid charge, content, or orientation (with respect to other molecules in the biological membranes) is the important factor, is not yet clear.

That various hepatic membranes aggregate with microtubule protein and interfere with microtubule assembly seems certain. However, the reason why some membranes appear to be so much more potent than others in this regard may be a function of true differences in the biochemical or structural makeup of the individual membranes, or it may be a function of changes

occurring in the membranes during the isolation procedures. For example, nuclear membrane preparations appear exceptionally clean (the starting material appears to be exclusively isolated nuclei) with only occasional clusters of material that resembles chromatin. Yet, we know that chromatin contains the basic protein, histone, and it is conceivable that what appears to be a small amount of contaminating chromatin may be sufficient, in fact, to lower the critical concentration of microtubule protein necessary for assembly, resulting in relatively less inhibition of microtubule assembly with nuclear membranes than with similar amounts of plasma or Golgi membranes. The more substantial inhibitory effect seen with intact nuclei (in which the nuclear contents are protected from coming in contact with microtubule protein) or with the lipids derived from nuclear membranes is consistent with this view. On the other hand, neither the intact membrane nor the lipids from the SER and RER (even when derived from the same preparation as Golgi membranes) seem particularly inhibitory to microtubule assembly. In this situation, where two independent techniques show the same result (i.e., intact membranes and liposomes from these membranes), we are less likely to believe that changes induced by the isolation procedures are responsible for the observed differences.

What are the in vivo implications of these experiments? To begin with, a number of reports have already emphasized the relationship between microtubule protein (i.e., colchicine-binding protein) and cell surface phenomena (1, 27, 36, 37). Other studies have shown the importance of membrane phospholipid turnover during cell secretion (12), and have demonstrated that when microtubule protein is bound to colchicine, membrane phospholipid turnover ceases (33). The work of Caron and Berlin (11) with lecithin liposomes, and this current work with various membrane-derived phospholipids, suggest that there is a direct interaction of microtubule protein (at least from brain) with phospholipids from different sources. Our current view is that microtubule protein may be involved in the delivery of phospholipids to membranes during membrane synthesis or reconstruction. Colchicine may interfere with this process by competitively binding to microtubule protein and, in this way, membrane reconstruction (and associated events, such as packaging of secretory proteins) may be halted. The fact that hepatic Golgi membranes are disrupted (along with microtubules and lipoprotein secretion) following colchicine treatment (32) is consistent with this thinking. However, we believe it possible that structural or organizational changes may be more visible in Golgi complexes than in other membranes (because of the characteristic stacking of the Golgi cisternae), and that other cell membranes may, in fact, share in this interaction with microtubule protein. As a consequence, the availability of microtubule protein for binding to phospholipids may be important for a large variety of membrane-associated events.

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