Aggregation and Calcium-induced Fusion of Phosphatidylcholine Vesicle-Tubulin Complexes*

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Insertion of tubulin into the bilayer of dipalmitoyl phosphatidylcholine vesicles at the phase transition results in the formation of stable vesicle-tubulin complexes (Klausner, R. D., Kumar, N., Weinstein, J. N., Blumenthal, R., and Flavin, M. (1981) J. Biol. Chem. 256, 5879-5885). These complexes aggregated when maintained below phase transition for 19-20 min. Addition of millimolar concentrations of Ca²⁺, Mn²⁺, Zn²⁺, and Co²⁺, but not Mg²⁺, caused the vesicle-tubulin complexes to fuse into larger structures as shown by (a) electron microscopy, (b) increased trapped volume, and (c) changes in resonance energy transfer between two fluorescent lipid probes incorporated into the same vesicle. There was no loss of internal aqueous contents from the vesicle-tubulin complexes during Ca²⁺-induced fusion. Anti-tubulin drugs had no effect on the aggregation or fusion, and vesicle-bound tubulin did not associate with microtubules when tubulin was assembled in vitro. Trypsin-treated vesicle-tubulin complexes were incapable of supporting Ca²⁺-induced fusion. This system provides a model for Ca²⁺-induced and protein-mediated nonleaky fusion of uncharged lipid bilayers.

Although tubulin, the basic structural unit of microtubules, is generally considered to be a cytoplasmic protein, there are data which suggest that it is also associated with various organelles and membranes (1-3). In addition to their primary role in mitosis, microtubules have also been implicated in a number of membrane-linked processes such as cell motility, secretion, transport, redistribution of membrane proteins, and maintenance of cell shape and size. So far, there is little knowledge regarding the specific role of membrane-associated tubulin. It has been suggested that membrane-bound tubulin could be involved in interactions between membranous vesicles and cytoskeletal elements or between vesicles and the plasma membrane (4). Of equal interest is the question of how tubulin becomes an integral component of the membrane. Soifer and Cosnefry (5) have reported that tubulin is synthesized on rough microsomes, incorporated into microsomal membranes, and translocated to the plasma membrane as a component of vesicles.

We have recently shown (6, 7) that purified tubulin can be incorporated into dipalmitoyl phosphatidylcholine vesicles at the lipid phase transition temperature, without any requirement for detergent or sonication, and results in the formation of stable vesicles. Similar results had been reported by Caron and Berlin (8) using dimyristoyl phosphatidylcholine vesicles. The insertion process is accompanied by structural perturbations of both the lipid bilayer and the tubulin (6, 7). In this paper we examine the properties of the vesicle-tubulin complexes. These complexes aggregated even in the absence of divalent cations; in the presence of millimolar Ca²⁺ or Mn²⁺ but not Mg²⁺, they fused to form much larger closed structures without release of the water-soluble internal marker, carboxyfluorescein. We have also compared free and vesicle-bound tubulin for their ability to bind colchicine and MAPs† or to be tyrosinated and detyrosinated by specific enzymes.

MATERIALS AND METHODS

Preparation of Tubulin—Microtubule protein was purified from freshly obtained bovine brains by three cycles of assembly and dis-assembly without glycerol, according to the procedure of Aanes and Wilson (9). Tubulin* was further purified by phosphocellulose chromatography as described elsewhere (6) and stored in buffer containing 200 mM K⁺-2-(N-morpholino)ethanesulfonic acid, 1 mM MgSO₄, 1 mM EGTA, 2 mM dithiothreitol, and 0.1 mM GTP, pH 6.8, at -70 °C. MAPs which remain bound in the column were subsequently eluted with 1.0 M KCl in the above buffer, concentrated using an Amicon PM-30 filter, and stored at -70 °C. Protein was determined according to the method of Lowry et al. (10) using bovine serum albumin as standard.

Preparation of Vesicles—Small unilamellar vesicles of DPPC labeled with either [³⁵S]DPPC or [¹⁴C]DPPC but without carboxyfluorescein were prepared as described elsewhere (6). DPPC vesicles labeled with N,N-dibenzylethylenediamine and N-8-hydroxynaphthalene-1,8-diylphosphatidylethanolamine; N-Rh-PE, N-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; Heps, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.

The abbreviations used are: MAPs, microtubule-associated proteins; DPPC, dipalmitoyl phosphatidylcholine; Tc, phase transition temperature; EGTA, ethylene glycol bis-(β-aminoethyl ether)-N,N′-tetraacetic acid; N,N,N,N′-tetraacetic acid; N,N-dibenzylethylenediamine; N-8-hydroxynaphthalene-1,8-diylphosphatidylethanolamine; N-Rh-PE, N-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; Heps, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.

*This refers to tubulin purified by phosphocellulose chromatography. 3 X tubulin refers to three assembly cycles purified tubulin.
transition temperature, i.e. 40 °C for 2 min. We have shown earlier that tubulin is inserted into the bilayer of these vesicles at the phase transition to form stable complexes (6).

The trapped volume of vesicles (volume of the total internal aqueous compartment) was determined by preparing and incubating the vesicles at 40 °C in the presence of 20 mM carboxyfluorescein. The vesicles and vesicle-tubulin complexes were washed seven times with buffer containing 150 mM NaCl, 10 mM Hepes, and 1 mM EGTA, pH 7, dissolved with Triton X-100, and carboxyfluorescein and lipid were measured. The volume of the aqueous compartment of vesicles relative to total volume was determined by measuring the ratio between fluorescence before and after separating the vesicles from the medium containing 20 mM carboxyfluorescein. The phospholipid concentration (mol/liter) was measured after the separation. The trapped volume (liters/mol) was then determined by dividing the fluorescence ratio by the phospholipid concentration.

**Centrifugation Assay for Aggregation/Fusion of Vesicles or Vesicle-Tubulin Complexes—**This assay made use of the change in sedimentation constant as a result of aggregation and/or fusion of vesicles. Routinely, vesicles alone or vesicle-tubulin complexes were incubated for 15–30 min below the phase transition temperature of DPPC (usually at 28–30 °C) in tubes (7 × 50 mm; cellulose acetate) containing 100 mM KCl-2-(N-morpholino)ethanesulfonic acid (pH 6.8) containing 0.5 mM NaCl and 0.75 mM EGTA. At the end of incubation, tubes were centrifuged (using adaptors no. 408) at 40,000 x g for 20 min at 25 °C (Sorvall Rotor SS-34). Supernatants were removed for counting. The bottoms of the tubes were cut off and dropped into scintillation vials for counting.

The recovery of total radioactivity (pellet and supernatant) was >98%.

Results are expressed as the ratio of radioactivity in the pellet/total radioactivity. The actual amount pelleted varied between 20 and 65% (at a tubulin to vesicle molar ratio of 10:1) with several vesicle and/or tubulin preparations. In general, this variability was seen only with the pelleting of vesicle-tubulin complexes in the absence of added Ca2+. However, any single preparation gave extremely reproducible results.

**Resonance Energy Transfer between Lipid Probes—**Mixing of lipids was assayed according to the procedure developed by Pagano and his co-workers (11) involving resonance energy transfer between two fluorophores incorporated into the vesicle bilayer. The energy donor NBD and the energy acceptor rhodamine are coupled to the free amino group of phosphatidylethanolamine to form N-NBD-PE and N-Rh-PE (11). The two lipid probes were obtained from Avanti Polar Lipids (Alabaster, Ala.) and the phospholipid concentration was assayed according to the procedure developed by Pagano et al. (11). The two lipid probes were obtained from Avanti Polar Lipids (Alabaster, Ala.) using a Perkin-Elmer MPF 44b spectrofluorometer, the excitation band slit was at 10 nm, and the emission slit at 10 nm. Excitation was at 450 nm, showing emission maxima at 530 nm and 585 nm (see Fig. 5 under "Results"). Essentially all of the fluorescence at 530 nm comes from N-NBD-PE, whereas the fluorescence at 585 nm arises from resonance energy transfer between the donor and acceptor pair.

The efficiency of energy transfer (quenching of the energy donor) in such samples is defined by the relationship (12)

\[ E = 1 - \frac{F}{F_0} \]

where \( F \) is the fluorescence at 530 nm in the presence of N-Rh-PE and \( F_0 \) is the fluorescence at 530 nm in the absence of N-Rh-PE. With this assay, energy transfer between the donor and acceptor pair is determined by measuring the fluorescence of the donor in the absence of detergent, the rhodamine fluorescence at 585 nm is completely abolished when excited at 450 nm, indicating complete mixing of the fluorophores with excess DPPC lipid. We define the NBD fluorescence at 530 nm in the presence of detergent as Fo. The energy transfer efficiency for the DPPC-NBD-PE-N-Rh-PE = 98:1:1 vesicles (88%) was about equal to that reported by Struck et al. (11) for phosphorylcholine vesicles containing the same mol per cent N-NBD-PE and N-Rh-PE.

**Electron Microscopy—**For electron microscopy, DPPC vesicles and vesicle-tubulin complexes with or without Ca2+ were negatively stained with 1% uranyl acetate and viewed with a Phillips 400 microscope at 60 kV.

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**RESULTS**

**Aggregation of Vesicle-Tubulin Complexes**

Fig. 1 shows the typical turbidity changes (indicated by changes in 90° light scattering at 470 nm) of DPPC vesicles upon addition of various concentrations of tubulin at 40 °C. Without tubulin, there was no change in the light scattered by vesicles, whereas increasing amounts of tubulin resulted in an increase in the scattering, probably as a result of aggregation of the small unilamellar DPPC vesicles. There was very close correspondence in the time course for the turbidity increase and the release of encapsulated carboxyfluorescein reported previously (6). The change in turbidity takes place only when tubulin is incubated with vesicles around 37–40 °C (which is also the transition temperature). A likely interpretation of the observed increase in the turbidity is that tubulin promoted the aggregation of vesicles under conditions in which it is inserted into the lipid bilayer (6). A variety of other soluble proteins such as hemoglobin, immunoglobulin, albumin, and ovalbumin do not produce an effect similar to that of tubulin on these vesicles. Formation of larger structures was also indicated by the relative increase in the sedimentation of vesicles as shown in Table I and Fig. 2. The pelleting of the vesicle-tubulin complexes allowed us to study further the effect of divalent cations. Fig. 2 shows the effect of 2.5 mM CaCl2 on the pelleting of DPPC vesicles. Under these conditions, Ca2+ did not increase the pelleting of DPPC vesicles.
was also a substantial population of free small vesicles. Within the clumps, individual small vesicles of the original size were clearly distinguishable (Fig. 4C). Fig. 4C was selected from an area including some of the largest aggregates found. Tubulin-containing vesicles incubated in the presence of 5 mM Ca\(^{2+}\) completely gathered into clumps with no free vesicles apparent. Within these clumps in addition to some distinguishable small vesicles there were also vesicles of a variety of larger sizes as well as much larger structures apparently composed of sheets of membrane folded into topologically complex forms (Fig. 4D). Fig. 4D was selected from an area showing the highest proportion of distinguishable small vesicles in addition to the larger membrane forms. The most obvious interpretation is that the larger vesicles and complex membrane structures are formed by fusion of the small vesicles. The differences between vesicles and vesicle-tubulin complexes with or without Ca\(^{2+}\) were obvious and reproducible.

**Trapped Volume**—Small unilamellar vesicles as well as vesicle-tubulin complexes at a 30:1 ratio (tubulin to vesicle) were formed in 150 mM NaCl-10 mM Hepes buffer, pH 7.0, containing 20 mM carboxyfluorescein and 1 mM EGTA. Fusion was induced by adding Ca\(^{2+}\) to a final concentration of 10 mM to the vesicle-tubulin complexes in a medium containing 20 mM carboxyfluorescein. The trapped volume was measured after separation of the vesicles from the medium. As shown in Table II, vesicle-tubulin complexes had a 2-fold increase in trapped volume over the original vesicles, and Ca\(^{2+}\)-induced fusion produced a 10-fold increase in trapped volume. This result indicates that at least some of the large structures seen in Fig. 4D are closed vesicles.

**Mixing of Lipid Probes**—In Fig. 5 we show spectra of a tubulin-vesicle preparation containing both N-NBD-PE (donor) and N-Rh-PE (acceptor) (DPPC:N-NBD-PE:N-Rh-PE = 98:1:1), excited at 450 nm. Emission maxima at 530 nm and 585 nm are observed. Essentially all of the fluorescence at 530 nm comes from N-NBD-PE, whereas the fluorescence at 585 nm arises from fluorescence energy transfer between the donor and acceptor. The tubulin-vesicle complexes were mixed with tubulin-vesicle complexes (without probes) at a (vesicle/vesicle) ratio of 1:20, and fusion was initiated by addition of Ca\(^{2+}\). As shown in Fig. 5 there was a marked reduction in the emission peak at 585 nm and a concomitant increase in fluorescent yield from N-NBD-PE at 530 nm upon addition of Ca\(^{2+}\). These changes indicate a reduction in the efficiency of energy transfer between N-NBD-PE and N-Rh-PE. This is consistent with fusion between the DPPC/N-NBD-PE/N-Rh-PE-tubulin complexes and pure DPPC-tubulin complexes, followed by lateral diffusion of the fluorescent lipids in the

**Evidence for Ca\(^{2+}\)-induced Fusion**

**Electron Microscopy**—Fig. 4 is a series of electron micrographs prepared by the technique described under "Materials and Methods." It illustrates the effect of Ca\(^{2+}\) on vesicle-tubulin complexes. The population of DPPC vesicles eluted from the Sepharose 4B column contained primarily single spherical vesicles 18–30 nm in diameter. Among these vesicles, there was a small number of larger vesicles (up to 10 nm in diameter) each of which appeared to contain several of the small vesicles (Fig. 4A). DPPC vesicles incubated with 5 mM Ca\(^{2+}\) were indistinguishable from those without Ca\(^{2+}\) (Fig. 4B). DPPC vesicles into which tubulin had been incorporated by passage through Tc showed marked clumping, but there

**TABLE I**

Effect of varying tubulin/vesicle ratio on the pelleting of vesicles

| Tubulin/vesicle | (%)([^c]DPPC (pellet/total) |
|-----------------|----------------------------|
| mol/mol         |                            |
| 0               | 0.09                       |
| 3               | 0.17                       |
| 6               | 0.25                       |
| 15              | 0.36                       |
| 30              | 0.48                       |

**Fig. 2. Effect of Ca\(^{2+}\) on pelleting of vesicles and vesicle-tubulin complexes.** Lipid concentration was 2.7 mM. 1, vesicles alone; 2, vesicles + 2.5 mM CaCl\(_2\); 3, vesicles + tubulin (6 µM) without passage through the phase transition; 4, vesicle-tubulin complexes; 5, vesicle-tubulin complexes + 2.5 mM CaCl\(_2\). All the incubations and centrifugation were at 30 °C. Numbers in parentheses represent the results obtained when incubation and centrifugation were done at 0 °C.

alone nor was there any significant effect on the pelleting when tubulin and vesicles were mixed below Tc. When tubulin was inserted into the lipid bilayer at the endothermic Tc approximately 40 to 65% of the vesicles could be pelleted in the absence of Ca\(^{2+}\). Addition of Ca\(^{2+}\) to the vesicle-tubulin complexes, however, caused nearly complete pelleting of the vesicles (Fig. 2). The effect of Ca\(^{2+}\) was dependent upon the final concentrations added to vesicle-tubulin complexes. As shown in Fig. 3, maximum pelleting occurred above 2.5 mM concentration. Since these assays were performed in buffer containing 0.75 mM EGTA, the threshold concentration of free Ca\(^{2+}\) for maximum pelleting was above 1.0 mM. The inset of Fig. 3 also shows that Mg\(^{2+}\) was without any effect, whereas Mn\(^{2+}\), Zn\(^{2+}\), and Co\(^{2+}\) enhanced the pelleting of the vesicle-tubulin complexes. Effectiveness of various cations tested was Mn\(^{2+}\) ≥ Ca\(^{2+}\) ≥ Zn\(^{2+}\) > Co\(^{2+}\) > Mg\(^{2+}\).

**Fig. 3. Effect of varying CaCl\(_2\) concentration on vesicle-tubulin complexes.** Vesicle-tubulin complexes were incubated for 30 min at 30 °C in the presence of various concentrations of CaCl\(_2\). Data in the inset shows the pelleting of vesicles in the presence of 2.5 mM concentration of other cations. Assay buffer contained 0.75 mM EGTA.
plane of the newly formed membrane. Struck et al. (11) have shown that the energy transfer efficiency of vesicles containing 1% N-NBD-PE increases linearly as a function of mol % (surface density) N-Rh-PE up to about 1 mol % N-Rh-PE. Therefore, a 2-fold decrease in energy transfer efficiency represents a 2-fold decrease in surface density, consistent with fusion of one probe-containing vesicle with one pure vesicle.

In Fig. 6B we have plotted the energy transfer efficiency calculated according to Equation 1 as a function of Ca$^{2+}$ concentration. At about 1 mM Ca$^{2+}$ there is a 2-fold decrease in transfer efficiency, indicating that on the average each fluorescent vesicle has fused with an unlabeled vesicle. The Ca$^{2+}$ dependence shown in Fig. 6 is quite similar to the Ca$^{2+}$ dependence of pelleting shown in Fig. 1. In accordance with the divalent cation specificity of pelleting we also see that Mg$^{2+}$ had little effect on energy transfer, whereas the effect of Mn$^{2+}$ was similar to that of Ca$^{2+}$.

When the probe-containing vesicles were incubated with Ca$^{2+}$ in the absence of pure vesicles there was no change in energy transfer, indicating that the effect was not due to, for instance, a change in the radius of curvature of the vesicles. However, when we incubated probe-containing vesicle-tubulin complexes with DPPC vesicles without tubulin we did see Ca$^{2+}$-dependent changes in energy transfer (Fig. 6). This

### TABLE II

|                          | Trapped volume/liter/mol |
|--------------------------|--------------------------|
| Vesicles                 | 0.36                     |
| Vesicle-tubulin complexes| 0.85                     |
| Vesicle-tubulin complexes + 10 mM Ca$^{2+}$ | 4.33                     |

FIG. 4. Electron micrographs of negatively stained vesicles. 165,000 ×: A, DPPC vesicles; B, DPPC vesicles in the presence of 5 mM CaCl$_2$; C, DPPC vesicle-tubulin complexes containing 10 tubulin molecules per vesicle in the presence of 5 mM EGTA; D, DPPC vesicle-tubulin complexes containing 10 tubulin molecules per vesicle in the presence of 5 mM CaCl$_2$. The bar indicates 200 nm.
were mixed with the unlabeled DPPC vesicle-tubulin complexes labeled complexes (DPPC/N-NBD-PE/N-Rh-PE (98:1:1) and tubulin complexes were formed at a tubulin to vesicle ratio of 30:1. The unlabeled complexes (DPPC/N-NBD-PE/N-Rh-PE (98:1:1) and tubulin complexes were formed at a vesicle:vesicle ratio of 1:20, and CaCl₂ was added to induce fusion. The numbers indicate final Ca²⁺ concentrations.

Methods." The numbers were not corrected for the effect of Triton X-100 was 0.23. Leakage was assessed by measuring fluorescence of the rechromatographed complexes before and after disrupting them with Triton X-100 was 0.23. This is consistent with 30 mM carboxyfluorescein releasing from the vesicles during complex formation. All this was done in a buffer containing 1 mM EDTA. The ratio between the fluorescence of the rechromatographed complexes before and after disrupting them with Triton X-100 was 0.23. This is consistent with 30 mM carboxyfluorescein releasing from the vesicles during complex formation. All this was done in a buffer containing 1 mM EDTA. The ratio of fluorescence of the rechromatographed complexes before and after treatment with 0.1% Triton X-100 was 0.23. Leakage was assessed by measuring fluorescence after mixing with Ca²⁺ and subtracting the background fluorescence of the complexes (no Ca²⁺). B, energy transfer was assessed as described in the legend to Fig. 5. Transfer efficiency was calculated according to Equation 1 (see under "Materials and Methods"). The numbers were not corrected for the effect of Triton X-100 on NBD fluorescence. DPPC-tubulin complexes containing the probe were mixed with unlabeled DPPC-tubulin complexes (O) and DPPC vesicles alone (O) in the presence of CaCl₂. Energy transfer efficiency in the presence of Mn²⁺ (△) or Mg²⁺ (▽) is also shown. Energy transfer in the presence of Ca²⁺ but without any acceptor vesicles is indicated by □.

result indicates that Ca²⁺-induced fusion can also occur between vesicle-tubulin complexes and protein-free vesicles, as was suggested by the pelleting experiments (see below).

Mixing of vesicle-tubulin complexes at 44 °C produced a similar Ca²⁺ effect on energy transfer as that seen at room temperature (below transition).

The probe-containing vesicle-tubulin complexes needed to be mixed immediately after formation with the unlabeled vesicles to produce efficient Ca²⁺-dependent energy transfer changes. When they were allowed to stand for a while in the absence of Ca²⁺ they presumably aggregated. When those were mixed with unlabeled vesicle-tubulin complexes there was no marked change in energy transfer efficiency upon addition of Ca²⁺ (not shown). The aggregated labeled complexes had presumably fused with each other and not with the unlabeled complexes.

Other controls (not shown) which did not produce Ca²⁺-dependent energy transfer changes include mixing vesicles with Ca²⁺ without tubulin and mixing vesicles with tubulin (without phase transition) and Ca²⁺.

Ca²⁺-induced Fusion of Vesicle-Tubulin Complexes was Nonleaky—An important question about vesicle-vesicle fusion is whether the fusion process is accompanied by leakage of vesicle contents. This is the case with Ca²⁺-induced fusion of phosphatidylserine vesicles (19). In order to assess leakage we formed vesicle tubulin complexes by bringing DPPC vesicles containing 120 mM carboxyfluorescein to Tc in the presence of tubulin at a tubulin-vesicle ratio of 9:1. We then immediately rechromatographed the complexes on a Sephadex G-25 (PD-10) column to remove dye released during complex formation. All this was done in a buffer containing 1 mM EDTA. The ratio between the fluorescence of the rechromatographed complexes before and after disrupting them with Triton X-100 was 0.23. This is consistent with 30 mM carboxyfluorescein releasing from the vesicles during complex formation. All this was done in a buffer containing 1 mM EDTA. The ratio of fluorescence of the rechromatographed complexes before and after treatment with 0.1% Triton X-100 was 0.23. Leakage was assessed by measuring fluorescence after mixing with Ca²⁺ and subtracting the background fluorescence of the complexes (no Ca²⁺). B, energy transfer was assessed as described in the legend to Fig. 5. Transfer efficiency was calculated according to Equation 1 (see under "Materials and Methods"). The numbers were not corrected for the effect of Triton X-100 on NBD fluorescence. DPPC-tubulin complexes containing the probe were mixed with unlabeled DPPC-tubulin complexes (O) and DPPC vesicles alone (O) in the presence of CaCl₂. Energy transfer efficiency in the presence of Mn²⁺ (△) or Mg²⁺ (▽) is also shown. Energy transfer in the presence of Ca²⁺ but without any acceptor vesicles is indicated by □.

Further Characterization of Aggregation and Ca²⁺-induced Fusion—In the next set of experiments we tested whether the pelleting of lipid is also accompanied by a proportional amount of tubulin in the pellets. Results of one such experiment in which we used [³H]DPPC vesicles and tyrosinolated [³H]tubulin are shown in Table III. There is no difference between tyrosinolated and detyrosinolated tubulins in their interaction with DPPC vesicles (see below). Tyrosinolated [³H]tubulin was prepared according to the method described elsewhere (13). This modification of tubulin involves addition of a tyrosine residue at the COOH terminus of the α subunit, catalyzed by tubulin tyrosine ligase in the presence of ATP. No significant differences in the in vitro microtubule assembly properties so far have been observed between maximally tyrosinolated and detyrosinolated tubulins (14). Tubulin alone under these conditions did not pellet, with or without Ca²⁺.

| Condition         | [³H]DPPC | Tyrosinolated [³H]tubulin |
|-------------------|---------|--------------------------|
| Pellet/total      |         |                          |
| Vesicles          | 0.17    |                          |
| Vesicles + Ca²⁺   | 0.19    |                          |
| Vesicles + Ca²⁺ + EGTA | 0.33 |
| Vesicle-tubulin + Ca²⁺ | 0.95 |
| Vesicle-tubulin + Mn²⁺ | 0.99 |

**Table III**

Co-pelleting of [³H]DPPC and tyrosinolated [³H]tubulin

Tubulin to vesicle ratio was 9:1. Concentration of Mn²⁺ or Ca²⁺ was 2.5 mM and that of EGTA was 5 mM. Tyrosinolated [³H]tubulin was prepared as described elsewhere (13).
without Ca\(^{2+}\). There was no pelleting of the tyrosinolated \(^{14}\)C tubulin if the tubulin was added to vesicles below \(T_c\). However, tubulin did pellet with the lipid when it was inserted into vesicles. When the vast majority of the lipid was pelleted in the presence of divalent cation, a comparable amount of tubulin accompanied the lipid.

Results in Table IV show that actin, another cytoskeletal protein which interacts with DPPC vesicles, also mediated the Ca\(^{2+}\)-induced sedimentation of vesicles. On the other hand, serum apolipoprotein A1, which also inserts into these vesicles, did not behave like tubulin and actin.

Agents which affect polymerization of tubulin (polylysine, colchicine, and podophyllotoxin) did not have any significant effect on the tubulin-mediated or Ca\(^{2+}\)-enhanced aggregation and fusion of vesicles. MAPs reproducibly caused 10–15% inhibition of pelleting of vesicles (Table V). Bovine serum albumin and NaCl had no effect on the vesicle sedimentation with or without added Ca\(^{2+}\). Brief treatment with trypsin abolished the Ca\(^{2+}\)-induced sedimentation of tubulin-containing vesicles (Table VI). This result suggests a role for the exposed portion of the tubulin molecule (i.e. that is not buried in the bilayer) in mediating the Ca\(^{2+}\) effect.

To test whether the Ca\(^{2+}\) effect reflected enhanced protein-protein interaction or whether tubulin could interact with pure lipid, we made vesicle-tubulin complexes with unlabeled and examined the pelleting of \(^{3}C\)-labeled (protein-free DPPC) vesicles. Data in Table VII show that Ca\(^{2+}\)-induced pelleting of vesicles can take place between tubulin-containing and protein-free vesicles. However, it is necessary that tubulin be inserted in at least some participating vesicles.

Finally we have examined whether vesicle-tubulin complexes interact with microtubules during polymerization of tubulin. Results shown in Table VIII suggest that neither vesicles nor tubulin-containing vesicles bind tightly to microtubules. Assembled microtubules were separated from the bulk of unassembled contents by centrifugation through a 50% sucrose cushion. In these experiments more than 60% of the tubulin was recovered in the microtubule pellet, and 10 \(\mu\)M colchicine resulted in 90% inhibition of this polymerization. Radioactive lipid in the pellets using either vesicles or vesicle-tubulin complexes was the same with or without colchicine and represented only about 6% of the total lipid.

Further Characterization of Tubulin-Vesicle Interaction— Association of tubulin with DPPC vesicles was described earlier (6) by measuring the release of encapsulated dye (carboxyfluorescein) into the medium. The amounts of protein required to cause 50% (of total) release of the dye are shown in Table IX. Three assembly-cycle purified tubulin (3 \(\times\) tubulin) which has approximately 80% tubulin and 20% MAPs was 9 to 10 times less effective than purified tubulin, and MAPs (up to 3 mg/ml) were totally ineffective in inducing dye-release. In fact, addition of MAPs to tubulin during phase transition release resulted in the partial inhibition of release (not shown). There was no difference in the ability of maximally tyrosinolated and detyrosinolated tubulins to cause release of the dye from DPPC vesicles. Tyrosinolated and detyrosinolated tubulins were prepared by incubating tubulin with tubulin tyrosine ligase and carboxypeptidase A as described (13). There was no significant effect of GDP and various anti-tubulin drugs such as colchicine and podophyllotoxin in the phase transition release assay. On the other hand, heat inactivation of the tubulin (90 min at 45 \(^\circ\)C) dramatically destroyed phase transition release activity. Since there was no difference between tyrosinolated and detyrosinolated tubulins in their interaction with vesicles, we also...
TABLE VIII

| Assembly condition | Protein | Radio-lipid % total |
|--------------------|---------|-------------------|
| (a) 3 × tubulin    | 61      |                   |
| (b) (a) + 10 μM colchicine | 8      |                   |
| (c) 3 × tubulin + vesicle-tubulin complexes (10% v/v) | 60 | 6.6 |
| (d) (c) + 10 μM colchicine | 9 | 6.5 |
| (e) (a) + vesicles (10% v/v) | 62 | 4.9 |
| (f) Vesicles alone | 10 | 4.0 |

*Similar concentration range was observed for maximally tyrosinolated (45% tyrosine) and detyrosinolated (0-2% tyrosine) tubulins.

The studies reported in this paper show that the insertion of tubulin into lipid bilayers of uncharged sonicated small unilamellar vesicles results in aggregation of the vesicles, measured by light scattering and increased pelleting of the vesicles. Simply mixing the protein and vesicles was not sufficient to cause the aggregation of vesicles. In the presence of millimolar Ca\(^{2+}\) or Mg\(^{2+}\) but not Mg\(^{2+}\), there was nearly complete pelleting of the vesicles. The effects of Ca\(^{2+}\) on the DPPC vesicle-tubulin complexes could be related to the fact that tubulin is a Ca\(^{2+}\)-binding protein (20). Data in Table V suggest that protein-protein interaction during aggregation of vesicles does not involve polymerization of tubulin as judged by the lack of effect of polymerization inhibitors and that vesicle-tubulin complexes do not associate with microtubules.

Results in Table VIII, however, do not rule out the possibility of dissociation of weakly bound vesicles from microtubules during centrifugation.

Actin, another component of the cytoskeletal system when incubated with lipid vesicles and passed through the phase transition also promoted increased pelleting of vesicles, which was enhanced by Ca\(^{2+}\). We have recently observed that clathrin, a component of coated vesicles can produce fusion of dioleoyl phosphatidylcholine vesicles at pH below 6. Apolipoproteins, on the other hand, while they form vesicular recombinants with DPPC by passage through the phase transition, do not promote increased pelleting.

Based on pelleting and light-scattering data, it is not possible to differentiate between aggregation and fusion of vesicles.

Electron microscopy, however, shows that inserted tubulin results in the aggregation of vesicles, and only the addition of divalent cations induces membrane fusion. Although these results with electron microscopy were clear and reproducible, a number of authors have pointed out a variety of artifacts that can be produced by negative staining of phospholipid preparations (see for example reference 21); and it is perhaps warranted to discuss the question of the reliability of our results in more detail. The results are internally consistent and show clear differences between the controls: vesicles alone, vesicles with Ca\(^{2+}\), and tubulin-vesicle complexes without Ca\(^{2+}\) which did not fuse. If the final fusion of vesicles were caused by phosphotungstic acid it would have been a phosphotungstic acid-induced fusion dependent upon the insertion of tubulin into the DPPC vesicles and requiring calcium. In our protocol the effects of the stain on aggregation and fusion of the vesicles were minimized by applying the vesicles or vesicle-tubulin complexes to grids and blotting or washing off the excess fluid before applying the stain. This should have left primarily vesicles that were adsorbed to the substrate to be outlined by the stain. (Washing the grids bearing the vesicles or complexes with water before application of the stain or rinsing away floating vesicles in a stream of stain gave essentially the same results as those shown in Fig. 4). On the other hand, these modifications. In several experiments, we did not see any differences between the ability of free and vesicle-bound tyrosinolated tubulin to be detyrosinolated by purified carboxypeptidase (13) or in the ability of detyrosinolated tubulins to act as substrates for the enzymes which cause these modifications. In several experiments, we did not see any differences between the ability of free and vesicle-bound tyrosinolated tubulin to be detyrosinolated by purified carboxypeptidase (13) or in the ability of detyrosinolated tubulins to act as substrates for the enzymes which cause these modifications. In several experiments, we did not see any differences between the ability of free and vesicle-bound tyrosinolated tubulin to be detyrosinolated by purified carboxypeptidase (13) or in the ability of detyrosinolated tubulins to act as substrates for the enzymes which cause these modifications. In several experiments, we did not see any differences between the ability of free and vesicle-bound tyrosinolated tubulin to be detyrosinolated by purified carboxypeptidase (13) or in the ability of detyrosinolated tubulins to act as substrates for the enzymes which cause these modifications. In several experiments, we did not see any differences between the ability of free and vesicle-bound tyrosinolated tubulin to be detyrosinolated by purified carboxypeptidase (13) or in the ability of detyrosinolated tubulins to act as substrates for the enzymes which cause these modifications. In several experiments, we did not see any differences between the ability of free and vesicle-bound tyrosinolated tubulin to be detyrosinolated by purified carboxypeptidase (13) or in the ability of detyrosinolated tubulins to act as substrates for the enzymes which cause these modifications. In several experiments, we did not see any differences between the ability of free and vesicle-bound tyrosinolated tubulin to be detyrosinolated by purified carboxypeptidase (13) or in the ability of detyrosinolated tubulins to act as substrates for the enzymes which cause these modifications.
other hand, when DPPC vesicles were mixed with phosphotungstic acid in suspension, we observed stacking of the vesicles as described by Melchior et al. (21). Uranyl acetate which is generally considered the negative stain of choice for studies of microtubules was not useful for this study because the divalent cation UO₂²⁺ is known in many systems to mimic the effects of Ca²⁺ which was the object of these experiments. In fact, with uranyl acetate staining there was extensive reorganization of the tubulin-vesicle complexes without calcium while the DPPC vesicles alone or with calcium resembled those stained with phosphotungstic acid. The electron microscope shows morphologic evidence of fusion but does not allow us to distinguish between the fusion of small vesicles and the somewhat less likely possibility that the small vesicles disintegrate and then form large vesicles. The increased trapped volume (see Table II) and the conservation of encapsulated carboxyfluorescein upon addition of Ca²⁺ to vesicle-tubulin complexes (see Fig. 6A), however, indicate that fusion is the most likely mechanism for the formation of the large structures.

Additional evidence for Ca²⁺-induced fusion of DPPC vesicle-tubulin complexes was provided by the lipid mixing assay developed by Pagano and his coworkers (11). Efficient energy transfer was observed between two fluorescent lipid analogues incorporated into the same vesicle containing one of each probe molecule per 100 phospholipid molecules. Upon fusion of these vesicles with a second vesicle population containing no fluorescent lipid, the efficiency of energy transfer was reduced since lateral diffusion of the probes in the plane of the newly formed larger membrane effectively lowers its surface density. In the absence of added Ca²⁺ the vesicle-tubulin complexes aggregated but did not fuse, and we did not see any changes in energy transfer efficiency. However, upon addition of Ca²⁺ we observed marked changes in energy transfer. This observation provides a built-in control that aggregation of vesicles does not change the energy transfer pattern. We could not use the resonance energy transfer assay developed by Wilscnitz et al. (22) based on mixing of intravesicular compartments during fusion; one of the probes used in that assay bound too strongly to tubulin, interfering with this assay.

The energy transfer assay strengthens the interpretation of our data in terms of fusion. Both gel phase and fluid phase (at 44 °C) DPPC vesicles exhibit tubulin-mediated Ca²⁺-dependent fusion. It is interesting that tubulin, which has been inserted into vesicles, is capable of inducing fusion with pure lipid vesicles. This suggests that the tubulin is altered by its insertion such that the exposed region is more capable of Ca²⁺-dependent perturbation of another lipid bilayer. Such an alteration is consistent with our previous findings of an overall change in the conformation of the molecule upon insertion into the lipid bilayer.

In the majority of earlier studies (22) the effect of divalent cations, mainly Ca²⁺, in inducing aggregation and fusion of vesicles has been studied with vesicles made of negatively charged phosphatidylserine or of mixed phospholipids (phosphatidylserine, phosphatidylcholine, and phosphatidyethanolamine). Small unilamellar DPPC vesicles have been reported to fuse (in the absence of Ca²⁺) to vesicles about 70 nm in diameter, when held below Tc (23). This spontaneous fusion does not produce the large structures seen in Fig. 4D. We have earlier reported that the presence of Ca²⁺ in the medium did not affect the insertion of tubulin into DPPC vesicles at the phase transition (6).

In a recent paper, Hong et al. (24) have presented evidence for the enhancement of Ca²⁺-dependent fusion of vesicles made of phosphatidylserine and phosphatidylethanolamine (1:3) by synexin (M₀ = 47,000), a water-soluble protein isolated from the adrenal medulla. In another study Zimmerberg et al. (25) observed fusion of phospholipid multilamellar vesicles with a planar phospholipid bilayer membrane that contained a water-soluble Ca²⁺-binding protein (M₀ = 16,000) purified from calf brain. This is, however, the first example of nonleaky fusion of phosphatidylcholine vesicles mediated by a protein and induced by Ca²⁺.

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REFERENCES

1. Zisapel, N., Levi, M., and Gozes, I. (1980). J. Neurochem. 34, 26-32
2. Bhattacharyya, B., and Wolfi, J. (1975). J. Biol. Chem. 250, 7639-7646
3. Walters, B. B., and Matus, A. J. (1975) Nature (Lond.) 257, 496-498
4. Soifer, D., and Czoscenk, H. (1986) in Microtubules and Microtubule Inhibitors (DeBrabander, M., and DeMey, J., eds) pp. 429-447. Elsevier/North-Holland Biomedical Press, Amsterdam
5. Soifer, D., and Czoscenk, H. (1980). J. Neurochem. 35, 1128-1136
6. Klausner, R. D., Kumar, N., Weinstein, J. N., Blumenthal, R., and Flavin, M. (1981) J. Biol. Chem. 256, 5879-5885
7. Kumar, N., Klausner, R. D., Weinstein, J. N., Blumenthal, R., and Flavin, M. (1981) J. Biol. Chem. 256, 5886-5889
8. Caron, J. M., and Berlin, R. D. (1979) J. Cell Biol. 81, 665-671
9. Asnes, C. F., and Wilson, L. (1979) Anal. Biochem. 95, 64-73
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
11. Struck, D. K., Hoekstra, D., and Pagano, R. E. (1981) Biochemistry 20, 4093-4099
12. Pung, B. K.-K., and Stryer, L. (1978) Biochemistry. 17, 5241-5248
13. Kumar, N., and Flavin, M. (1981) J. Biol. Chem. 256, 7678-7686
14. Kumar, N., and Flavin, M. (1982) Eur. J. Biochem., in press
15. Kobayashi, T., and Flavin, M. (1978) J. Cell Biol. 79, 285a
16. Garland, D., and Teller, D. C. (1975) Ann. N. Y. Acad. Sci. 253, 232-238
17. Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L., and Greenberg, P. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 177-181
18. Vallee, R. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3206-3210
19. Kim, H., Binder, K. L., and Rosenbaum, J. L. (1979) J. Cell Biol. 80, 265-276
20. Solomon, F. (1977) Biochemistry 16, 358-363
21. Melchior, V., Hollingshead, C. J., and Cahoon, M. E. (1980) J. Cell Biol. 86, 881-884
22. Wilschut, J., Duzgunes, N., Fraley, R., and Papahadjopoulos, D. (1980) Biochemistry 19, 6011-6021
23. Schullery, S. E., Schmidt, C. F., Felger, P., Tilleke, T. W., and Thompson, T. E. (1980) Biochemistry 19, 3919-3923
24. Hong, K., Duzgunes, N., and Papahadjopoulos, D. (1981) J. Biol. Chem. 256, 3641-3644
25. Zimmerberg, J., Cohen, F. S., and Finkelstein, A. (1980) Science 210, 906-908