Stacking in Lipid Vesicle-Tubulin Mixtures is an Artifact of Negative Staining

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ABSTRACT Multilamellar stacking seen in negatively stained lipid vesicle-tubulin mixtures has been attributed to lipid-protein interactions (Caron, J. M., and R. D. Berlin, 1979, *J. Cell Biol.* 81:665–671). We show that this stacking is produced by the phosphotungstic acid used for staining, independent of the presence of tubulin in the sample. The morphology of negatively stained single bilayer vesicles obtained from dimyristoyl phosphatidylcholine or egg lecithin is specifically dependent upon the choice of metal stain. Uranyl oxalate maintains the appearance of unilamellar vesicles. After staining with sodium tungstate, the lipids form a network of multilayered lamellae with a periodicity of ~55 Å. Phosphotungstic acid produces stacks of flattened vesicles with a period of ~115 Å as well as broader multilamellar structures having a 55 Å repeat. The stain-determined morphology is not markedly altered by sample concentration, incubation time, or temperature, or by the presence of tubulin.

Caron and Berlin (4) recently reported that tubulin and the high molecular weight proteins associated with microtubule assembly selectively adsorb to unilamellar dimyristoyl phosphatidylcholine liposomes. Negative staining of the protein-lipid mixtures with phosphotungstic acid (PTA) showed stacks of flattened liposomes and/or larger multilamellar structures resulting from fusion of the stacks.

We have found that identical images of stacked vesicles and multilamellar structures can be produced with negatively stained liposomes that contain no tubulin. The appearance of these negatively stained lipid preparations is specifically dependent on the metal stain used, rather than on the presence of protein. The possibility of a morphology being induced by the stain must be critically evaluated in this and other negatively stained lipoprotein systems.

MATERIALS AND METHODS

Preparation of Lipids

Dimyristoyl phosphatidylcholine (DMPC) and egg lecithin were obtained from Sigma Chemical Co., St. Louis, Mo. Lipids were suspended in chloroform, dried overnight under vacuum to remove solvent, and swollen for 6–18 h in phosphate-buffered 0.1 N saline (pH 6.75; 2 mM EDTA). The multilayered liposomes (10 mg of lipid/0.6 ml of buffer) were sonicated in a bath sonicator at 26°–30°C until nearly optically clear.

Preparation of Tubulin

Once- and twice-cycled tubulin proteins were prepared from homogenates of gray matter from adult cattle, following the procedures of Caron and Berlin (4).

Lipid-Tubulin Mixtures

The cold-soluble proteins resulting from one or two cycles of microtubule assembly were incubated with sonicated DMPC or egg lecithin vesicles at 30°C for at least 30 min. Lipid and protein were each suspended in 0.1 M NaCl, 0.02 M phosphate, pH 6.75, 2 mM EDTA, and mixed in ratios of 10 mg of lipid/0.5 mg of protein. Several specimens were prepared with higher protein content (10 mg of lipid/5 mg of protein).

Gel Electrophoresis

Lipid-protein suspensions were purified from nonadsorbed proteins and lipids by washing upwards through discontinuous Ficoll step gradients (Sigma Chemical Co.). The procedures of Caron and Berlin (4) were followed, except that the Ficoll gradients contained buffered saline. Gel electrophoresis on polyacrylamide slabs was done according to the Laemmli procedure, using 12% gels and 5% stacking gels (7). Samples of tubulin proteins, tubulin-protein mixtures, and protein standards were boiled in SDS for 2 min before application.

Protein and Lipid Concentrations

Protein was measured by Folin-Lowry assay (1). Lipid concentrations were determined by assaying total phosphorus (11).

Electron Microscopy

For electron microscopy, a drop of sample was placed on a Formvar-carbon-coated grid rendered hydrophilic by glow discharge before use. After 30 s, 3–4 drops of negative-staining solution were washed over the grid and the excess fluid was drained off with filter paper. The variables in preparative procedure included: (*a*) Negative stain: 1% phosphotungstic acid, 1% sodium tungstate, and 1% uranyl oxalate, all adjusted to pH 6.6 with sodium hydroxide. (*b*) Dilution: samples were

examined without dilution or diluted 1:10 or 1:20 with buffer. (*c*) Incubation time: some specimens of DMPC vesicles were examined immediately after sonication. All other samples were examined after 30–60 min at 30°C. Several samples were reexamined after 22 h and again after 3 d, at 30°C. (*d*) Temperature: after incubation at 30°C, some samples were examined after further incubation at room temperature (20°C) or after cooling on ice.

RESULTS

Analysis by gel electrophoresis of the protein content of lipid-protein mixtures confirmed the observation of Caron and Berlin (4) that the adsorbed protein patterns closely resemble those of the cold-soluble supernate from once- or twice-cycled tubulin. No differences exist in the protein populations adsorbed by DMPC or egg lecithin vesicles.

When lipid controls or the lipid-protein aggregates are negatively stained, their appearance varies remarkably with the choice of metal stain. With uranyl oxalate, the grid is covered with unilamellar vesicles and an occasional closed multilayered vesicle (Fig. 1 *a* and *b*). Many of the single vesicles appear to be collapsed and some are flattened. Flattened vesicles rarely associate with each other and form stacks limited to three or four members. These small stacks are only seen where the liposomes and the stain have been concentrated into pools by the drying process. The presence of tubulin in the sample does not alter this appearance of primarily single bilayer liposomes (Fig. 1 *b*).

If sodium tungstate is used as the negative stain, the picture changes dramatically. No single bilayer liposomes can be found. The lipid forms an anastomosing network of multilayered lamellae with a periodicity of ~55 Å that covers the surface of the grid (Fig. 1 *c* and *d*). This appearance results whenever sodium tungstate is used for staining, whether or not tubulin is present, whether or not the sample is diluted, and whether the sample is kept at 30°C or cooled on ice.

Phosphotungstic acid produces stacking of single vesicles and multilamellar structures whether tubulin is present or absent (Fig. 2). The extent of stacking induced by PTA staining is somewhat variable, however, and is not always consistent even on a single grid. Generally, stacking is apparent in all grid squares. In many samples, single liposomes coexist with stacks; in others, virtually all liposomes are stacked. With the conditions we used (i.e., pH 6.6), it was difficult to make grids that showed no stacking at all, though in several cases it was necessary to search the grid for stacked areas. After longer incubation times, stacking is reduced. Cooling on ice after incubation at 30°C also greatly increases the number of single bilayer liposomes. When liposomes are formed from DMPC, two types of stacking as reported by Caron and Berlin (4) are observed: columns of flattened single vesicles with a periodicity of ~115 Å (Fig. 2 *a* and *b*) and broader multilamellar sheets with a period of ~55 Å (Fig. 2 *c* and *d*). In most samples, the stacks are predominantly of one type or the other, but they may coexist (Fig. 2 *e*). When liposomes are formed from egg lecithin, the two types of stacking are also observed but the lamellar arrays with a period of 55 Å curve around the surfaces of clusters of unilamellar liposomes rather than form flat stacks (Fig. 2 *f*). We have found no single variable that predicts the type of stacking seen on PTA-stained grids, although increased lipid concentration and increased stain density seem to produce more of the multilayered structures relative to the columns of flattened vesicles.

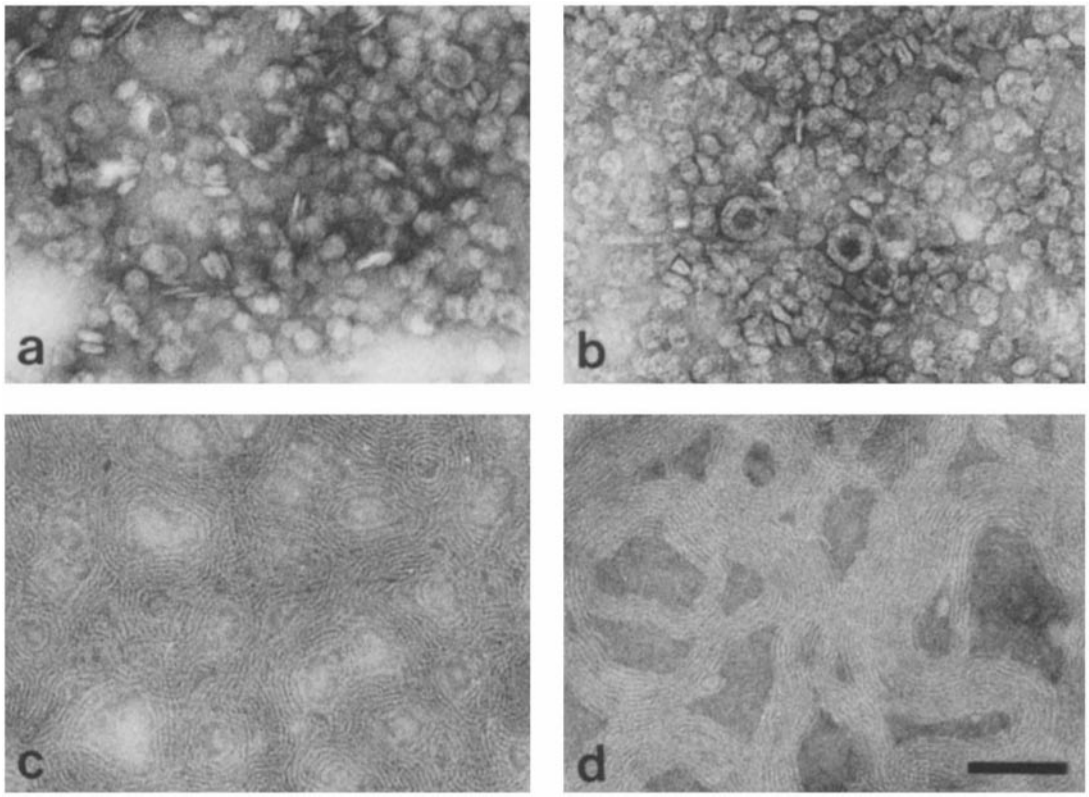

FIGURE 1 Liposome preparations negatively stained with uranyl oxalate (*a* and *b*) or sodium tungstate (*c* and *d*). (*a*) DMPC, incubated 30 min at 30°C, undiluted. (*b*) DMPC plus tubulin, incubated 30 min at 30°C, undiluted. (*c*) Egg lecithin, incubated 30 min at 30°C, diluted 1:10. (*d*) DMPC plus tubulin, incubated 30 min at 30°C, diluted 1:10. The appearance of unilamellar liposomes is maintained in uranyl oxalate. In sodium tungstate, the lipids form a multilamellar network with a 55 Å periodicity. The presence of tubulin (*b* and *d*) does not affect the stain-determined morphology. Bar, 0.1 μm. × 126,000.

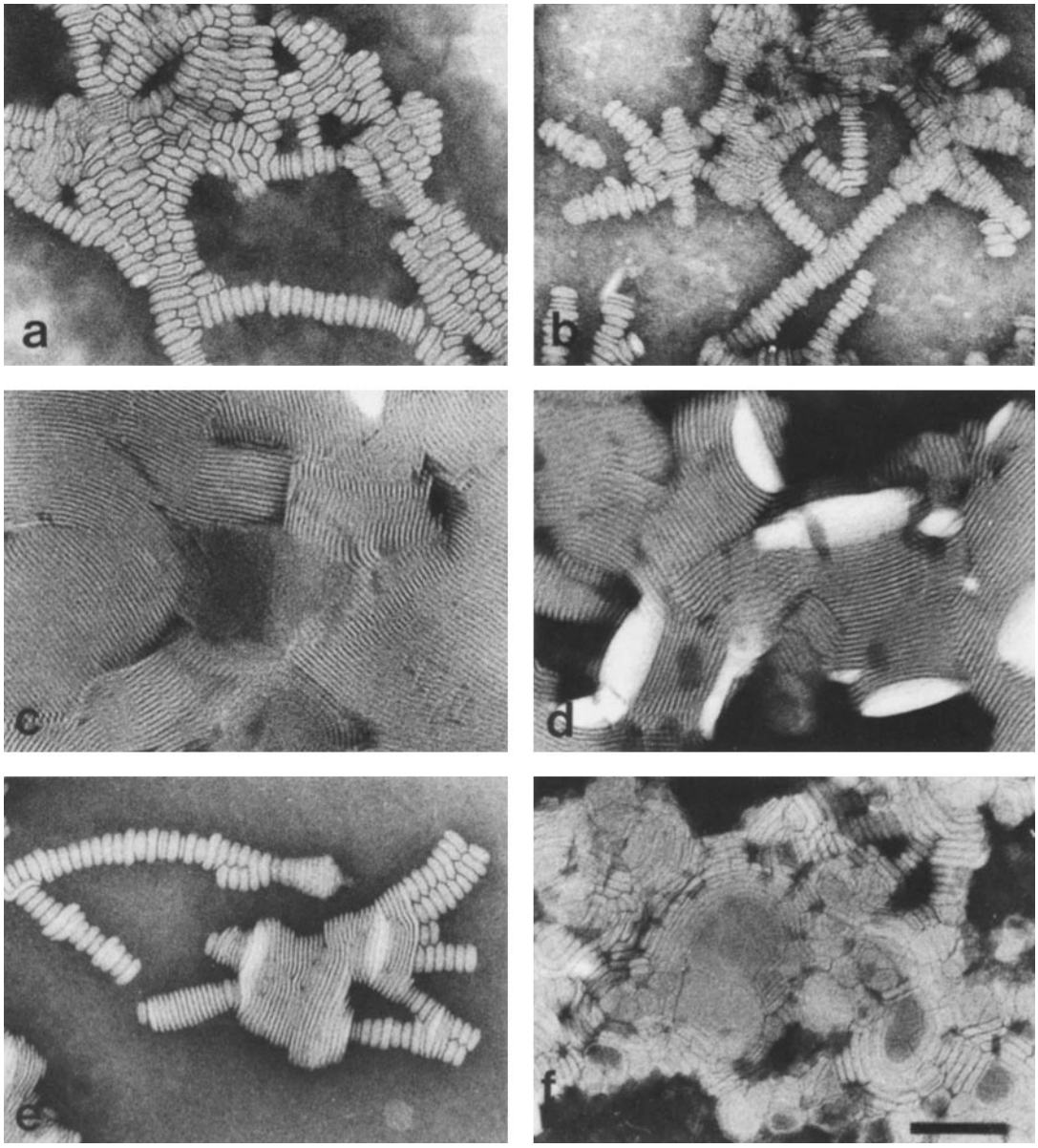

FIGURE 2 Liposome preparations negatively stained with PTA. (*a*) DMPC, incubated 30 min at 30°C, undiluted. (*b*) DMPC plus tubulin, incubated 30 min at 30°C, undiluted. (*c*) DMPC, incubated 30 min at 30°C, undiluted. (*d*) DMPC plus tubulin, incubated 30 min at 30°C, diluted 1:20. (*e*) DMPC, fresh from sonication, undiluted. (*f*) Egg lecithin plus tubulin, incubated 30 min at 30°C, undiluted. When DMPC liposomes (*a–e*) are negatively stained with PTA, two types of stacking are seen. Columns of flattened vesicles have a periodicity of 115 Å (*a* and *b*), whereas broader multilamellar structures have a periodicity of 55 Å (*c* and *d*). Both types of stacking can coexist (*e*). Liposomes made from egg lecithin (*f*) show similar columns of flattened vesicles but not the broader stacks. Instead, lamellae of 55 Å periodicity curve around clusters of single liposomes. Again, the presence of tubulin does not affect the morphology. It is interesting that Plate IX in reference 3 looks remarkably like *f* although the stacked vesicles were obtained from a bulk lipid dispersion treated with lysolecithin before staining. Bar, 0.1 μm. × 126,000.

DISCUSSION

We have confirmed that tubulin proteins are adsorbed onto single bilayer vesicles formed from neutral lipids. Our results show that the stacking seen after negative staining with PTA, however, is produced by interaction of the negative stain with lipid vesicles. When lipid-tubulin vesicles are stained with uranyl oxalate they appear to be unstacked unilamellar liposomes. Thus, the interactions between tubulin proteins and lipids may be less strong and specific than suggested by Caron and Berlin.

The results reported here serve to illustrate, perhaps more directly than has been done previously, the hazards involved in the interpretation of negatively stained specimens, particularly those of lipid phases in aqueous media. Warnings have been issued previously about possible artifacts resulting from the technique (2, 3, 5, 6). It has been suggested that the ionic constitution of the negative-staining solution may determine whether a lipid assumes a lamellar or micellar configuration, and that changes in pH, which are uncontrollable during drying, may be of importance (3, 5, 6). Local changes in concentration of both lipid and stain are also a consequence of

drying and appear to affect morphology in the PTA-stained samples. PTA has been implicated previously in the formation of multilayered structures (12). Both ammonium molybdate (10) and potassium phosphotungstate (8, 9) have been used successfully by other workers to preserve the appearance of unilamellar liposomes.

The lipid structures in these experiments are clearly labile and respond with rapid morphological changes to changes in their environment. Because multilayered stacking is seen with both DMPC (melting temperature, $T_m = 23°C$) and egg lecithin ($T_m = 0°C$), this morphology is not strongly affected by phase changes produced by cooling of the specimen during grid preparation. Deliberate cooling of the lipids below their phase transition temperatures before grid preparation reduces but does not eliminate the stacking induced by PTA and does not alter the multilayered network seen with sodium tungstate. Our results reemphasize the suggestion by Bangham et al. (2) that it is prudent to use as many different negative stains as possible, as well as freeze-fracture, when examining lipid-containing samples.

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REFERENCES

1. Bailey, J. L. 1967. Techniques in Protein Chemistry. Elsevier North Holland, Amsterdam. 340–341.
2. Bangham, A. D., M. W. Hill, and N. G. A. Miller. 1974. Preparation and use of liposomes as models of biological membranes. *Meth. Membr. Biol.* 1:1–68.
3. Bangham, A. D., and R. W. Horne. 1964. Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. *J. Mol. Biol.* 8:660–668.
4. Caron, J. M., and R. D. Berlin. 1979. Interaction of microtubule proteins with phospholipid vesicles. *J. Cell Biol.* 81:665–671.
5. Glauert, A. M. 1965. Factors influencing the appearance of biologic specimens in negatively stained preparations. *Lab. Invest.* 14:331–341.
6. Glauert, A. M., and J. A. Lucy. 1968. Electron microscopy of lipids: Effects of pH and fixatives on the appearance of a macromolecular assembly of lipid micelles in negatively stained preparations. *J. Microsc. (Oxf.).* 89:1–18.
7. Greenfield, S., W. T. Norton, and P. Morell. 1971. Quaking mouse: Isolation and characterization of myelin protein. *J. Neurochem.* 18:2119–2128.
8. Hauser, H., M. C. Phillips, and M. Stubbs. 1972. Ion permeability of phospholipid bilayers. *Nature (Lond.).* 239:342–344.
9. Huang, C. 1969. Studies of phosphatidylcholine vesicles. Formation and physical characteristics. *Biochemistry.* 8:344–351.
10. Johnson, S. M., A. D. Bangham, M. W. Hill, and E. D. Korn. 1971. Single bilayer liposomes. *Biochim. Biophys. Acta.* 233:820–826.
11. Marinetti, G. V. 1962. Chromatographic separation, identification and analysis of phosphatides. *J. Lipid Res.* 3:1–20.
12. Miyamoto, V. K., and W. Stoeckenius. 1971. Preparation and characteristics of lipid vesicles. *J. Membr. Biol.* 4:252–269.