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Association of the Membrane-Penetrating Polypeptide Segment of the Human Erythrocyte MN-Glycoprotein with Phospholipid Bilayers. I. Formation of Freeze-Etch Intramembranous Particles

(electron microscopy/protein-lipid interactions/protein-protein interactions/liposomes/micelles)

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ABSTRACT **The membrane-penetrating segment of the surface MN-glycoprotein of the human erythrocyte is contained intact within the tryptic peptide T(is). We report here on the association of this peptide with hydrated phospholipid vesicles. Under these conditions 80 Å intramembranous particles, as seen by freeze-etch electron microscopy, are produced that are similar in size to those seen in the native erythrocyte membrane. These particles increase in number as a linear function of T(is) concentration and a plot of particle number versus concentration is compatible with a micelle-like phenomenon; from this curve the critical concentration for the formation of particles is estimated to be approximately one mole of T(is) to 120 moles of lecithin. These data suggest that the membrane-penetrating peptide T(is) is being incorporated, monomerically and multimerically, within the hydrocarbon phase of lecithin bilayers.**

From these data it can be calculated that each intramembranous particle contains between 10 and 20 T(is) monomers. The peptide portion of each particle, therefore, has a molecular weight of 45,000–85,000.

An exact analogy cannot be drawn at this time between the *in vivo* structure of erythrocyte intramembranous particles and the reconstituted particles described here, although an argument has been constructed to support this possibility. What is clear is that the reconstituted system promises to be useful for further examination of protein-lipid interactions in membranes.

It is accepted by most workers that the so-called intramembranous particles seen in freeze-etch electron microscopic preparations of most biological membranes represent real entities *in vivo* that are localized predominantly in the hydrophobic plane of the phospholipid bilayer of biological membranes (1–3) and are at least partly composed of protein (4, 5). These particles have a diameter of 70–80 Å in the erythrocyte membrane (6). Changes in particle number and distribution are related to pinocytotic activity (7), the cell growth cycle (8), cell transformation (9), and the conformation of the paraffin chains of lipids (10).

Questions concerning intramembranous particles remain. We know neither the relative role played by lipids, as opposed to proteins, in their formation nor the physical, chemical, and structural nature of their protein components (including molecular weight). Even to be able to say that a protein with a molecular weight of 100,000 forms intramembranous particles is relatively meaningless without knowledge of how many copies of the protein are involved per particle and what portion of a single copy is involved.

One approach to the further understanding of intramembranous particles is to reconstitute these particles from component elements. For example, intramembranous particles have been observed in freeze-etch preparations of purified rhodopsin from rod outer segment membranes associated with phospholipid liposomes (5). Unfortunately, relatively little is known about the detailed structure of the rhodopsin molecule *in situ* (11) and when associated with lipids (5, 12), other than evidence that it penetrates the bilayer.

The membrane protein whose *in situ* structure is perhaps most clearly defined is the major surface glycoprotein of the human erythrocyte, the MN-glycoprotein (4, 6, 13). The MN-glycoprotein has been shown to penetrate the erythrocyte membrane (13–15). The intramembranous portion of this molecule has a known amino acid sequence (16) with a linear distribution of polar and nonpolar residues identical to the polar–nonpolar–polar cross section of a phospholipid bilayer. The 23 residue central hydrophobic portion or nonpolar domain of this sequence appears to belong to a special class of membrane-penetrating protein segments on the basis of degree of hydrophobicity (17, 18; §). There is evidence that this nonpolar domain is at least partially helical *in situ* (18; §).

The nonpolar domain is contained intact within a hydrophobic tryptic peptide (35 residues and a residue weight of 3700) from the MN-glycoprotein (13, 16, 19). The amino-acid sequence of this peptide is as follows:

Val-Gln-Leu-Pro-His-Pro-Phe-Ser-Glu-Ile-Glu-Ile-Thr-Leu-Ile-
5 10 15
Gly-Phe-Gly-Val-Met-Ala-Gly-Val-Ile-Gly-Thr-Ile-Leu-Leu-Ile-
20 25 30
Ser-Tyr-Gly-Ile-Arg.
35

The nonpolar domain extends from residue 12 to residue 34. The experiments reported here involve the complexing of this peptide with hydrated phospholipid vesicles. Under these conditions intramembranous particles are produced similar in size to those seen in intact erythrocytes and other cell membranes.

MATERIALS AND METHODS

Preparation of the Hydrophobic Peptide T(is) and Phospholipid. MN-glycoprotein was purified as described elsewhere (20). The solubilized glycoprotein was hydrolyzed with Tos-PheCH$_2$Cl treated trypsin (Worthington Biochemical Corp.).

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§ J. P. Segrest, D. G. Dearborn, and L. D. Kohn, manuscript submitted for publication.

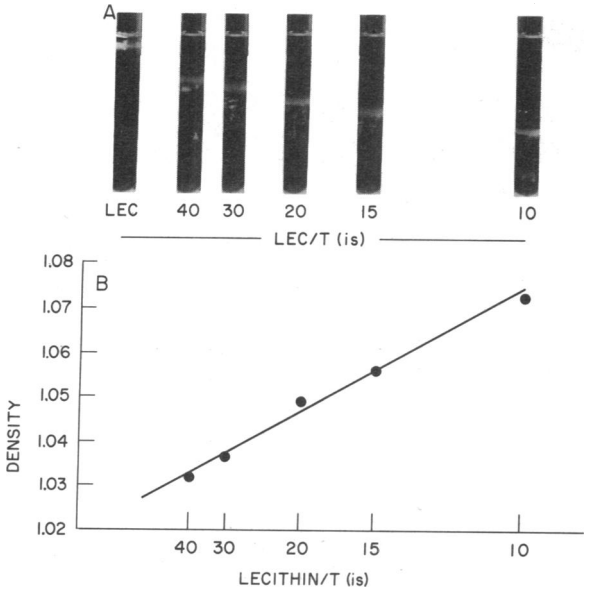

FIG. 1. Sucrose density gradients of complexes formed between the membrane-penetrating peptide T(is) and lecithin as concentration of T(is) is increased. (*A*) Gradients. Samples are from *left:* Lecithin (*LEC*) alone and lecithin/T(is): 40, 30, 20, 15, and 10 moles/mole. (*B*) Plot of equilibrium density (g/ml) of each T(is)–lecithin complex versus concentration of T(is).

The hydrophobic peptide, T(is), precipitated from solution. This precipitate was centrifuged at 43,000 × *g* for 30 min at 4° and washed three times with 15% formic acid for removal of residual sialoglycopeptides. The peptide has been characterized elsewhere (13, 16).

T(is) can be solubilized in 2,2,2-trifluoroethanol (spectro quality, Matheson, Coleman, and Bell) by the following procedure. One milligram of T(is) at the bottom of a conical tube is rapidly solubilized in 20 μl of trifluoroacetic acid (Aldrich Chemical Co.) under anhydrous conditions. Immediately the trifluoroacetic acid is made to a concentration of 0.5% in trifluoroethanol and the solution dried *in vacuo* for 24 hr over a P_2O_5 trap. T(is) can now be solubilized in 100% trifluoroethanol to a maximum concentration of approximately 0.5 mg/ml.

Egg lecithin was prepared by the procedure of Singleton *et al.* (21).

Preparation of Hydrated Phospholipid Liposomes Containing T(is). In a typical experiment 0.1–2 mg of T(is) in trifluoroethanol was mixed with 4 mg of egg lecithin in absolute alcohol and taken to dryness on a rotary evaporator with aspirator vacuum at 30°. After the last residual solvent was removed under oil diffusion vacuum with a P_2O_5 trap for 30 min, 0.3 ml of distilled water was added and the lecithin–peptide complex suspended by agitation with a pasteur pipette.

Sucrose Density Gradients. Sucrose gradients were prepared in 5-ml centrifuge tubes between the densities 1.01 g/ml and 1.12 g/ml. Each hydrated lecithin-peptide associate (0.3 ml) was layered on top of a gradient and the tubes were centrifuged at 20° for 15 hr at 300,000 × *g*. The lecithin–peptide association bands are easily seen as the result of light scattering.

Freeze-Etch Electron Microscopy. Peptide–lecithin bands were removed from the gradient tubes by aspiration with micro syringes. The aspirated material was resuspended in 2 ml of distilled water in conical centrifuge tubes and centrifuged three times at 24,000 × *g*. The peptide–lecithin associations form a cloudy suspension at the bottom of the tubes.

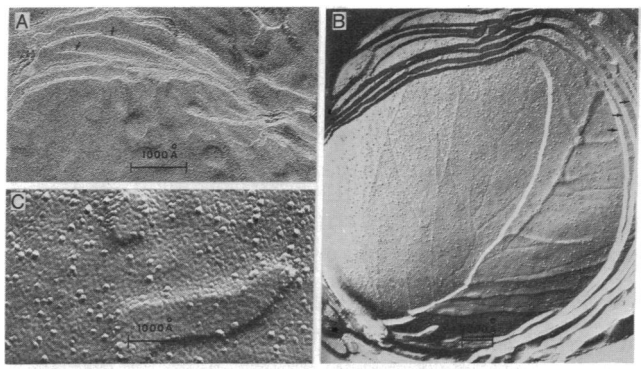

FIG. 2. Freeze-cleavage replicas of multilamellar structures formed by lecithin and T(is)–lecithin association. (*A*) Lecithin showing a multilamellar structure and smooth fracture face. (*B*) T(is)–lecithin complex [1 mole of T(is) to 20 moles of lecithin] showing a multilamellar structure and a particulate fracture face. (*C*) Higher magnification of T(is)–lecithin complex of same peptide concentration.

The washed liposomes were resuspended in from 0.1 to 0.3 ml of distilled water at room temperature. Small droplets of these preparations were placed on 3-mm copper planchets and were rapidly frozen in liquid Freon 22. The specimens were freeze-cleaved at −100° without etching and shadowed with platinum-carbon in a Balzer's BAF 300 freeze-etch apparatus (Balzer High Vacuum Corp.). The platinum-carbon replicas were floated off the planchets with distilled water, cleaned with Clorox, picked up on electron microscope grids, and examined in a Philips EM 200 electron microscope.

RESULTS

Sucrose density gradient equilibrium runs on mixtures of peptide and lecithin of varying proportions are shown in Fig 1*A*. A single band representing the association product is formed at each peptide concentration. Fig. 1*B* shows that the density of the association product formed in each case is a linear function of peptide concentration.

Fig. 2*A* shows the appearance of hydrated egg lecithin multilamellae when examined by freeze-etch electron microscopy. The surfaces exposed by freeze-cleavage without etching represent the hydrophobic surface of one leaflet of an individual bilayer making up the multilamellae (1–3, 22). These surfaces are smooth. The stairstep effect (*arrows*) represents points where the fracture plane has jumped to adjacent bilayers. The thickness of each step is less than 100 Å and represents two adjacent bilayer halves in contact along their polar surfaces.

By contrast, a T(is)–lecithin complex (1 mole/20 moles) is shown in Fig. 2*B*. The multilamellar nature of this complex can be clearly seen, strongly suggesting that the bilayer structure of hydrated lecithin has been maintained. There are two major differences in appearance of this complex from lecithin alone. First, the thickness between lamallae has increased to 400–500 Å and suggests that considerable water is present between adjacent bilayers, probably due, in part, to the presence of charged residues in the incorporated T(is) (12). Second, the fracture faces of the bilayers are no longer smooth but contain particulate structures. Fig. 2*C* shows a higher magnification of these particles. Under the conditions of this

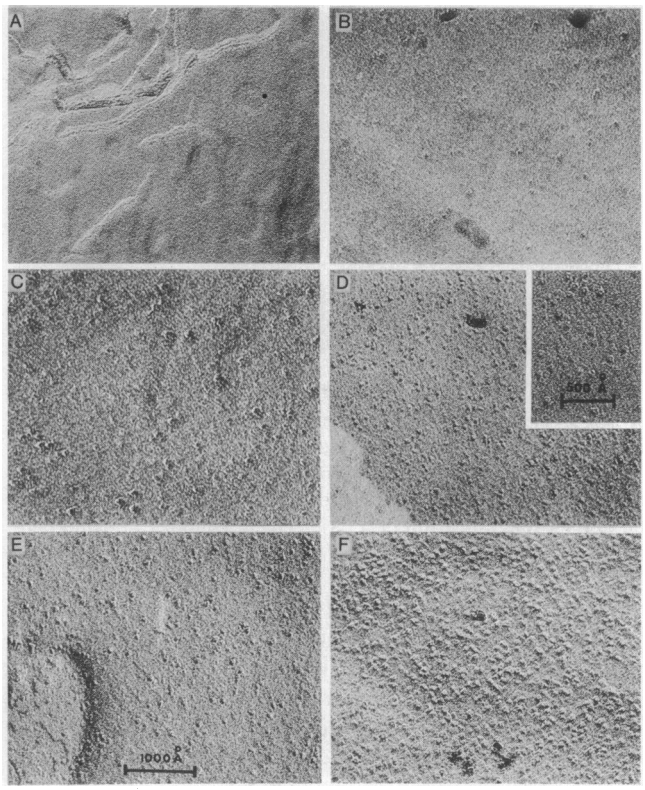

FIG. 3. Freeze-cleavage replicas of T(is)–lecithin complexes at increasing T(is) concentrations. (*A*) Lecithin. (*B*) 1 mole of T(is) in 40 moles of lecithin. (*C*) 1 mole of T(is) in 30 moles of lecithin. (*D*) 1 mole of T(is) in 20 moles of lecithin. (*E*) 1 mole of T(is) in 15 moles of lecithin. (*F*) 1 mole of T(is) in 10 moles of lecithin. The *insert* in (*D*) at a higher magnification demonstrates that many of the particles have a central cavity, i.e., are torus-shaped.

particular replica two distinct populations of particulate objects are seen. There is one population of quite distinct particles (major particles) which are separate from one another, and a second population of spherical irregularities which form a granular background. The major particles all project sufficiently far above the fracture plane (30 Å or so) to have penetrated through the complementary half of the bilayer that has been removed by cleavage. The cobblestone-like granularity is seen only when the angle formed by the direction of platinum-carbon deposition and the replica surface is sufficiently acute to project long shadows.

Fig. 3 shows the freeze-cleavage appearance of peptide–lecithin complexes as peptide concentration is increased. Only the major particles are apparent in these replicas. Clearly, the number of these particles increases with peptide concentration. The number of major particles per μm^2 has been plotted against peptide concentration in Fig. 4. Within the limits of experimental error the increase in particles is a linear function of peptide concentration. These results strongly indicate that the major particles are at least partially constructed of peptide material and, together with the results of Figs. 1 and 2, suggest that the hydrophobic peptide from the MN-glycoprotein is being incorporated within the hydrocarbon phase of lecithin bilayers.

The distribution of major particle diameters has been plotted for lecithin:peptide molar ratios of 40, 20, 15, and 10 in Fig. 5. From these plots it is clear that the major particles have a reasonably constant diameter with a mean of between

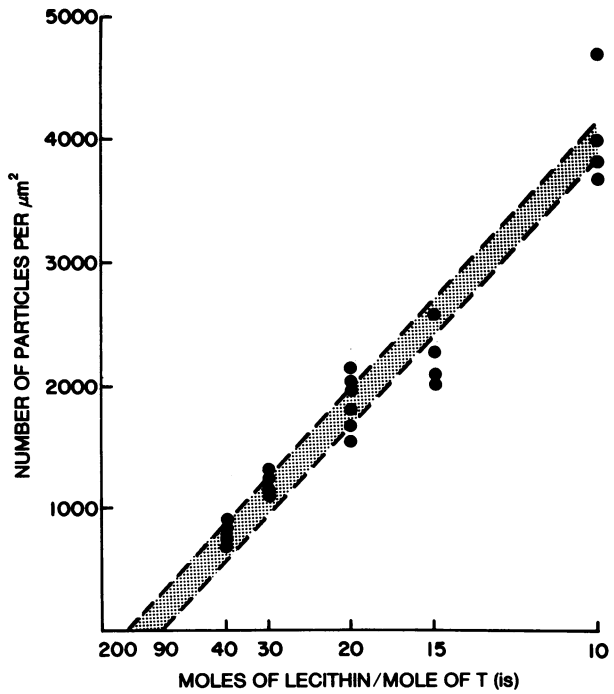

FIG. 4. Particle count of T(is)–lecithin associations plotted against T(is) concentration. Each point represents a particle count done over an area of 103 cm^2 (4 $inches^2$) on each print extrapolated to particles per μm^2. Rather than attempting to fit a unique straight line to the data points, we have indicated a range of possible straight lines (*shaded area* within *broken lines*).

70 and 80 Å. This diameter is independent of peptide concentration within the limits of experimental error. On high quality replicas a significant fraction of the major particles exhibit a central cavity (see *insert* to Fig. 3*D*) which gives them a torus-like shape.

DISCUSSION

The curve of particle number versus peptide concentration (Fig. 4) is indistinguishable from that of a micellization curve, such as the micelle concentration of sodium dodecyl sulfate in water plotted as a function of the concentration of the compound (23). Micellization has been shown to also occur for amphipathic molecules in nonaqueous solvents (e.g., phospholipid molecules in hexane). It is characteristic of micelle formation that aggregation begins at a specific concentration for a given micelle-forming compound and a given solvent at a constant temperature (24). This specific concentration, called the critical micelle concentration, can be calculated by extrapolating the straight line of a micellization curve to zero micelle concentration. From Fig. 4 one can see that the curve of particle number versus T(is) concentration extrapolates to zero at a concentration range of 1 mole T(is) to 90–200 moles of lecithin. This will be referred to as the critical multimer concentration for T(is) in egg lecithin.

Note from Fig. 5 that the diameters of the particles with a definite torus shape (Fig. 3*D*) lie within a narrower range than that of the complete spectrum of major particles, and the torus-like particles have a mean diameter of 80 Å.

It is now possible on the basis of Figs. 4 and 5 to calculate the approximate number of T(is) monomers present in each major particle. It has been determined that a phosphatidyl choline molecule takes up an area of approximately 65 $Å^2$

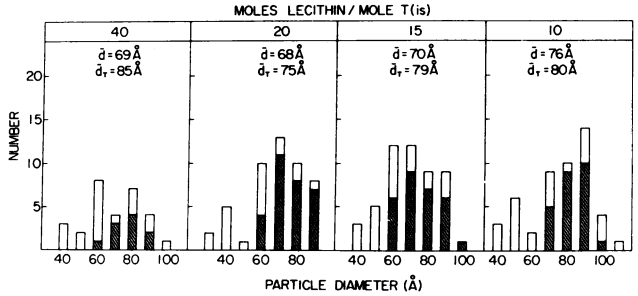

FIG. 5. Bar graphs showing particle diameter distribution for the concentrations of 1 mole of T(is) in 40, 20, 15, and 10 moles of lecithin. The *hatched bars* represent the distribution of diameters for the particles in which central cavities can be clearly identified (i.e., torus-shaped particles shown in Fig. 3*D*). $\bar{d}$, mean diameter of all major particles; $\bar{d}_T$, mean diameter of torus-shaped particles (*hatched bars*).

when above its phase transition temperature (24). Since it has been shown that rapid freezing of lipid–water preparations preserves the structure of the high-temperature phases (22), the number of lecithin molecules present in a given area of bilayer can be calculated. By circular dichroism, T(is) has been shown to be at least partially helical when incorporated into lecithin bilayers (18).§ The cross-sectional area of an α-helix is approximately equivalent to three lecithin molecules. Therefore, the following expression can be derived:

$$65([L] + 3\,[L]/M_T) = A$$

where $[L]$ = number of molecules of lecithin in area A
A = area in Å² of bilayer for which a particle count has been performed
M_T = molar ratio of lecithin/T(is) (e.g., 10, 15, etc.)
and $[L]/M_T$ = number of T(is) molecules in area A.

Using this formula it can be calculated that there are approximately 25 ± 2 T(is) monomers per major particle.

This is, of course, an approximation. Several possible factors have not been taken into account. First, there likely is a certain amount of T(is) present as the monomer even at the highest concentration. The theory is that once micelle formation begins the concentration of monomer is thereafter approximately constant (23). Assuming, then, that residual monomer concentration is equal to the monomer concentration at the critical multimer concentration (determined by extrapolation of Fig. 4), one arrives at a new value of 23 ± 3 T(is) monomers per major particle or a molecular weight of 85,000 ± 11,000 for the multimer.

A second factor affecting the calculation of monomers per particle is the assumption made for T(is) cross-sectional area. If it is assumed that T(is) takes up zero space, an unlikely possibility, then the calculation is 33 ± 3 monomers per particle. On the other hand, if one assumes that each T(is) helix is oriented parallel to the plane of the bilayer (i.e., takes up an area equivalent to eight lecithin molecules), then the calculation is 18 ± 3 monomers per particle (see proposed model in Fig. 6).

There is an independent way of calculating the number of monomers per particle. An 80 Å particle takes up an area of approximately 5000 Å². An α-helix has a cross-sectional area of approximately 200 Å². Therefore, into a particle 5000 A² in cross section one could pack approximately 25 α-helixes.

The question of the origin of the cobblestone-like granularity to the cleavage face, so clearly seen in Fig. 2*B* and *C*,

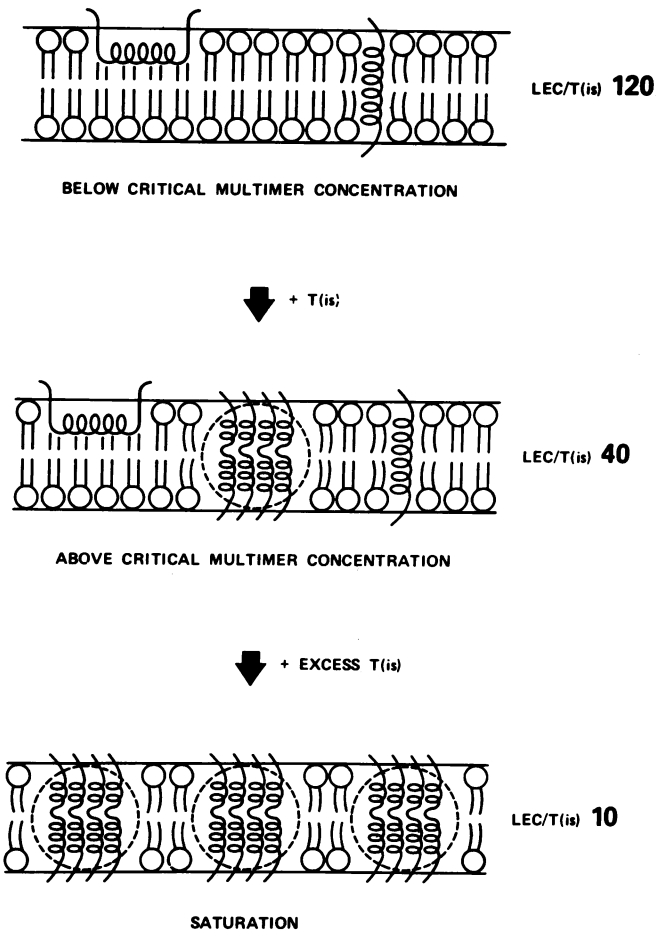

FIG. 6. Schematic diagram of a proposed model for interactions of the membrane-penetrating peptide T(is) with hydrated lecithin (*LEC*) vesicles. The peptide, which is partially helical when associated with lecithin (18; §), could be either oriented perpendicular or parallel to the paraffin chains of the phospholipid bilayer. However, because the intramembranous particles appear to penetrate the bilayer and because of the topographical distribution of charge along the T(is) molecule, the orientation of the peptides forming these structures likely is parallel to the hydrocarbon chains.

cannot be completely answered. It seems most likely that this phenomenon is due to some alteration in the packing of the lecithin molecules by the presence of monomeric or oligomeric T(is) intercalated into the bilayer. Using the formula derived previously, one finds that at a concentration range of 1 mole T(is) to 90–200 moles of lecithin (the postulated critical multimer concentration), there would be from 7,600–16,000 T(is) monomers per μm^2 of lecithin bilayer. A count of the cobblestone-like background particles from Fig. 2*C* shows there are approximately 12,000 of these structures per μm^2. The overlap of these calculations is compatible with the possibility that each T(is) monomer incorporated into a lecithin bilayer corresponds to one background particle.

If, however, the background particles represent incomplete multimers or oligomers of T(is), an exact calculation of the degree of multimerization of the "mature" torus-shaped particles is precluded, given the present data. One can only calculate a multimeric range: 8–20 T(is) molecules/"mature" particle.

The data suggest the model shown in Fig. 6 for the formation of intramembranous particles of 80 Å diameter as the

intramembranous peptide T(is) is solubilized in hydrated egg lecithin bilayers.

At this point any relationship suggested between the 80 Å particles present in T(is)-lecithin complexes and the *in vivo* 80 Å freeze-etch particles present in erythrocyte membranes is speculative. It is interesting that both have similar diameters and that the MN-glycoprotein has been shown to be associated with the intramembranous particles of the erythrocyte (6).

The number of MN-glycoprotein molecules per intramembranous particle of the human erythrocyte membrane can be estimated by the methods described. Pertinent data for this calculation are as follows: (*a*) there are 4,200 particles/μm^2 in the whole membrane (25); (*b*) phospholipid represents 24% (26) and the glycoprotein 5% (20) of the membrane dry weight; and (*c*) the molecular weight of the MN-glycoprotein is approximately 30,000 (13, 27). Using the formula derived previously one calculates approximately 4 MN-glycoprotein molecules per particle.

However, this calculation and similar previous ones (4, 14) contain the unproven and, in retrospect, somewhat unlikely assumption that particles on both fracture faces of the erythrocyte membrane contain MN-glycoprotein. It seems more likely that the MN-glycoprotein, with a bulky and highly charged outer glycoprotein coat, would stay with the outer fracture face. There clearly is a marked difference in the particle distribution between the outer and inner fracture faces [1400 versus 2800 particles/μm^2, respectively (25)], suggesting at least two different types of particles. Further, when the intact erythrocyte is treated with trypsin, preliminary results suggest that the outer fracture face particles are slightly altered in size and distribution but not the inner (unpublished results). This suggests a correspondence between the MN-glycoprotein and the outer particles, since the *N*-terminal (outer) end of the MN-glycoprotein is markedly degraded under these conditions (13, 19).

Calculating as before but assuming 1400 particles/μm^2 one arrives at a new number of 12 MN-glycoprotein molecules/particle. This calculation is within the lower limits of the multimeric range of T(is) in egg lecithin calculated previously assuming the presence of oligomers.

The MN-glycoprotein, isolated by the lithium diiodosalicylate procedure (20), exists as multimers with molecular weight of 3–4 $\times$ 10^5 when dissolved in aqueous solvents (personal observation). Each multimer thus corresponds to 10–14 molecules of glycoprotein. The close correspondence of this multimeric number to that calculated in the previous paragraph for the MN-glycoprotein *in situ* raises the possibility, although speculative, that the *in situ* functional unit of the MN-glycoprotein is a multimer of 12 or so molecules and is isolated intact by the lithium diiodosalicylate extraction procedure.

Final speculations aside, our results show that the large particles that are observed on the fracture faces of most biological membranes can arise from the association of relatively small peptides. Further, the techniques described provide a useful model system for further study of the nature of protein–lipid interactions in membranes.

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