ence in perturbation of ^{13}C NMR spectra does not occur at the carbonyl but at unsaturated sites. Although a localized hydrocarbon interaction is possible, it is also possible that perturbations by GPA to the inner half of the bilayer are greater and that this difference results in preferential broadening of PE simply because of its preferential location on the inner half.

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REFERENCES

1. Chapman, D., J. C. Gomez-Fernandez, and F. M. Goni. 1979. Intrinsic protein-lipid interactions. Physical and biochemical evidence. *Fed. Eur. Biochem. Soc. Lett.* 98:211–223.
2. Van Zoelen, E. J. J., R. F. A. Zwaal, F. A. M. Reuvers, R. A. Demel, and L. L. M. Van Deenen. 1977. Evidence for the preferential interaction of glycophorn with negatively charged phospholipids. *Biochim. Biophys. Acta.* 464:482–492.
3. Armitage, I. M., D. L. Shapiro, H. Furthmayr, and V. T. Marchesi. 1977. ^{31}P nuclear magnetic resonance evidence for polyphosphoinositide associated with the hydrophobic segment of glycophorin *A*. *Biochemistry.* 16:1317–1320.
4. Buckley, J. T. 1978. Co-isolation of glycophorin *A* and polyphosphoinositides from human erythrocyte membranes. *Can. J. Biochem.* 56:349–351.
5. Shukla, S. D., R. Coleman, J. B. Finean, and R. H. Michell. 1979. Are polyphosphoinositides associated with glycophorin in human erythrocyte membranes? *Biochem. J.* 179:441–444.
6. Jokinen, M., and C. G. Gahmberg. 1979. Phospholipid composition and external labeling of aminophospholipids of human En(a-) erythrocyte membranes which lack the major sialoglycoprotein (glycophorin *A*). *Biochim. Biophys. Acta.* 544:114–124.
7. Gerritsen, W. J., E. J. J. Van Zoelen, A. J. Verkleij, B. De Kruijff, and L. L. M. Van Deenen. 1979. A ^{13}C NMR method for determination of the transbilayer distribution of phosphatidylcholine in large unilamellar, protein-free and protein-containing vesicles. *Biochim. Biophys. Acta.* 551:248–259.
8. Longmuir, K. J., and F. W. Dahlquist. 1976. Direct spectroscopic observation of inner and outer hydrocarbon chains of lipid bilayer vesicles. *Proc. Natl. Acad. Sci. U.S.A.* 73:2716–2722.
9. Bretscher, M. S. 1975. *C*-terminal region of the major erythrocyte sialoglycoprotein is on the cytoplasmic side of the membrane. *J. Mol. Biol.* 98:831–833.

WHAT IS A SUCCESSFUL RECONSTITUTION OF A MEMBRANE GLYCOPROTEIN THAT LACKS AN ENZYMATIC ACTIVITY?

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Sindbis virus is an enveloped virus composed of two surface glycoproteins associated with a lipid bilayer, a nucleocapsid of RNA, and a core protein. The life cycle of the virus is undoubtedly very similar to that of Semliki Forest virus and includes events that occur at two pH's (1). Adsorption to host cells is observed at neutral pH (1). In contrast, at pH below 6.7, Sindbis and Semliki Forest viruses adsorb to lipid vesicles containing cholesterol (2) and to red cells (3), cause hemagglutination and, in some cases, lead to hemolysis (3). During infection, intact viruses are internalized by cell membranes and transported into lysozomal vesicles. The low pH functions of a viral coat protein are activated by the pH inside the lysozomes (pH 5–6) so that the viral membrane fuses with that of the lysozome to release uncoated nucleocapsids into the cell cytoplasm. It has been postulated that one of the Semliki Forest virus glycoproteins, E1, is responsible for hemagglutination and fusion activities while the other, E2, exhibits the ability to bind to host cell surfaces at neutral pH (1, 3).

The mechanisms of the varied functions of the Sindbis virus coat proteins can be studied by using reconstituted membranes in which such parameters as lipid composition and lipid:protein ratio are manipulated and with which the functions of the viral coat can be separated from infection since the RNA core is absent. However, because no enzymatic activities are known to be associated with the Sindbis coat proteins, a question arises as to what constitutes a functional assay for successful reconstitution of the membrane glycoproteins.

RESULTS AND DISCUSSION

We have previously reported reconstitution of egg lecithin membranes containing the E1 and E2 glycoproteins of Sindbis virus (4). We demonstrated that the reconstituted structures resembled native viral membranes in lipid:protein ratio and in size, that the reconstituted membranes retained solutes such as 6-carboxyfluorescein (6-CF) and that the 6-CF-loaded membranes were internalized by macrophages. However, the uptake of the reconstituted membranes is difficult to compare quantitatively with that of the native virus. As a more precise test of functional reconstitution, we compare here hemagglutination by intact Sindbis virus and reconstituted membranes containing E1 and E2. When the results are expressed in terms of the number of virus particles per erythrocyte, it is apparent that this is an extremely sensitive assay because as

TABLE I

AGGLUTINATION OF GOOSE ERYTHROCYTES BY SINDBIS VIRUS AND BY RECONSTITUTED MEMBRANES CONTAINING THE SINDBIS COAT PROTEINS

Agglutinin*	P-lipid: protein	Number of virus particle equivalents§/ Number of red cells
	(*mol:mol*)	
Sindbis	25	<2
Fraction I	435	7
Fraction II	47	60
Fraction III	38	60

*Fractions I, II and III refer to reconstituted Sindbis E-protein membranes collected from a Sepharose 2B column. Fractions I, II and III are the early, middle and late thirds (by fraction number) of the elution profile.

‡The estimate of moles of lipid is based on phosphate assay and an assumed phospholipid mol wt of 750. For intact Sindbis, the stated P-Lipid:Protein refers to phospholipid only. With cholesterol counted, the Sindbis Lipid:Protein is 45. Protein assays were done by the Lowry procedure using bovine serum albumin as standard. All samples were prepared in saline weakly buffered with bicarbonate at pH 8.0. P-Lipid:Protein ratios are ± 10%.

§Calculated at the last serial dilution to show agglutination.

little as one virus particle (or 240 pairs of E proteins) per erythrocyte is required for hemagglutination to be observed.

Hemagglutination assays were carried out by a modification of the Clarke and Casals procedure (5). Goose erythrocytes were washed three times in three vol of a barbital buffer which differed from the Clarke and Casals buffer only in the omission of gelatin. Also, bovine serum albumin was omitted from the agglutinin preparations. Serial dilutions of the agglutinins in 100μl of isotonic phosphate buffer at the desired pH's were made in glass or plastic tubes. A volume of 100μl containing 10^6 goose erythrocytes was then added to each tube, the tubes were gently rocked, and the cells were allowed to settle for at least 1 h at 25°C.

Table I compares the ability of reconstituted membranes and native Sindbis virus to cause hemagglutination. The results are expressed as the number of virus particle equivalents (240 copies each of E1 and E2) required for the minimally detectable agglutination response. Interestingly, a fraction of reconstituted membranes which has about a 10-fold higher lipid:protein ratio than does the native virus membrane resembles the virus more closely (on a mole of protein basis) in this assay than do membranes of lipid:protein ratio approximating that of the intact virus. The reason for the lower activity of the latter reconstituted membrane fractions is under further investigation. Enzymatic iodination (6) of the viral coat proteins provides one test for the native conformation of the membrane-bound proteins because E1 is protected from iodination while in the membrane but not in detergent-solubilized form. The effects of lipid composition on the function are also being tested.

What event is involved in agglutination if so few viral proteins are required? A positive agglutination response is based on the erythrocytes settling in a thin film over the bottom of a test tube, or microtiter plate well, whereas a negative response shows cells that have settled into a more compact "button" (5). We find that cells which are positive for agglutination by these criteria do not appear at all aggregated when transferred to a microscope slide. Thus, agglutination may result from an alteration in the surface of the red cell membranes. It is possible that the answer to the question posed in the title of this manuscript is, for Sindbis virus, that there is in fact an enzymatic activity associated with the agglutination response. We are testing that possibility: low pH agglutination by Sindbis is inhibited by pretreatment at 62°C for 10 min. Alternatively, failure to observe aggregated cells under the microscope may mean that the forces between the cells are very weak.

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REFERENCES

1. Helenius, A., J. Kartenbeck, K. Simons, and E. Fries. 1980. On the entry of Semliki Forest Virus into BHK-21 cells. *J. Cell Biol.* 84:404–420.
2. Mooney, J. J., J. M. Dalrymple, C. R. Alving, and P. K. Russell. 1975. Interaction of Sindbis Virus with liposomal model membranes. *J. Virol.* 225–231.
3. Vaananen, P., C. G. Gahmberg, and L. Kaarianen. 1981. Fusion of Semliki Forest Virus with red cell membranes. *Virology.* 110:366–374.
4. Scheule, R. K., and B. J. Gaffney. 1981. Reconstituted vesicles containing the Sindbis Virus glycoproteins and entrapped markers. *In* Liposomes in the Study of Drug Activity and Immunocompetent Cell Functions. C. Nicolau editor. Academic Press, London.
5. Clarke, D. H., and J. Casals. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am. J. Trop. Med. Hyg.* 7:561–573.
6. Sefton, B. M., G. G. Wickus, and B. W. Burge. 1973. Enzymatic iodination of Sindbis Virus proteins. *J. Virol.* 730–735.