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DISCUSSION

Session Chairman: Patricia C. Jost *Scribe*: William J. Pjura

Jost: We'll begin with three questions from an anonymous referee: First, "In the data analysis you assume $r(0) = 0.40$. Hence, what is the point of listing fitted $r(0)$ values in Table I? In addition, $r(0)$ may be 0.40, but the observed value is likely to be dependent on the instrument and the alignment of its optics. Have you determined $r(0)$ for the fluorophores in your instrument?"

HUDSON: We have done this in our apparatus by collecting data for viscous solutions. The value of $r(0)$ is found to be 0.40. Values for $r(0)$ for the bilayer experiments are reported in our paper (Table I) because the values (0.35 to 0.41) demonstrate that we are not missing any part of the decay.

JOST: The other questions are (*a*) "The *cis* and *trans* isomers of parinaric acid yield distinct values for the limiting anisotropy ($r\infty$). Thus it appears that you may be measuring the order parameters of the probes and not of the membranes. How does this affect your analysis of the data, and your comparison of your order parameters with those found by NMR?" (*b*) "At 39°C the DMPC vesicles are above their phase-transition temperature. What is the effect of M13 coat protein when $T<T_c$? What is the effect on the order parameter of an unsaturated lipid like DOPC?"

HUDSON: The first question concerns experiments in the absence of protein where the asymptotic anisotropy observed for *cis* parinaric acid has a lower asymptotic anisotropy than that observed for *trans* parinaric acid. We believe that is simply due to the fact that *cis* parinaric acid has an extra depolarizing motion consisting of a rotation of the chain about its axis in addition to the other motions which occur. That means that if we're making comparisons to NMR experiments we should, in this case, compare to an oleic acid-like chain with the deuterium in position 9 rather than to a stearic acid-type chain with a deuterium in this position.

With respect to the second question, we haven't looked at any unsaturated lipids. We have looked at the low temperature phase primarily by static fluorescence measurements. The effect that we see is a small decrease in the order parameter when coat protein is in gel-phase lipids. Changes in static anisotropy are weighted in favor of species with higher quantum yield. DMPC bilayers containing coat protein appear to have a short lifetime component associated with the protein. This environmental lifetime effect results in a decreased importance for the perturbed region near the protein, and therefore the decrease in the order parameter may be larger than the observations suggest.

FEIGENSON: The state of aggregation of coat protein could influence the effect of the protein on the lipid-motional state. Have you determined whether the coat protein in your preparations was in the α-form or in the (polymeric) β-form?

HUDSON: We believe that the protein is in the monomeric state on the basis of an energy transfer experiment involving the tryptophan in the hydrophobic segment of this protein, reported in Kimelman et al. (1979. *Biochemistry.* 18:5874–5880). The fluorescence of this tryptophan acts as a label for measuring the distribution of the parinaric acid chromophore with respect to the protein. We get two pieces of information from this. The first is that in the quenching experiment, the decrease in tryptophan fluorescence as parinaric acid is added can be quantitatively described using the assumption that the parinaric acid and the protein are randomly distributed in the bilayer. If the protein were extensively aggregated, there would be tryptophan residues that would be too far from lipids to be quenched by parinaric acid. This does not exclude the possibility of dimers or trimers in the sample. There are no large aggregates.

FEIGENSON: I believe that the protein could still be aggregated and still be surrounded by lipid.

HUDSON: Yes, that is possible. In fact that is probably what happens at low temperatures. When you freeze the acyl chain the system probably phase-separates into one in which the proteins surrounded by ~20 acyl chains phase-separate from a pure lipid phase. We know that there is lipid surrounding that protein because we can still quench tryptophan. Phase-transition data (Kimelman et al., 1979) show that at high protein:lipid ratio the sharp transition of the pure lipid disappears. This indicates to us that there is no separate phase of pure lipid at high temperatures. Also, the static anisotropy is roughly proportional to the protein content.

FEIGENSON: Do you know whether your protein is 50% β-pleated sheet or 50% α-helical?

HUDSON: No, we haven't done the CD measurements.

FEIGENSON: But don't you know whether your protein is 50% α-helical or not? The conformation of the protein could affect the lipid that's around it.

HUDSON: Yes, it could.

WOLBER: While we can't say absolutely that we don't have aggregation problems in the fluid phase, we can make relevant probabilistic arguments. These involve the use of the two isomers of parinaric acid. The natural product, which is *cis-trans-trans-cis*, shows a slight preference for fluid lipids over gel-phase lipids. The *all-trans* isomer, which can be made from the natural product, shows a marked preference for

gel-phase lipids. So if we had a highly aggregated system, one would then make the argument that *trans* parinaric acid ought to partition preferentially into that system.

FEIGENSON: If the annular lipid is like a gel, in this case.

WOLBER: That is correct. If, on the other hand, the adsorbed lipid is not at all like the gel, I find it hard to believe that the protein could be highly aggregated, given the kinds of things that have been discussed.

FEIGENSON: There was an experiment discussed earlier where the rhodopsin in a highly-aggregated state was associated with very fluid lipid. So, I don't know if that line of reasoning is valid or not.

But here's another question. Is it possible that your protein is in a U-shape?

WOLBER: Not if we believe the characterization of these vesicles that Wickner has done. His characterization of vesicles made by this method demonstrates, at least to my satisfaction, that the protein is oriented and is *trans* membrane.

HUDSON: One other point concerning the energy-transfer measurement is that the agreement with a random distribution demonstrates that the probe molecules are partitioning randomly with respect to the protein so that there is no preferential binding.

LAKOWICZ: You indicated that about five lipids are bound around M13 coat protein. From your data can you estimate the correlation times and degree of hindrance for these particular lipids?

HUDSON: There is about a fourfold decrease in the rotational rate and a 30% decrease in the mean-square cone angle of the motion. This behavior is consistent with a perturbation of the acyl chains due to a blockage of the motion in the sense of a static barrier rather than in the sense of binding. We conclude that there are ~12–14 perturbed lipids/protein molecule in the fluid phase, corresponding to six on one side and six on the other, roughly the number of lipid molecules which would just surround the molecule. This analysis is based on the anisotropy curve shown in Fig. 3, where the anisotropy decays to a minimum value and then rises again. The only physically reasonable interpretation of an anisotropy decay that falls to a minimum and then recovers to a higher value is that there are two different populations in the sample that have two different lifetimes. At long times, the anisotropy is due to the remaining fluorescing species. In this case these are the molecules near the protein and as a result one gets an independent measurement of the limiting anisotropy of the molecules near the protein.

GEORGHIOU: The analysis of your fluorescence emission anisotropy data assumes that the binding of the coat protein to the phospholipid does not alter the location of the probe in the bilayer. Because there is a gradient of fluidity in the bilayer, changes in the location of the probe will affect the values of the extracted parameters, in particular those of the rotational correlation time.

HUDSON: I think that this can be excluded by a qualitative consideration. We are looking at the orientational behavior of a vector connected to position 9 of the acyl chain and, therefore, the order parameter associated with position 9. The order parameter gradient is essentially flat from the top of the bilayer down to position 9; consequently, moving the molecule upwards will not affect the order parameter. Moving it down will have an effect opposite from what is experimentally observed.

GEORGHIOU: Looking at your Table I, it appears that the reduction in the rotational correlation time in the presence of the protein depends on the parinaric acid isomer you use. For the *trans* isomer ϕ increases by a factor of ~3 whereas for the *cis* isomer, it increases by a factor of only ~1.25. However, the order parameter appears to change proportionally in both cases.

HUDSON: But the order parameter for the *trans* molecule is not a reflection of the average in the bilayer. It is a reflection of only the long lifetime species, which happens to be those molecules right next to the protein, so you can't really compare the order parameter for the *cis* and *trans* probes in the presence of the protein.

GEORGHIOU: What that means then is that one has to use more than one probe?

HUDSON: Yes. That's right.

LAKOWICZ: Have you done or are you doing the time-resolved decays for the tryptophan fluorescence?

HUDSON: We have started looking at the tryptophan polarization anisotropy in order to make comparisons with magnetic resonance measurements. This system is a particularly good case for doing that.

PRENDERGAST: Your data show an increase in the order parameter for *trans* parinaric acid when coat protein is added to the vesicle but no concommitant increase in lifetime. Usually an increase in lipid order correlates well with an increase in τ for the probe. How would you explain this, and does DPH yield data similar to those of tPNA?

HUDSON: It is true that there is usually a correlation between order and fluorescence lifetime. The basis of this correlation is not clear to me and, therefore, I do not feel particularly obliged to explain deviation from this correlation.

WEINSTEIN: What is the evidence for the location of parinaric acid in the bilayer, and is there any evidence regarding sidedness?

HUDSON: There is a good deal of evidence that it is on both sides of the bilayer (Sklar et al. 1977. *Biochemistry.* 16:819–828). The most relevant point on the location is that we get the same results with the labeled phospholipids as with pure fatty acids (Wolber and Hudson. 1981. *Biochemistry.* 20:2800–2810).

WEINSTEIN: So, with respect at least to the energy transfer and assuming an asymmetrically-disposed protein, there are two populations of parinaric acid.

HUDSON: That's correct.

EDELMAN: There are two important technical details that I wish you would explain in more detail. First, how clear is it that the excitation transfer experiments really indicate a uniform distribution of parinaric acid rather than only that parinaric acid gets in and out of the annulus in times short compared to its excitation lifetime? And, second, can you explain more carefully how you get the asymptotic anisotropy of the annular probe? As your discussion of the *cis* parinaric acid makes clear, the estimate of the annulus size is extremely sensitive to that anisotropy.

HUDSON: On the first point, the lifetime that's relevant to the energy transfer experiments is the tryptophan lifetime, which is on the order of nanoseconds; the translational hop time is $\sim 10^{-7}$ or 10^{-8} s, so that you can't move a fraction of the nearest-neighbor distance in the lifetime of the tryptophan. You're looking at a static-time-average distribution in that experiment. The asymptotic anisotropy for the *trans* parinaric acid experiment is directly related to the experimental value at long times, and that value is related to the annular region values because of the fact that the probe molecule in the annular region has the longer lifetime. If you wait long enough, the only thing left in your sample that's fluorescing and

is polarized to a certain extent is the species with the longer lifetime. We can show that it is the species close to the protein because that lifetime component increases when you increase the protein. In principle we can do energy transfer experiments to make sure that we're only looking at the species near the protein, but we haven't done that yet.

EDELMAN: Specifically, in the energy transfer experiment, how much heterogeneity would there have to be for you to see it? How much would the partition coefficient of parinaric acid between annular and bulk lipid have to differ from one?

HUDSON: I think that a 20% deviation of the partition coefficient from one would suffice. A factor of three really shifts the energy transfer quenching curve a lot, so we have a pretty good number on the partition coefficient. If there is any preference, the quenching responds very quickly.

T. THOMPSON: It seems to me that from the biological standpoint there are two important questions being discussed in this session and the previous one. (*a*) Why is it that *trans* membrane proteins do not cause leaks in the bilayer? (*b*) How can the interaction between the bilayer and the protein influence biological activity? I haven't heard anybody tell me anything that addresses these questions.

To put it differently, "so what?" So there is a boundary layer and the lipids bump against the protein because the bilayer stops there. What has that got to do with any problem of interest in biological membranes?

HUDSON: My conclusion, based on our measurements and the comparison with deuterium NMR measurements, is that the effect of a protein on the lipid around it is purely a surface effect with no specific chemical interest. It probably serves no real functional interest, except as modulation of the structure of the bilayer can influence things that may be functionally important, like the relative distribution of two protein subunits. I think that the more interesting protein-lipid interactions from a functional point of view probably involve specificity of particular lipid type for the protein. Our contribution is that we have taken a careful look at the acyl-chain dynamics with this powerful new method. It tells us something about other spectroscopic methods. For instance, in the absence of protein, we find no large amplitude relaxations in the 10^{-8}–10^{-7} s time region, although they had been postulated on the basis of analysis of proton NMR and, independently, from a comparison of deuterium and spin-label ESR measurements. That's not in agreement with our measurements on pure lipid systems. Whether this detailed dynamic information will lead to any subsequent functional information, I don't know.

LITMAN: Tom Thompson's comment is that we've been focusing on lipid dynamics and have been missing the point that there are protein dynamics in the membrane, and that the lipid may be there modulating the protein dynamics. There's very good evidence that a large number of functional proteins have to undergo a conformational change to be functional in a membrane. There's also evidence that the variability in the hydrocarbon region will control the kinetics of protin conformation changes. This has been shown very nicely by David O'Brien in his studies of the dependence of the metarhodopsin I - metarhodopsin II transition on the lipid matrix. I think that it is more than just a function of looking at the lipid dynamics. If we focus more attention on the effects of the lipids on the protein dynamics, we'll find that the lipid hydrocarbon region is, in fact, controlling vibrational fluctuation modes of the protein, preventing excursions out into high energy states that lead to denaturation, but allowing energy excursions to a range of conformations that allows activation of the enzyme. In fact, too restrictive an environment won't allow the fluctuations that lead to activation of the protein.

WOLBER: If it turns out that there is not anything particularly interesting going on between the lipids and the proteins, other than just the proteins getting in the way of the lipids, then the "so what" comment is justified, but the point is that the experiment had to be done properly to realize that the "wrong" question was being asked. How relevant all of this is can't be evaluated until all the evidence is in. I thought that that was the purpose of this conference, to try to collect a lot of the evidence in one place and to ask that kind of question, hoping that there is enough evidence available now to support some tentative conclusions as to what kind of experiments need to be done in the future.

WILLIAMS: In the last three years Steve Fodor and I have been doing work in Keith Dunker's lab that shows that you can take the fd coat protein, or the M13 coat protein and put it into a solution of lipids; by adding one lipid or another lipid you can titrate the structure of this protein between an α-helical form and a β-sheet form. You can change the lipid composition of this membrane and cause this protein to switch conformation in a complex.

FLEISCHER: The "so what" question can help us refocus. Actually, I believe that a tremendous amount has been learned from biophysical studies to help us think about membrane function. We can now say that lipid is arranged in membranes to a first approximation in the form of a bilayer. The insertion of protein into the bilayer of a lipid vesicle causes only a small amount of disordering and a small decrease in the rotational motion of the lipid. That is fundamental information. Although most of the discussion here has been with regard to rotational motion, we have learned in the past decade that there is lateral motion of membrane components within the plane of the membrane. The phospholipid bilayer serves as a two-dimensional matrix into which membrane components are vectorially inserted and organized. The membrane serves to coordinate all function in time and space by making possible interactions of membrane components with one another and with constituents in the two different compartments which it separates. An understanding of lipid-protein interactions requires a description of motional characteristics of membrane constituents so that they can be correlated with the exercise of membrane function.