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PARAMAGNETIC SPIN LABEL INTERACTIONS WITH THE ENVELOPE OF A GROUP A ARBOVIRUS

LIPID ORGANIZATION*

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SUMMARY

Electron paramagnetic resonance observations were made on nitroxide spin-labeled molecules which were bound to the TC-83 vaccine strain of Venezuelan equine encephalomyelitis virus. Paramagnetic resonance parameters derived from the observations and their dependence on sample temperature were similar but not identical to those which have been reported for these labels dissolved in lipid bilayer membranes of mammalian and bacterial origin. The data are consistent with the existence of a bilayer lipid structure in the virion envelope which has a mechanical rigidity substantially greater than that of bilayers in cellular membranes. A model is presented which assumes the location of the lipid bilayer outside the nucleoprotein capsid and inside a spherical layer of envelope proteins. The model is in accord with Harrison's X-ray diffraction results for Sindbis virus. The model is discussed in terms of its implications with respect to the role played by lipid in viral maturation and infectivity.

INTRODUCTION

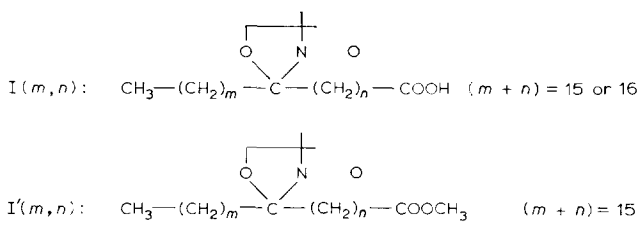
Electron micrographs have shown that some enveloped viruses are released from the host cell plasma membrane by a process ("budding") resembling reverse pinocytosis. These viruses have been found to have high lipid content with a molecular lipid distribution which is similar to that of the host cell. These results led other investigators [1] to the conclusion that such viruses matured by enveloping themselves in a portion of the host cell membrane. With increased viral yields from tissue culture, more precise lipid assays by thin-layer chromatography and improved serological methods, information is now available which renders this conclusion questionable.

Analysis of a group A arbovirus, Venezuelan equine encephalomyelitis (VEE) virus, grown on a number of different host cells has shown that lipid compositions of normal host cell, infected host cell, and VEE virus are different from one another

* A preliminary account of this work was presented at the Biochemistry-Biophysical Societies Meeting in Minneapolis, June 6, 1974.

[2–4]. These results indicate that the virus interferes with, and may direct, host cell lipid metabolism during infection. Examination of data [2–4] for lipid composition of virus derived from different host cells indicates that, at most, three lipid components may be host dependent. Another group A arbovirus, the structurally similar and serologically related Sindbis virus, also has a lipid composition which appears to be independent of the host cell [5]. Thus, for group A arboviruses there is strong implication that viral lipid composition is under secondary control of the viral genome. The finding that Sindbis virus lipid appears to be organized as a well-defined and highly symmetrical structure resembling a bilayer in form [6] supports this hypothesis.

With this background knowledge and the understanding that the integrity of VEE virus is dependent upon the presence of lipid, it was desirable to investigate the lipid organizational structure. For such an investigation, we have used a relatively new technique developed by McConnell [7]. The method requires the electron paramagnetic resonance (EPR) observation of paramagnetically labeled molecules dissolved in the lipid of biological preparations. The labels used in this study have the formulas:



They are fatty acids (I) and methyl stearates (I′) with the paramagnetic radical, *N*-oxyloxazolidine, [7] placed in various positions along the aliphatic chain. In anticipation of the results below, it has been found that the molecules intercalate as flexible rods into lipid that is in bilayer form. The principal motions consist of a high speed rotation of the molecule about its long axis and a slower translational motion of the whole molecule parallel to the plane of the bilayer [8]. Paramagnetic resonance observation can detect not only differences in motion for the molecules with the varying (m, n) indices indicated here, but can distinguish lipid-bound labels from those bound to defatted serum albumin or to membranes from which the lipid has been extracted. Further, the method can distinguish between label bound to a bilayer and that dissolved in a pure, disordered lipid phase. The first reported use of this method for virus was in the observation of spin labels bound to influenza virus [9].

MATERIALS AND METHODS

Virus preparation and assay

The TC-83 strain of VEE virus used in this study and virus titrations performed in chick embryo monolayer cells have been described elsewhere [10].

Baby hamster kidney (BHK-21) cells were propagated in roller bottles. Confluent monolayers were washed with medium 199 and infected with $\approx 10^6$ plaque forming units (p.f.u.) in 10 ml of medium 199. After absorption for 60 min at 37 °C, 150 ml of complete medium (Medium 199 containing Earle's balanced salts, 100

units/ml of penicillin, 50 μg/ml of streptomycin and 1 % human serum albumin) were added. Virus was harvested after incubation for 18–20 h at 37 °C.

Cell debris was removed from the medium by centrifugation at $650 \times g$ for 30 min followed by centrifugation at $10\,400 \times g$ for 30 min. Virions were concentrated by differential centrifugation for 2.5 h at $59\,000 \times g$ through a cushion of 10 % sucrose in 0.1 M NaCl, 0.02 M Tris · HCl, 0.001 M EDTA, pH 7.6. The virus pellet was suspended in a minimal amount of the NaCl/Tris · HCl/EDTA buffer and shaken overnight at 4 °C. This preparation was then treated with trichlorotrifluoroethane (Genetron 113, Allied Chemical Co., Morrison, N. J.) and subjected to agitation for 30 s on a vortex mixer. Genetron was removed by centrifugation at $1000 \times g$ for 15 min and the virus-containing supernatant was purified by rate-zonal centrifugation for 2 h at $90\,000 \times g$ in a 10–30 % linear sucrose gradient. Purified infectious virus was collected from the visible opalescent band, pelleted at $90\,000 \times g$ for 90 min through a 10 % sucrose cushion, suspended in 1.5 ml of NaCl/Tris · HCl/EDTA buffer ($\approx 10^{12}$ p.f.u./ml) and stored at −70 °C until used.

The effect of Genetron 113 (which has been commonly used for purification of cell-bound virus) on viral lipid was checked by exposing one purified virus preparation to a series of extractions with the halocarbon. The starting material was virus which had been purified as indicated above but with the first Genetron treatment omitted. After each sequential extraction virus was assayed for concentration (p.f.u./ml), serological activity (hemagglutination assay), and lipid structural change by EPR.

The purified virus produced infection in animals, replicated in tissue culture cells, and reacted serologically with antisera prepared against VEE virus in assays for hemagglutination inhibition, complement fixation, and plaque neutralization. The virus preparation did not react with antisera produced in rabbits hyperimmunized with homogenized normal host cells.

Paramagnetic resonance

Absorption measurements were made with a Varian EPR Spectrometer (Varian Associates, Palo Alto, Calif.) and dual cavity (TE_{104}) operating near 9.5 GHz. Although spin labels in buffer exhibited inhomogeneous power saturation, labels bound to virus had EPR absorption curves and derived hyperfine constants which were independent of microwave power in the range 1–100 mW. All data were taken at 100 mW and with 5 G field modulation at 100 KHz. The sample temperatures, which were controlled by a Varian attachment, are reproducible to $\pm$ 1 °C; the temperatures, by thermocouple check, are expected to be accurate to $\pm$ 2 °C. The spin labeled materials were obtained from Syva Inc., Palo Alto, Calif. and used as received. Thin-layer chromatography and ultraviolet absorbance assays indicated impurities in the 1–3 % range.

Virus was labeled by incubating suspensions $\approx 1 \cdot 10^{12}$–$6 \cdot 10^{12}$ p.f.u./ml in NaCl/Tris · HCl/EDTA buffer with thin films of spin label which had been deposited on the bottom of the incubation tube by evaporation from hexane solutions. The incubation was carried out overnight at 4 °C. This method was chosen in preference to the more conventional use of labeled bovine serum albumin as transfer agent since serum albumin binds to VEE virus and unbound labeled serum albumin has an EPR resonance pattern which resembles that of labeled virus. Label concentrations

are indicated by r = [label]/([label]+1/3 [viral protein]), where the protein concentration is measured by the method of Lowry et al. [11]. For virus with 25 % lipid content [4], r is nearly equal to the label-lipid concentration ratio in the incubation mixture; assuming this lipid content, virion mass was $\approx$ 1.5 mg/10^{12} p.f.u.

RESULTS AND DISCUSSION

Paramagnetic resonance

Fig. 1 shows the first derivative of EPR absorption vs applied magnetic field for the label I (12, 3) bound to VEE virus. Sample temperatures from calibration charts are within one or two degrees of T_{set}. The system changes were reversible with respect to temperature. The form of the curves and their temperature dependence resembles label bound to the lipid bilayer of microsomal membranes [12]. The form at lower temperatures is similar to that for label bound to bovine serum albumin but the latter system does not have the observed temperature dependence. Both form and temperature are very different from that for labels dissolved in bulk lipid [13]. Thus, analysis of the data in terms of a bilayer structure seemed warranted.

For quantitation of our experiments, we used the "order parameter",S, which can be derived from paramagnetic resonance absorption data. This parameter can be a measure of how well the label symmetry axis remains aligned parallel to a bilayer membrane-normal during excursions of the label under thermal influence. For rigid membranes or for labels placed very near the membrane surface, the alignment is closely parallel and S approaches unity. For less rigid membranes, or for labels placed near the methyl terminals of the phospholipids, the value of S is lowered. Work with synthetic lipid bilayers indicates that membrane stiffness can be related to measured values of S [14].

For the general case, extraction of S values from data such as these requires computer fitting. For curves where the hyperfine constants, $T_{||}$ and $T_{\perp}$, (see Fig. 1) are experimentally accessible, one may evaluate S by using the constants and approxi-

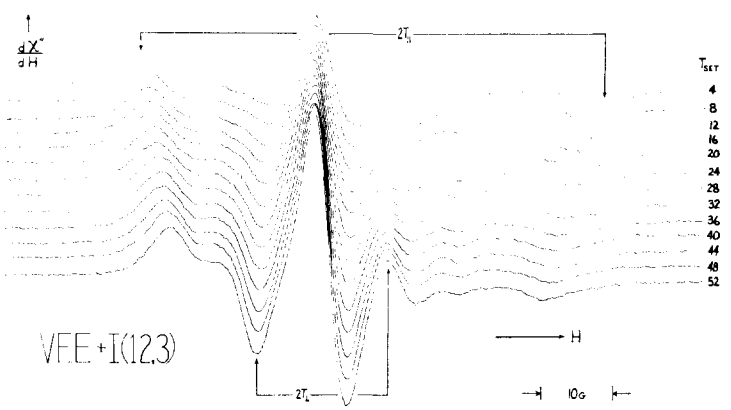


Fig. 1. First derivative of EPR absorption with respect to magnetic field vs applied magnetic field for I(12,3) bound to VEE virus; the curve parameter is related to sample temperature. The 5 G field modulation produces a central section broadening of 0.5 G and an increase of 3 % in S.

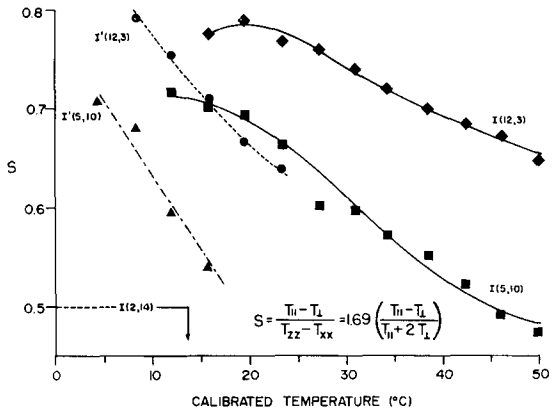


Fig. 2. Computed "order parameter" S vs T for five spin labeled molecules. Label/viral lipid sus pension ratios are $r = 0.0032$ for I(5,10) and $r = 0.022$ for the other labels.

mations derived from work with spin labels in single-crystal environments. The equation of Fig. 2 follows from equations 6–9 of Seelig [15].

Fig. 2 shows computed "order parameters" for the labels used here. The notation in the lower left corner indicates that label I(2,14) had an estimated $S < 0.5$ for all measured temperatures. The temperature dependences for each label and the changes produced by varying the distance of the label from the carboxyl or ester group (n-value) are qualitatively similar to those observed by other investigators for labels bound to synthetic and cellular bilayer structures. Quantitative comparisons are given by room temperature S values for I(12,3) bound to VEE virus and to a number of membranes: 0.77, VEE virus; 0.65, host BHK cells in NaCl/Tris · HCl/EDTA buffer; 0.55, microsomal membranes [10]; 0.76, 0.69, 0.67 for erythrocytes, L-cells, and lymphocytes [16]; 0.61, cardiolipin vesicles [17]. Kaplan et al. [16] and Hegner et al. [17] used a different approximation for computing S. The values here were recalculated from their data using the equation of Fig. 2 above. The results are consistent with the conclusion that VEE virus lipid has a structure resembling a lipid bilayer but with a stiffness which is generally greater than that of cellular and synthetic bilayers. Although no direct relation between stiffness and S values has been determined, it has been found [14] that addition of $\approx 20\,\%$ cholesterol to synthetic bilayers can produce an increase of $\approx 30\,\%$ in S as monitored by I(5,10).

Label solubility and concentration effects on S

Because of the small amount of viral lipid available for observation (virus pelleted at $90\,000 \times g$ has a lipid concentration of ≈ 1 mg/ml) it was necessary to prepare samples at relatively high label/lipid ratios to attain adequate EPR signal/noise ratios. The procedure adopted was to lower the value of r for each label until little or no effect on S vs T plots was detected. In the data of Fig. 2, only the lowest temperature points of the I(12,3) and I(5,10) results are expected to change with further lowering of r values. These findings do not affect the conclusion given above as to viral lipid organization.

The data at higher label concentrations are only indirectly related to the ques-

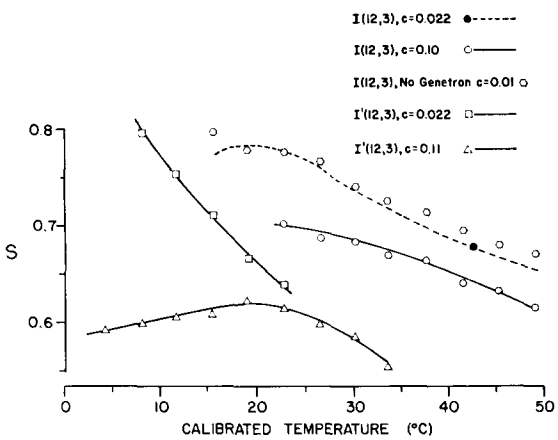


Fig. 3. S vs T curves illustrating label/membrane lipid concentration (c) effects for I(12,3) and I'(12,3). A comparison of the effects of Genetron extraction is also indicated.

tion of lipid organization. They will be presented here because similar data have not been reported for other membranes. It has been found for other systems [12] that, when the bound label/membrane lipid ratio, c, is greater than 0.01–0.05, broadening of EPR curves of the type of Fig. 1 occurs. This effect is due to interaction between adjacent spin labels. Fig. 3 shows S vs T for I(12,3) and I'(12,3) labels at lower and higher concentrations. For all labels except I(5,10), label molecules in the incubation suspension were almost completely bound to virus ($> 99\,\%$) for r values less than 0.1. This was indicated by the absence or minimal appearance of the EPR spectrum of the buffer-phase label [14]. Thus, for completely bound label, $r \approx c/(1+c)$.

The drastic low temperature drop in S for I' (12, 3) and the lesser drop of S for I(12,3) occurs through an increase of $T_{\perp}$, with $T_{\parallel}$ being relatively insensitive to concentration. Thus the concentration effects do not appear to be due to a breakdown [18] in the theory for computation of S. The drop in S for samples with high c values can be accounted for by a temperature-dependent line broadening mechanism similar to that reported for the oxazolidine radical on a phospholipid [19]. At present there seems to be no basis for attributing the effects to a real decrease in bilayer rigidity. The most interesting result of this portion of the work was the finding that the binding capacity of VEE virus for I(15,10) was at least one order of magnitude less than that of the other labels. Label in the incubation suspensions was $\approx 99\,\%$ bound for I(12,3) at $r = 0.102$ and for I(5,10) at $r = 0.0032$. Similar binding capacity ratios (> 10) for these two labels were observed for BHK cells and human erythrocytes (Hughes, F., unpublished). For sarcoplasmic reticulum vesicles from rabbit skeletal muscle prepared by a method similar to that of Meissner and Fleischer [20], the binding capacity ratio for the two labels was approximately unity.

Effects of trichlorotrifluoroethane

This lipophilic solvent was used for VEE virus preparation because its use permitted a better separation of virus from cellular protein [10]. In terms of the present work, it was necessary to check the effect of this solvent on bilayer lipid.

TABLE I

PROPERTIES OF VEE VIRUS AFTER GENETRON TREATMENT

Number of extractions	Plaque count (p.f.u./ml)	Hemagglutination units	Protein (mg/ml)
0	$103 \cdot 10^{10}$	4 000	2.3
1	$105 \cdot 10^{10}$	4 000	1.9
2	$107 \cdot 10^{10}$	8 000	1.7
3	$56 \cdot 10^{10}$	16 000	1.8
4	*	*	1.7

* Not measured to conserve material for EPR samples.

Virus was prepared and purified as indicated above except for Genetron 113 treatment. Table I shows the effects on the purified virus during a series of sequential extractions with Genetron 113.

Label I(12,3) was used to monitor the samples of Table I. Fig. 3 compares results on virus prepared with and without an extraction. Samples with zero to three extractions had S vs T curves which were the same within experimental error. The sample which had four extractions had S vs T values paralleling those of Fig. 3 but displaced downward by $\Delta S \approx -0.2$. The change can be accounted for by a loss of 60–80 % of intact virus bilayer with an effective increase in label/lipid concentration (see Fig. 3). Thus, the single extraction used for virus for the rest of this work produces no undue effects on viral lipid. The change in S vs T occurred at approximately the same Genetron exposure X time level for loss in infectivity and concomitant increase in released hemagglutination proteins of the VEE envelope.

CONCLUSION

In considering the lipid organization of VEE virus, it seems necessary to relate the present research to published results on Sindbis and Simliki Forest virus (SFV) since all three viruses have many common biochemical features (above and beyond the morphological and serological similarities which led to their taxonomic assignment to group A of the arboviruses).

Low angle X-ray diffraction experiments [6] on Sindbis virus showed a deep electron charge density minimum at a radius substantially less than the known virus radius. The charge density minimum had a magnitude compatible with its assignment as a hollow, spherical shell of lipid. Although the diffraction experiment cannot directly determine lipid order, collateral considerations suggested that the lipid was a bilayer form. Fig. 4 shows a model similar to that of Harrison et al. [6] except that the two envelope proteins of VEE virus [10] are indicated schematically.

The EPR results for VEE virus are strongly indicative of a bilayer organization of viral lipid but the data provide little evidence as to the position of the bilayer with respect to viral geometry. Taken together, the data for high VEE virus bilayer rigidity and resistance to Genetron treatment, favor the assignment of bilayer position to the interior position of Fig. 4 rather than to an exterior position suggested by earlier maturation models [1].

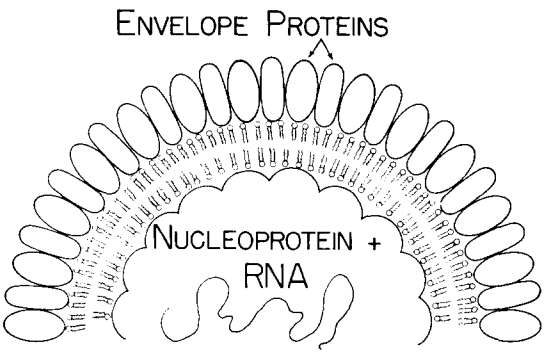


Fig. 4. Upper half of a central section of a proposed model for structure of group A arboviruses. The model indicates schematically a single protein associated with the RNA core, a lipid bilayer and an envelope consisting of two glycoproteins.

SFV has been treated with phospholipase C [21]. After hydrolysis of 60 % of viral lipid, the viral infectivity and sedimentation density in sucrose gradients were unchanged. The results are incompatible with an SFV structure similar to Fig. 4, not only because of the lack of physical accessibility of lipid to enzyme, but also, because such lipid losses would release envelope proteins, with a large change in density of the virus.

One may reconcile the above results by assuming that (1) SFV lipid is dissimilar in structure to the lipid in Sindbis or VEE viruses, (2) all three viruses have equivalent lipid structures but the SFV phospholipase C experiments are in some way anomalous or (3) the group A arbovirus model of Fig. 4 is essentially correct but instead of a tightly packed sheath of envelope proteins, the outer structure should resemble the relatively open protein array of a recent influenza model [22]. Optimum agreement with all present information is obtained with this last possibility if one includes the proviso that envelope protein packing density is different for the three arboviruses. This restriction would require that the relative densities of the proteins of the three viruses be Sindbis > VEE $\gg$ SFV. Further work will be required not only to check this aspect of structure but also to explain how reverse pinocytosis can result in a submerged virus bilayer. The current state of knowledge does not appear to be so complete as to warrant the assumption [22] that all enveloped viruses possess similar envelope structures.

The agreement between Sindbis and VEE virus information is sufficiently great to warrant discussion of the implications of Fig. 4 in terms of current concepts of arbovirus maturation. First, the model provides insight into mechanisms wherein the viral genome control of protein structure can be translated into secondary control of lipid distribution. The primary-to-quarternary structure of the RNA-nucleoprotein on the interior, and the envelope proteins on the exterior of the bilayer can exercise strong influence on the kinds and distribution of lipids which sterically "fit" the hollow, spherical shell. Of course, the specificity for fixed lipid composition cannot be absolute (present results suggest that mature virus bilayers can dissolve labeled stearic acid up to a 10–15 % increase in lipid). The lipid specificity requirement would be most obviously expressed in terms of yield and infectivity of the virus. A virus-host

cell combination, with cellular lipid metabolism which can be induced to produce the required specific lipids, might be expected to have a high yield of virions with structure perfect enough to permit a high level of secondary infection. These considerations fit well with the finding (Pedersen, C. E., unpublished) that in BHK cells the VEE virus yield per cell is of an order of magnitude greater than can be obtained with a number of other tissue culture cells. The concept may be part of the explanation for the clinically observed localization of VEE virus in brain and cord cells during infection.

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