Biochimica et Biophysica Acta, 433 (1976) 63–74

BBA 77255

PERMEABILITY PROPERTIES OF THE MEMBRANE OF VESICULAR STOMATITIS VIRIONS

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(Received September 22nd, 1975)

SUMMARY

Observations of the light-scattering properties of several enveloped viruses indicate that virions (vesicular stomatitis, SV5 and influenza), in common with other membrane systems, are osmotically active, responding to NaCl gradients by swelling in hypo-osmolar solutions and shrinking in hyperosmolar solutions. The permeability barrier responsible for this osmotic response in vesicular stomatitis virions was modified both by protease treatment to remove the viral glycoprotein and by treatment with the polyene antibiotic filipin, an agent known to interact with cholesterol in liposomes and membranes. Filipin altered the kinetic and equilibrium permeability behavior of virions but the extent of leakage of osmotic shocking agent was less than that in lecithin/cholesterol and lecithin/ergosterol liposomes and in ergosterol-containing ciliary membranes. Negative-staining electron microscopy revealed that filipin treatment caused structural changes in the viral membrane. Intact virions exhibited appreciably larger responses to osmotic change than did protease-treated virus particles. Thus, the osmotic barrier in intact vesicular stomatitis virions may not be exclusively lipid in nature.

INTRODUCTION

Lipid-containing viruses that are formed from plasma membranes of host cells by a budding process are of interest in studies of membrane structure and assembly. The lipids of these enveloped viruses generally appear to be derived from the lipids of the host plasma membrane [1–5] and to be arranged in a bilayer [6–8]. The viral polypeptides, which are few in number, are coded by the viral genome. The arrangement and types of proteins appear to be very similar in various lipid-containing viruses, even though the total number of major polypeptides may differ [9, 10].

Abbreviation: VS, vesicular stomatitis.

Glycosylated protein(s) are present on the external surface of the viral envelope, whereas a carbohydrate-free polypeptide is apparently associated with the inner surface of the viral membrane.

Studies of permeability properties offer the advantage that no extrinsic probe need be incorporated in the membrane. Light-scattering changes have been used to record the changes in volume of intact cells and cell membranes that occur when the osmotic pressure of the medium is changed. The kinetics of water diffusion into or out of multiconcentric bilayer membranes (liposomes) has been measured in a stopped-flow spectrophotometer as the osmotic equilibrium between the membrane interior and the medium is restored [11]. Factors that complicate study of osmotic water permeability in liposomes, such as the presence of multiple bilayers and marked heterogeneity with respect to size and surface area, do not arise in most enveloped viruses. Vesicular stomatitis (VS) virus particles are uniform in size (approx. 750 × 1750 Å); electron spin resonance studies have indicated that the lipids of this virus appear to be arranged in a bilayer [36]. The present study was undertaken to compare the osmotic permeability properties of lipid-containing virus particles with other biological membranes and with artificial lipid membranes. Since relatively large amounts of membrane suspensions are required for studies of initial rates of water permeability, VS virus was chosen because high yields of this virus may be obtained.

MATERIALS AND METHODS

Materials

The sources of egg lecithin, cholesterol, ergosterol and dicetyl phosphoric acid were cited previously [11]. Phosphate-buffered salt solution was prepared as described by Dulbecco and Vogt [12]. The osmolarity of phosphate-buffered salt solution is 0.311. All reagents were of analytical grade. Filipin complex (lot no. 8393-DEG-11-8) was generously supplied by Dr. G. B. Whitfield of the Upjohn Co., Kalamazoo, Mich. Stock solutions of filipin were prepared in dimethylformamide and aliquots were added to phosphate-buffered salt solution (or other buffer solutions specified below) to give the desired concentrations of antibiotic and dimethylformamide. The concentrations of antibiotic solutions were measured spectrophotometrically using a molar absorbancy of $5.0 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 338 nm for pure filipin. Cholesterol was assayed at 610 nm by the modified Liebermann-Burchard method of Huang et al. [13]. Phosphorus was measured by the method of Fiske and SubbaRow [14] and protein by the method of Lowry et al. [15] using bovine serum albumin as standard. Ergosterol and tetrahymanol were analyzed by gas-liquid chromatography as described by Conner et al. [16].

Preparation of liposomes and membranes

Liposomes were prepared from egg lecithin and cholesterol or ergosterol as described previously [11]. Liposomes contained 4 mol % of dicetyl phosphoric acid. The total lipid concentrations cited in the tables are those obtained after mixing in the stopped-flow apparatus. Ciliary membranes from *Tetrahymena pyriformis* W were kindly supplied by Dr. Robert L. Conner and his colleagues of Bryn Mawr College, Bryn Mawr, Pa.

Virus

VS virus, Indiana strain, was grown in BHK 21-F cell monolayer cultures and purified by precipitation with polyethyleneglycol and equilibrium zonal centrifugation in a potassium tartrate gradient as described previously [17].

Spikeless virus particles

Treatment of the virus with protease type VI from *Streptomyces griseus* (purchased from Sigma Chemical Co.) was performed as described previously [17]. Because of the possibility that impurities such as lipases are present in protease type VI preparations, experiments using trypsin were also carried out. Trypsin (L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-treated, purchased from Worthington Biochemical Corp.) treatment was according to Cartwright et al. [18, 19]. Examination by electron microscopy indicated that these treatments removed the surface (or spike-like) projections. Relative virion concentrations were determined from the absorbance at 280 nm of a 5 % sodium dodecyl sulfate solution (obtained by a five-fold dilution of the viral suspension), which was converted to the protein content as determined by the procedure of Lowry et al. [15].

Assay of osmotic permeability

Permeability measurements were performed at 25.0 °C on a Durrum-Gibson stopped-flow spectrophotometer operated in the transmission mode. Equal volumes of virions, dialyzed against phosphate-buffered salt solution, and osmotic shrinking or swelling solutions were mixed in the stopped-flow apparatus within approx. 3 ms. The osmotic shocking solutions resembled phosphate-buffered salt solution except for the concentration of NaCl, which was varied to give the desired osmolarity. Measurements of transmittance changes in the presence of filipin were made at wavelengths where the antibiotic does not absorb. The changes in transmittance as a function of time and at equilibrium (within about 4 min of mixing) were recorded on a Tektronix storage oscilloscope. Oscilloscope traces for permeability measurements of virions were similar to those shown for liposomes [11], but the direction of the transmittance changes associated with shrinking and swelling conditions were opposite to those found with liposomes. Initial rates of change of transmittance, dT/dt, were measured from slopes of the initial linear portion that followed the region of disturbance (up to approx. 200 ms after mixing). The initial rate of absorbance change, $(dA/dt)_{t=0}$, and the total change in absorbance, ΔA, were calculated from the initial rate of change in light transmission, dT/dt, and the total change in transmittance, ΔT as follows. For virion swelling (decrease in transmittance),

$$dA/dt = \log \frac{S_0+\Delta T}{S_0+\Delta T-dT/dt} \text{ and } \Delta A = \log \frac{S_0}{S_0+\Delta T};$$

for virion shrinking (increase in transmittance),

$$dA/dt = \log \frac{S_0-\Delta T+dT/dt}{S_0-\Delta T} \text{ and } \Delta A = \log \frac{S_0}{S_0-\Delta T}.$$

S_0 is the signal on the oscilloscope screen, which was generally adjusted to 800 mV (in some experiments 1600 mV was used; see Table III) by varying the photomulti-

plier voltage when the osmotically swollen or shrunken membrane suspensions were present at equilibrium in the stopped-flow cuvet. For liposomes and ciliary membranes (whose initial rate of volume change is proportional to the initial rate of the reciprocal of absorbance change), $d(1/A)/dt$ was calculated from the product of dA/dt and $-(A_i A_f)^{-1}$, where A_i and A_f are the initial and final absorbance of the suspensions. A_i was measured on a Cary 14 spectrophotometer. Data reported for the initial rates of absorbance change represent the mean of 8–15 measurements of the same virion preparation exposed to a given osmotic shock, followed by the standard deviation. When virions were mixed with an equal volume of isoosmotic phosphate-buffered salt solution in the stopped-flow apparatus, no transmittance changes were observed after the initial 200-ms disturbance period had elapsed.

RESULTS

Osmotic permeability studies

When liposomes containing trapped solutes are exposed to hyper- and hypo-osmolar solutions, loss or gain of intraparticulate water occurs to equalize the concentration of the impermeant solute [20, 21]. The volume of liposomes in a given preparation is proportional to the reciprocal of the osmolarity of the resulting suspension. This volume, in turn, is proportional to the reciprocal of the absorbance of the liposomes [20, 21], where absorbance arises from scattered light only. Fig. 1 shows the relationship between the absorbance and the reciprocal of the osmolarity of the viral suspensions at equilibrium. The approximate linear dependence observed in this figure suggests that for virions that have undergone osmotic shrinking and swelling, volume is proportional to the absorbance arising from light scattering, rather than to the reciprocal of the absorbance. Thus the change in volume is directly proportional to the change in absorbance. Deviations from linearity became pronounced when absorbance changes were measured at changes in 1/osmolarity of more than 4.5

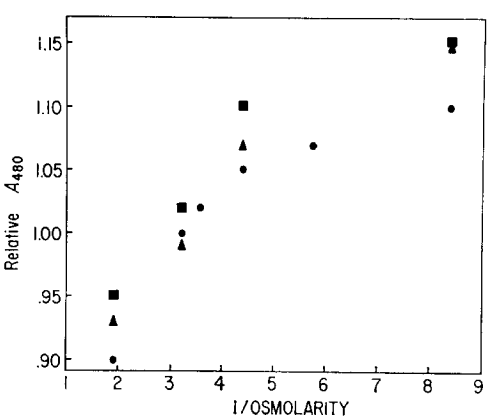

Fig. 1. Plot of the absorbance at 480 nm at equilibrium vs. reciprocal of the final osmolarity of the viral suspensions. One volume of virions suspended in phosphate-buffered salt solution was diluted with nine volumes of phosphate-buffered salt-like solutions made hyper-, iso- and hypo-osmolar in NaCl (●), NaI(▲) or CsCl (■). Absorbance values are relative to an A_{480} value of 1.00 in isosmolar NaCl solution.

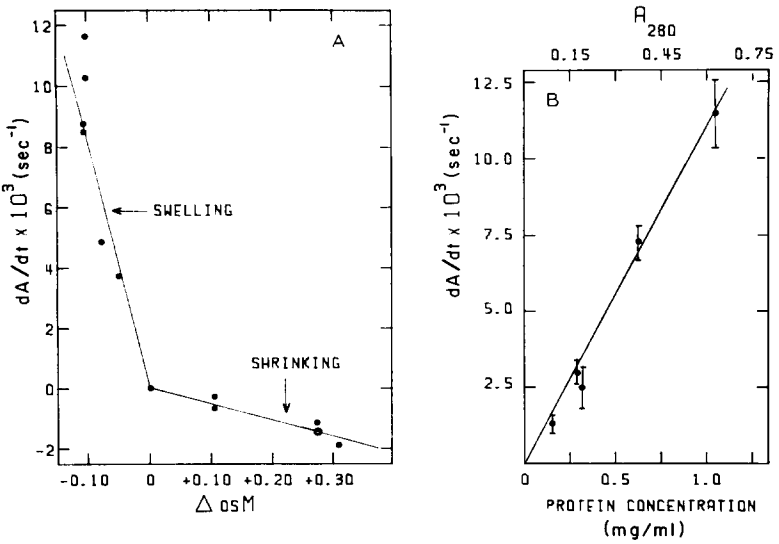

Fig. 2. (A) Plot of the initial rates of water permeability, dA/dt, vs. change in osmolarity. Virions were suspended in phosphate-buffered salt solution and mixed in the stopped-flow spectrophotometer with an equal volume of phosphate-buffered salt-like solution. The rates were normalized to a common virion concentration (total protein content of 1.0 mg per ml). In osmotic swelling experiments, hypotonic solutions were prepared by decreasing the concentration of NaCl in phosphate-buffered salt solution to give the desired osmolarity changes. In osmotic shrinking experiments, hypertonic solutions were prepared by increasing the concentration of NaCl in phosphate-buffered salt solution. In one experiment (○), NaI was used in place of NaCl in the suspending and hypertonic mixing solutions. (B) Plot of the initial rates of water permeability, dA/dt, vs. relative virion concentration, protein concentration or A_{280}, for virions subjected to a change of −0.104 osM. The initial rates of volume change, dA/dt, were recorded at 600 nm.

osM^{-1}. Preliminary results indicate that the response of intact influenza and parainfluenza SV5 virions to osmotic gradients of NaCl qualitatively resembles that of VS virions.

Fig. 2 shows that the relationship between the initial rate of absorbance change, dA/dt, and the change in osmolarity for various preparations of VS virions is approximately linear. For a membrane that is impermeable to solute, the initial rate of osmotic volume change is proportional to the concentration gradient of the solute in osmolar units [22, 23]. This behavior is predicted from an analysis of osmotic solvent and solute fluxes according to irreversible thermodynamics [24]; moreover, the relationships between changes in osmolarity and rates of volume changes, and between changes in reciprocal osmolarity and extent of volume changes, are also supported by a number of experimental observations in many biological membranes and isolated subcellular particles (e.g. refs. 25–27). Thus both the equilibrium values and the rates of change of light scattering from VS virions suggest that volume changes in virions are directly related to absorbance changes. Fig. 2 also shows that the initial rates of swelling of virions subjected to a given osmotic pressure change show a linear dependence on the concentration of VS virions.

In addition to the linear relationships displayed in Figs. 1 and 2, a number of other observations are consistent with the hypothesis that the absorbance changes are

not only osmotically induced but are also related to water transport across the viral membrane. First, the absorbance changes do not appear to arise from permeation of specific ions through the viral membrane. The equilibrium absorbance values obtained under swelling and shrinking conditions were identical when the osmotic gradient was produced with NaCl, NaI or CsCl (Fig. 1). The initial rate and extent of absorbance change was similar when NaI was used in place of NaCl in the suspending medium and osmotic shocking solution (Fig. 2). Thus, each of these ions produces a similar effect, i.e. net water movement across the membrane arising from the impermeability of the electrolyte. Second, when the solute mixed with the VS virions consisted of glycerol, ethyleneglycol, or xylose dissolved in phosphate-buffered salt solution, there was an initial shrinking phase (loss of water from the virion in response to the osmotic pressure gradient) followed by a swelling phase (permeation of nonelectrolyte, coupled with re-entry of water). When liposomes are exposed to the same hyperosmolar medium of glycerol or ethyleneglycol in phosphate-buffered salt solution, a 2-phase time course (shrinking followed by swelling) was also observed. However, the initial rates of permeation of the nonelectrolytes into virions were much greater than those for permeation into lecithin/cholesterol liposomes, and the absorbance change associated with each process was opposite in direction. When the virions were exposed to an isoosmolar medium of 0.20 osM glycerol in 0.11 osM phosphate-buffered salt solution, the only process observed was swelling, i.e. an increase in absorbance; as expected, liposomes exposed to an isosmolar glycerol solution also swelled, i.e. they exhibited an absorbance change in the opposite direction. The decrease in absorbance observed on shrinking of the virions in hyperosmolar solutions is reversible, i.e. exposure of pre-shrunken virions to swelling conditions gave absorbance increases. Conversely, exposure of pre-swollen virions to osmotic shrinking conditions gave absorbance decreases.

Effect of filipin on osmotic permeability

To further establish that the light-scattering changes represent osmotic water movement, a comparison of the effects of the polyene antibiotic, filipin, on liposomes, ciliary membranes and VS virions was made. Filipin interacts with sterol in cholesterol- and ergosterol-containing liposomes and in ciliary membranes obtained from *Tetrahymena* grown in the presence of ergosterol [28]. Table I shows that this membrane-active agent causes appreciable diminution of the initial rate and extent of the reciprocal absorbance change (volume change) that normally accompanies exposure of liposomes and ciliary membranes to an osmotic gradient of KCl. The effect of filipin on the osmotic water permeability of liposomes prepared from lecithin alone was not marked, and its effect on the initial rate of volume change of tetrahymanol-containing ciliary membranes is less than that on ergosterol-containing membranes. Thus, in order for filipin to exert an appreciable effect on both the extent ($\Delta 1/A$) and initial rate ($d(1/A)/dt$) of volume change, the presence of cholesterol or ergosterol in liposomes and ciliary membranes is required. The affinity of filipin for lecithin/cholesterol and lecithin/ergosterol liposomes far exceeds that for lecithin liposomes; in addition, the rate and extent of complex formation are functions of the accessibility, absolute concentration, and mol % of cholesterol in the bilayer [29]. Therefore, the observed permeability changes appear to be correlated with filipin-sterol complex formation. It is well known that the addition of filipin to cholesterol-containing

TABLE I

EFFECT OF FILIPIN ON THE INITIAL SHRINKING RATES AND EQUILIBRIUM VOLUME CHANGES OF LIPOSOMES AND CILIARY MEMBRANES

Liposomes were prepared in 0.04 M KCl/0.01 M Tris solution (pH 7.2) except for lecithin/cholesterol liposomes (1), which were prepared in 0.06 M KCl/0.01 M Tris solution. Membranes were suspended in 0.01 M KCl/0.01 M Tris solution (pH 7.2). Liposome and membrane suspensions contained 1% dimethylformamide by volume and were incubated with filipin in the dark at room temperature for approx. 1.5 h. Liposomes were mixed with 0.20 M KCl/0.01 M Tris solution containing 1% dimethylformamide by volume (except for lecithin/cholesterol liposomes (1), which were mixed with 0.18 M KCl/0.01 M Tris solution containing 1% dimethylformamide by volume). The final lipid concentration in the liposomes was 1 mM and the molar ratio of lecithin to sterol was 2 : 1. The final concentrations of tetrahymanol and ergosterol in the membranes were 0.068 and 1.08 mM, respectively. The absorbance was measured at 600 nm. The ratios shown are molar ratios of lecithin or solid alcohol (ergosterol, cholesterol and tetrahymanol) to filipin.

	Liposomes				Ciliary membranes	
	Lecithin	Lecithin/ergosterol	Lecithin/cholesterol		Tetrahymanol	Ergosterol
			(1)	(2)		
[Lecithin]/[Filipin]	7.7	–	–	–	–	–
[Alcohol]/[Filipin]	–	2.0	1.3	2.0	0.5	0.6
% decrease in d $(1/A)/dt$	14	78	73	40	27	88
% decrease in $\Delta(1/A)$	5	58	58	45	64	59

TABLE II

EFFECT OF FILIPIN ON THE INITIAL SWELLING RATES AND EQUILIBRIUM VOLUME CHANGES OF LIPOSOMES AND VIRIONS

Liposomes derived from lecithin and cholesterol in 2 : 1 molar ratio and virions were suspended in phosphate-buffered salt solution and mixed in the stopped-flow spectrophotometer with an equal volume of a phosphate-buffered salt-like solution whose NaCl concentration was lower than that of phosphate-buffered salt solution. The change in osmolarity was − 0.104. Light-scattering changes were measured at 500 nm. The final lipid concentrations of the liposomes and virions were 0.5 and 0.2 mM, respectively. The total protein content of the virions was 0.47 mg/ml. Liposomes and virions were treated with filipin as described in Table I.

	Cholesterol/filipin molar ratio	Liposomes				Virions			
		$\Delta(1/A)$	$d(1/A)/dt$	% Decrease		ΔA	dA/dt	% Decrease	
				$\Delta(1/A)$	$d(1/A)/dt$			ΔA	dA/dt
Untreated	–	0.073	0.0218±0.0020	–	–	0.085	0.0134±0.0013	–	–
Filipin-treated	1.1*	0.022	0.0073±0.0010	70	65	0.040	0.0086±0.0017	47	36
	0.56**	–	–	–	–	0.043	0.0050±0.0009	50	63

* The final concentration of filipin was 69 μM.
** The final concentration of filipin was 137 μM.

membranes results in rearrangements in lipid organization (e.g. refs. 28 and 30). An apparent consequence of this membrane disruption is that the concentration gradient of KCl across the liposomal and ciliary membranes is diminished.

Comparisons of the effect of filipin on lecithin/cholesterol liposomes and VS virions are given in Table II. Filipin diminishes the extent and initial rate of volume change, as measured by ΔA and dA/dt. Spectral measurements confirmed the interaction of filipin with cholesterol in the viral bilayer (unpublished observations from these laboratories). Thus, interaction of filipin with the viral membrane, which was found to contain a cholesterol : phospholipid molar ratio of 0.65 when obtained from virions grown on BHK cells, apparently results in rearrangements within the viral membrane, alteration of the permeability barrier, and concomitant solute passage. This indicates that cholesterol is accessible to filipin and is involved in the viral permeability barrier.

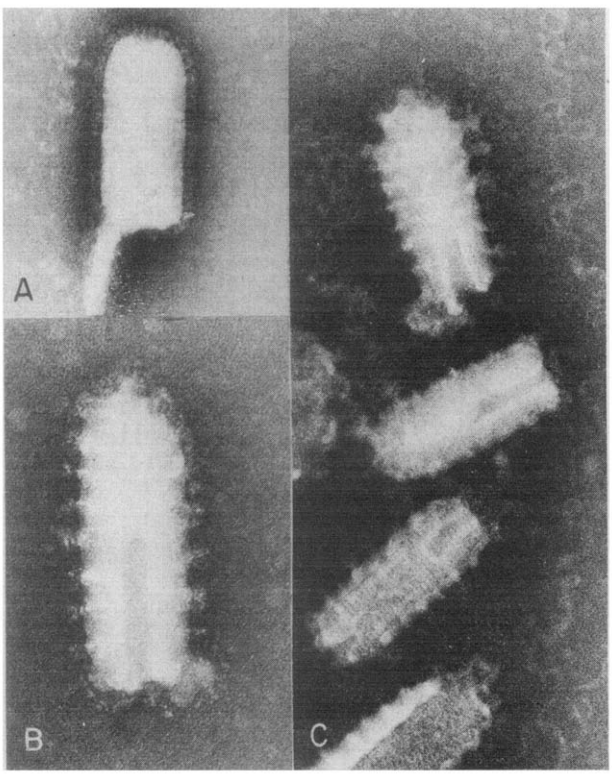

Fig. 3. Morphological alteration by filipin of the envelope of VS virions, negatively stained with sodium phosphotungstate, pH 6.2. (A) Virion from a control preparation, with projections distributed uniformly over the surface; (B, C) Virions from a preparation treated with filipin at a concentration of 0.25 μM for 30 min. The envelope exhibits a series of striations with a periodicity of approx. 150–200 Å. The surface spikes are redistributed, and along the periphery of the particle they are seen to be attached to the ridges in the envelope. The central core extending from the base of the bullet-shaped particles in B and C, and not in A, results from an infolding of the envelope. It is frequently observed in control preparations as well as in filipin-treated particles. Magnification: A and C × 150 000; B × 200 000.

Electron microscopy

Modification of the viral envelope by filipin was also indicated by a distinct morphological alteration. As shown in Fig. 3, filipin-treated virions exhibit a periodicity along their surfaces of 150–200 Å, consisting of a series of depressions and ridges in the envelope. These features extend across the particles, giving the envelope a striated appearance. It is of particular interest that the surface spikes are redistributed in the filipin-treated virions, being attached only to the ridges and not to the depressions in the viral envelope. Occasionally, virions are disrupted in the negatively stained preparations, and their membranes exhibit characteristic ring-like structures approx. 250 Å in diameter, of similar appearance to the "pits" described previously in filipin-treated liposomes [28].

Osmotic permeability properties of intact and protease-treated virions

The involvement of the envelope glycoprotein of VS virions in the osmotic barrier was examined. Table III shows results of osmotic permeability studies performed with intact virions and spikeless particles obtained by treatment with protease type VI or trypsin. The initial rate of osmotic water permeability of intact virions was appreciably greater than that of the spikeless particles. The extent of osmotic swelling was also larger in the intact virions. Treatment with either protease has been shown to be without effect on internal proteins of the VS virion [17, 19].

TABLE III

INITIAL SWELLING RATES AND EQUILIBRIUM VOLUME CHANGES OF INTACT AND SPIKELESS VIRIONS

Intact and spikeless virions from the same preparation were suspended in phosphate-buffered salt solution. Each virion suspension was exposed to an equal volume of hypoosmolar phosphate-buffered salt-like solution (0.137 osM) whose NaCl concentration differed from that present in phosphate-buffered salt solution. The change in osmolarity was (1) −0.087 and (2) −0.104 osM. Changes in light scattering at 375 nm were recorded on the stopped-flow spectrophotometer using a total signal of 800 mV for (1) and 1600 mV for (2). (This accounts for the larger changes in signal amplitude observed in (2) compared to (1)). The total protein concentrations of the virions were 0.31 mg/ml for preparations (1) and (2) of the intact particles, 0.14 mg/ml for the protease type VI-treated virions (spikeless preparation (1)), and 0.23 mg/ml for the trypsin-treated virions (spikeless preparation (2)).

Type of virion	$dA/dt \cdot 10^4$ (s^{-1})	ΔA
Intact (1)	30.8 ± 3.4	0.019
Intact (2)	50.9 ± 2.8	0.050
Spikeless (1)	6.4 ± 1.3	0.012
Spikeless (2)	8.4 ± 2.7	0.032

DISCUSSION

Analysis of the response of VS virions to osmotic pressure gradients suggests that the observed light-scattering changes represent water permeation through the viral membrane. Unlike the situation observed for osmotically driven volume changes in artificial bimolecular membranes (liposomes) and some naturally occurring systems (e.g. ciliary membranes, mitochondria, red blood cells), virions are found to possess a

direct rather than an inverse proportionality between volume change and absorbance change. Although a theoretical justification involving a Raleigh-Gans approximation has been made for treating absorbance increases as a function of particle shrinkage [31], there are many exceptions to this theoretical relation. They probably arise because the intensity of scattered light is a complicated function of the mass, size, shape, refractive index and volume of the particles. Experimental results and theoretical calculations show that particle size, internal structure, suspension concentration and scattering angle may be important variables in addition to volume in the relationship between optical change and volume change; shrinkage can result in increased light scattering (decreased transmission) for some particle sizes, and in decreased light scattering for other particle sizes [32–35]. Therefore, relationships between volume changes and absorbance changes need to be established empirically. The light-scattering changes we observe apparently do not arise from changes in the shape of the virus particle or in the conformation of glycoprotein because (a) electron microscopic investigations of virions collected from the stopped-flow apparatus after osmotic shock revealed no shape changes and (b) protease-treated virions produced the light-scattering data summarized in Table III. The results obtained with filipin-treated virions and with virions exposed to a variety of electrolytes and nonelectrolytes as the osmotically active species provide further support for the conclusion that the light-scattering measurements reflect osmotically driven volume changes.

The fact that the slope of the plot of initial rate vs. change in osmolarity is larger for swelling than for shrinking (Fig. 2) suggests that the nonglycosylated membrane protein(s) that appear to be associated with the inner surface of the viral membrane, and the nucleocapsid structure that underlies it, may limit the extent or rate of volume change resulting from osmotic shrinking. Gravimetric analyses of viral preparations centrifuged from solutions of different osmolarities suggested that the osmotically active space represented less than approx. 20 % of the isosmotic viral volume.

The present results indicate that the envelopes of VS virions exhibit permeability characteristics similar to those observed with other biological membranes. The permeability properties have been modified in the present studies using two specific perturbations, introduction of the antibiotic filipin into the lipid bilayer and removal of the major portion of the glycoprotein spike by two different proteolytic treatments. It therefore appears that both proteins and lipids are functional components of the viral permeability barrier. The lipid-containing viruses thus represent a biologically important system in which permeability measurements may provide insight into the role of lipid-protein interactions in membrane structure and function.

ACKNOWLEDGEMENTS

We thank Dianne L. Rosen and Linda N. Clark for excellent technical assistance. This work was supported by research grants HL 16660, AI 12680 and CA 18611 from the U.S. Public Health Service, VC 149 from the American Cancer Society, and BG 36789 and BMS 75-18199 from the National Science Foundation.

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