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Lipids of Rabies Virus and BHK-21 Cell Membranes

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The lipid composition of highly purified Flury strain of rabies virus (HEP) propagated in BHK-21 cells in a chemically defined medium was observed to be 6.7% neutral lipids, 15.8% phospholipids, and 1.5% glycolipids. In the virion, phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin were the most abundant phospholipids, accounting for 90% of the total, and the molar ratio of cholesterol to phospholipid was 0.48. Uninfected BHK-21 cell membranes were obtained by nitrogen cavitation techniques and separated by density gradient centrifugation, and the membranes were assayed for purity using 5'-nucleotidase, cytochrome oxidase, and reduced nicotinamide adenine dinucleotide phosphate diaphorase activities. Lipids of the plasma membrane were enriched in cholesterol, phosphatidylcholine, and phosphatidylethanolamine. In contrast, membranes of the endoplasmic reticulum were enriched in phosphatidylcholine, but contained smaller amounts of phosphatidylethanolamine and sphingomyelin. Comparison of the fatty acyl chains of virus and membranes from uninfected cells revealed the virion to have the lowest ratio of $C_{18:1}$ to $C_{18:0}$ (1.77), compared with values of about 3.0 for the plasma membrane and endoplasmic reticulum. Total polyenoic fatty acids were enriched in the plasma membrane, whereas the virus contained higher amounts of total saturates than either of the two membrane preparations. Analysis of the polar and neutral lipid fractions as well as the acyl chain analysis suggests the virion has a lipid composition that is intermediate to that of the plasma membrane and endoplasmic reticulum and is consistent with the view that numerous viral particles are synthesized de novo by not utilizing a preexisting membrane template. From the ratio of cholesterol to phospholipid of 0.48, we calculated that 1.92×10^5 molecules of lipid would cover 4.14×10^4 nm^2 in the form of a bilayer. Considerations of the molecular dimensions of the rabies envelope (total surface area, 5×10^4 nm^2) as a bilayer suggest that some penetration of lipids by envelope proteins (M and G) is necessary.

The rabies virion consists of a helical ribonucleoprotein capsid enclosed within a lipoprotein envelope (8, 29) containing one glycoprotein (G) and two "membrane" (M) proteins (22, 30). The viral lipid is an essential constituent of the virion since treatment with lipid solvents, detergents, or lipase results in loss of viral infectivity and hemagglutinating activity (18). Whereas the molecular properties of the structural proteins (22, 30) and RNA (1, 29) have been studied, a detailed study of the lipids of the virion and its host cell membrane has not been done. We describe the analysis of the lipids of rabies virus, the host cell membranes, and the possible arrangement of lipids within the envelope of this virus.

MATERIALS AND METHODS

Virus strain and propagation. The Flury high-egg-passage (HEP) strain of rabies virus was propagated in BHK-21 cells and concentrated by previously published methods (28), except that the maintenance medium used for virus propagation contained 0.2% (vol/vol) "fatty acid poor" bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.) as the only protein supplement to permit unrestricted lipid synthesis (7). With this technique, as with influenza virus, there was no loss of viral infectivity or hemagglutinating activity. Virus was quantified by hemagglutination (18), and protein was measured by the Lowry method (19). $^{32}PO_4^{3-}$-labeled rabies virus was prepared from equilibrium-labeled BHK-21 cells to insure the complete labeling of viral phospholipids (24). BHK-21 cells were grown to con-

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fluence in the presence of 10 μCi of $^{32}PO_4^{3-}$ per ml (New England Nuclear Corp., Boston, Mass.), and after infection the medium was supplemented with 5 μCi of $^{32}PO_4^{3-}$ per ml for the 72-h period of virus propagation.

Cell propagation and membrane isolation. BHK-21/C13 cells were grown in roller bottles and Blake bottles in minimal Eagle medium with 10% fetal calf serum containing appropriate antibiotics as described earlier (14), except that the cells were starved for 18 h prior to infection, and for 3 days during the propagation of rabies virus they were maintained in the presence of 0.2% fatty acid poor bovine serum albumin (3). Uninfected cell membranes were maintained in exactly the same way. In all cases, the same batches of serum were used for the propagation of the virus and for the isolation of membrane components from uninfected cells. A total of 9.5 to 10.3 g of wet cells was disrupted by the nitrogen cavitation technique, and individual plasma membrane and microsomal fractions were isolated by rate-velocity centrifugation as described by Gahmberg and Simons (13). Enzyme markers used for the characterization of membrane purity were reduced nicotinamide adenine dinucleotide phosphate diaphorase as a microsomal marker (33), cytochrome oxidase for mitochondria (26), and 5'-nucleotidase activity for plasma membranes (31).

Virus purification and isotope dilution techniques. Concentrated suspensions of rabies virus were partially purified by isopycnic centrifugation in a sucrose density gradient (1). For highly purified suspensions, the isopycnically banded virions were sedimented onto a glycerol cushion, and the resulting opalescent suspension was subjected to rate-velocity sedimentation through a sucrose density gradient (Fig. 1b). The final concentration of purified virions was achieved by sedimentation.

Rabies virions banded as a single zone at a buoyant density of 1.17 g/cm after isopycnic centrifugation and sedimented homogenously during rate-velocity centrifugation (Fig. 1a and b). Effective removal of radioactive cellular debris prepared from uninfected cells was achieved during virus purification as adjudged by isotope dilution techniques (8). When $^{32}PO_4^{3-}$-containing BHK-21 cell debris ($\geq 10^7$ cpm) was added to cell culture fluid containing unlabeled rabies virus, less than 0.007% of the radioactivity was detected on membrane filters as trichloroacetic acid-insoluble material in the purified virus suspension.

Quantification of lipid classes. Extraction of lipids and separation of neutral and polar lipid fractions were performed as previously described (4), except that phospholipids were separated into individual components by thin-layer chromatography on plates of Silica Gel H (14). Neutral lipids were quantified by the charring method (21), and phospholipids were measured spectrophotometrically (4) or, in the case of the rabies virus, radioisotopically (24). The sialoglycolipids were recovered from the upper phase of the Folch partition (12) after "washing"; the extracted lipids were extensively dialyzed, and neuraminic acid was quantified spectrofluorometrically

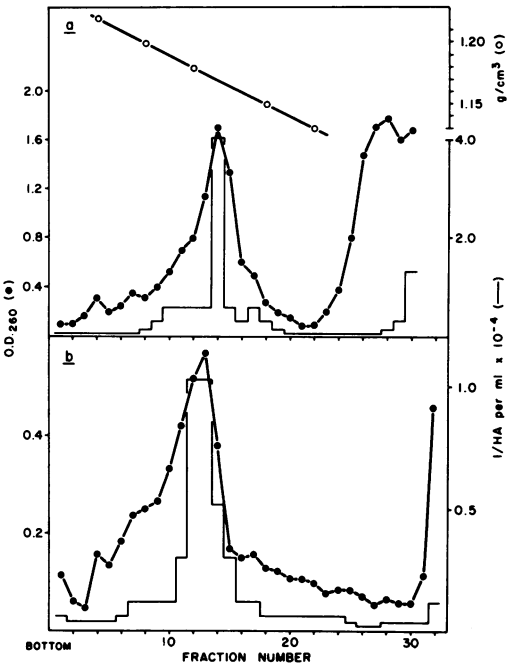

FIG. 1. *Purification of concentrated rabies virus (strain HEP). (a) Isopycnic centrifugation; (b) rate velocity centrifugation of isopycnically banded virus through a 15 to 30% sucrose gradient at 80, 000 × g for 50 min in a Spinco SW27 rotor. Symbols: ●, optical density at 260 nm; –, hemagglutinating activity; ○, density.*

with 3,5-diaminobenzoic acid (15). Neutral glycolipids were eluted from the thin-layer chromatography plates used for phospholipid separation and measured gravimetrically.

Fatty acid methyl esters were prepared as previously described (8) and analyzed by gas-liquid chromatography on 6-foot (ca. 183-cm) columns of 15% diethylene glycol succinate (Hi Stab) on Anakrom ABS with a Barber-Colman 5000 instrument equipped with dual-flame ionization detectors or by computer analysis on a Hewlett-Packard 5830A gas chromatograph equipped with dual-flame ionization detectors, using 6-foot coiled columns of 10% apolar 10C (silicone) on Gas-Chrom Q 100/120 mesh (2).

RESULTS

After purification the plasma membrane fraction was enriched eightfold in 5'-nucleotidase activity and was free from cytochrome oxidase, phosphatidylglycerol, and cardiolipin (mitochondrial markers); the endoplasmic reticulum (microsomal) fractions showed a 20-fold increase in reduced nicotinamide adenine dinucleotide phosphate diaphorase activity after purification and possessed no cardiolipin.

The total lipid composition of purified rabies virus and the profile of the individual components of the various lipid classes are presented in Table 1. Lipids constituted 24% of the weight of the virion, and the molar ratio of cholesterol to phospholipid was 0.48. Of the neutral lipids, cholesterol was the most abundant; however, the free fatty acid content, on a molar basis, was substantial. Phosphatidylethanolamine, the principal phospholipid, together with sphingomyelin and phosphatidylcholine, accounted for 90% of the phospholipids, in agreement with previous workers (10). No evidence was found that phospholipid was lost during purification of the Flury (HEP) strain as had been reported by other workers (27). The sialoglycolipids constituted about 60% of the glycolipids, assuming that the majority of the components contained only one sialic acid residue per molecule (23). A comparison of the phospholipids with neutral lipids of BHK-21 cell membrane is shown in Table 2.

The fatty acyl chain analysis of the polar lipids of the virion and its host cell membrane is shown in Table 3. In the virion, some long chain fatty acids and polyunsaturates were detected, viz., 7.4% of arachidonic acid ($C_{20:4}$) and $C_{22:5}$ and $C_{22:6}$, which collectively amounted to

TABLE 1. *Lipid composition of purified rabies virus (strain HEP)*

Component	% (wt/wt)	mol%[a]
Total lipids	24.0	
Neutral lipids	6.7	
Phospholipids	15.8	
Glycolipids[b]	1.5	
Neutral lipids[c]		
Free fatty acids		20.0 ± 2.0
Monoglycerides		2.4 ± 0.5
Diglycerides		7.8 ± 0.3
Triglycerides		5.2 ± 0.0
Cholesterol		63.4 ± 2.5
Cholesterol esters		1.2 ± 0.0
Phospholipids[d]		
Phosphatidylcholine		23.4 ± 2.0
Lysophosphatidylcholine		1.5 ± 0.1
Sphingomyelin		31.3 ± 0.4
Phosphatidylethanolamine		35.1 ± 1.2
Phosphatidylserine		7.8 ± 0.2
Phosphatidylinositol		0.25 ± 0.05
Phosphatidic acid		Trace ± 0.0
Lysobisphosphatidic acid		0.2 ± 0.0

[a] Calculated by the method of Blough and Merlie (4).
[b] Determined by addition of sialoglycolipids and neutral glycolipids (see text).
[c] Neutral lipids were separated by unidimensional thin-layer chromatography, using the two-solvent system (7), and identified by reference to lipid standards obtained from Applied Science Laboratory, State College, Pa.
[d] $^{32}PO_4^{3-}$-labeled phospholipids were separated by two-dimensional thin-layer chromatography as previously described (14) and spots were localized by exposure to iodine vapor or autoradiography and quantified after sublimation of the iodine and elution by the determination of radioactivity in a liquid scintillation spectrometer.

TABLE 2. *Neutral lipids and phospholipids of uninfected BHK-21 cell membrane fractions*[a]

Lipid	mol%[b]	
	Plasma membrane	Microsomal fraction
Free fatty acids	18.7 (±4.0)	19.3 (±0.3)
Monoglycerides	10.0 (±0.1)	11.3 (±0.2)
Diglycerides	11.5 (±0.8)	18.2 (±1.6)
Triglycerides	5.3 (±3.0)	11.1 (±0.3)
Cholesterol	41.3 (±3.4)	33.9 (±0.6)
Cholesterol esters	7.6 (±0.3)	7.3 (±0.1)
Phosphatidylcholine	34.2 (±3.0)	31.5 (±0.5)
Lysophosphatidylcholine	0.5 (±0.0)	5.9 (±0.3)
Sphingomyelin	14.3 (±0.3)	12.8 (±0.2)
Phosphatidylethanolamine	35.3 (±2.5)	20.8 (±0.6)
Phosphatidylserine	8.2 (±0.1)	7.7 (±0.5)
Phosphatidylinositol	0.8 (±0.0)	6.8 (±0.3)
Lysophosphatidylethanolamine	0.8 (±0.0)	5.3 (±0.2)
Phosphatidic acid	4.1 (±0.2)	4.0 (±0.1)
Lysobisphosphatidic acid	0.9 (±0.0)	ND[c]
Phosphatidylglycerol	ND	3.8 (±0.3)

[a] Mean determination of four separate experiments.
[b] Separations and quantification as described in the footnotes to Table 1.
[c] ND, Not detected.

TABLE 3. *Fatty acyl chain composition of the polar lipids of rabies virus and uninfected BHK-21 cell membranes*

Acyl group[a]	Composition (%)		
	Rabies virus	Plasma membrane	Microsomes
$C_{12:0}$	0.3	0.8	ND[b]
$C_{14:0}$	1.2	1.5	1.6
$C_{16:0}$	20.3	8.5	16.6
$C_{16:1}$	3.2	4.4	6.5
$C_{18:0}$	20.1	14.2	13.7
$C_{18:1}$	35.6	39.9	42.8
$C_{18:2}$	1.4	7.6	4.9
$C_{18:3}$	ND	Trace[c]	ND
$C_{20:0}$	ND	ND	2.1
$C_{20:1}$	0.7	0.4	1.3
$C_{20:4}$	7.4	13.1	ND
$C_{22:0}$	ND	0.7	0.9
$C_{22:1}$	ND	ND	2.2
$C_{24:0}$	ND	2.8	1.3
$C_{24:1}$	1.9	ND	1.5
$C_{22:3}$	ND	3.7	ND
$C_{22:4}$	ND	1.4	ND
$C_{22:5}$	2.8	0.5	ND
$C_{22:6}$	2.6	0.6	ND
Uncharacterized	2.4	None	4.6

[a] Methyl esters of the acyl chains were prepared and analyzed by gas-liquid chromatography on two or more stationary phases in a Barber-Colman 5000 instrument or by using programmable techniques in a Hewlett-Packard 5830A instrument.
[b] ND, Not detected.
[c] Less than 0.3%.

5.4%. Striking differences were apparent between membrane preparations: the ratio of $C_{18:1}$ to $C_{18:0}$ was 1.77 for the virion and 2.8 and 3.1 for the plasma membrane and endoplasmic reticulum, respectively. Total monoenoic fatty acids were approximately the same for the plasma membrane and the virion, whereas the plasma membrane was greatly enriched in polyenoic fatty acids (27%), with the virus (1) intermediate between the plasma membrane and the endoplasmic reticulum. The virus had a higher percentage of saturated fatty acyl chains than did either of the uninfected membranes.

DISCUSSION

Comparison of the lipids of rabies virus with those of other rhabdoviruses is difficult. Only vesicular stomatitus virus (VSV) has been similarly studied (20), and different cells were used in its propagation. Although the phospholipid profiles of VSV (Indiana strain) and rabies are dissimilar, they both contain a large amount of phosphatidylethanolamine and their cholesterol contents are similar. When the VSV neutral lipid data of McSharry and Wagner (20) are recalculated in terms of moles percent, the amounts of mono-, di-, and triglycerides present in VSV (Indiana strain, propagated in L cells) are quite similar to those of rabies virus (propagated in BHK-21 cells). Our results on the polar lipid composition of the virion are quite similar to those of Diringer et al. (10) on the Flury (HEP) strain of rabies propagated in BHK-21 cells, but they are different from those of Schlesinger et al. (25). It is unlikely that the lipid composition of the virion from two different assembly sites possessed similar polar and acyl chain composition, as suggested by the latter group. The ratio of cholesterol to phospholipid was much lower in our study (0.48) as compared with that of Diringer et al. (10) (0.87) or Schlesinger et al. (25) (0.87 to 0.92), which may reflect differences in methods of cell propagation. Neither Diringer et al. (10) nor Schlesinger et al. (25) analyzed the fatty acyl chains of the virion.

Extensive analysis of lipids in specific membrane preparations of BHK-21 cells was done by Renkonen et al. (23) and Klenk and Choppin (17) in an attempt to localize the site of viral membrane envelopment in the case of Semliki forest virus and simian virus 5. Our results with BHK-21 cell membranes (Table 2) agree with previous investigations (17, 23), except that the proportion of phosphatidylserine was less in our plasma membrane preparations; however, a high molar ratio of cholesterol to phospholipid was found in the plasma membrane. The compositional analysis of the rabies virus lipids appears intermediate to that of the endoplasmic reticulum and plasma membrane, and our data support electron microscopic observations that rabies virus envelopment (HEP strain) is mostly an intracellular event (16); i.e., the bulk of the viral particles appear to be synthesized de novo without any preexisting membrane template. In contrast, template viruses, e.g., paramyxoviruses, myxoviruses, and some rhabdoviruses, utilize preexisting membranes as a scaffolding. Both classes of viruses contain specific amino acid residues in the envelope proteins (M and G), which select out specific fatty acyl chains (5). These acyl chains are necessary for both the transport and insertion of the polypeptides into the viral membrane (6) and would be expected to have an essential role in viral maturation. Recent studies on the lipids of rabies virus propagated in Nil cells under different environmental conditions (monolayer versus Spinner cultures) have confirmed that environmental factors play an important part in viral lipid composition (8); thus, ceramide hexosides are responsible for maintaining the integrity of the viral envelope and determining the antigenic properties of the virion (J. Portoukalian, M. Bugan, G. Zwingelstein, and P. Precausta, submitted for publication).

Recent models of rhabdoviruses (9, 32; F. Brown and J. Crick, *in* H. A. Blough and J. M. Tiffany [ed.] *Cell Membranes and Viral Envelopes*, in press) have dealt with the arrangement of envelope proteins in some detail, but have failed to consider the arrangement of viral lipids. As the simplest arrangement of lipid would be bilayer, we have calculated the approximate extent of the lipid reported here as a bilayer. We assume that viral lipids are totally localized in the envelope and that cholesterol and phospholipids comprise all of the viral lipids (they account for 82% of the total lipid content). Our estimate of the total molecular weight of the virion lipids is based on the fact that the rabies nucleocapsid has been found to contain 4% RNA, and the nucleocapsid protein constitutes 33% of the total protein of the virion (29). Since the total protein content is 72 to 74% of the mass of the virion, the lipid composition is 24% (Table 1), and the molecular weight of the viral RNA is 4.5×10^6 (29), one may calculate that a total of 1.2×10^8 daltons of lipid is present in the virion. Assuming a uniform distribution of the fatty acyl chains (Table 3) among all classes of the phospholipids (Table 1), we calculate the mean molecular weight of

the phospholipid to be 737. Considering a molar ratio of cholesterol to phospholipid of 0.48, we determine that 1.92×10^5 molecules will cover an area of 4.1×10^4 nm^2 in the form of a bilayer. The envelope area of the virion (not including possible envelope invagination at the base) was calculated from the dimensions reported by Hummeler et al. (16) and found to be about 5×10^4 nm^2. We conclude that some penetration of the lipids by envelope proteins is necessary to extend the bilayer. From this study, we feel that no speculation as to the degree of protein penetration is yet possible, although the M and G proteins of VSV and rabies virus penetrate but do not traverse the membrane (11, 33); thus, our calculations in no way conflict with the proposed models of rhabdovirus (9, 32; Brown and Crick, in press). We have previously suggested that an important factor in rhabdovirus assembly is the time sequence of coiling of the nucleocapsid, which could be under the control of a morphopoetic factor. The characteristic curvature of the mature particle may be determined by this factor, aided by repulsion of the external projections of the virion. This is possible if the M protein acts as a recognition site for interaction with the G protein by direct hydrophobic interactions (6). Thus, if we assume that the M protein from one side and the G protein from the other side penetrate and interact to form a pillar of uniform diameter across the bilayer, for 790 spikes (5), the area per pillar would be 10.9 nm^2 or a disk of ~3.7 nm. This is larger than the stalk (about 2.5 nm) as visualized by electron microscopy (16); however, this can be accounted for it if the M protein occupies more of the inner leaflet or is shaped like an inverted toadstool (5).

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