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Lipid composition and fluidity of the human immunodeficiency virus

(acquired immunodeficiency syndrome/electron spin resonance/cholesterol/membrane/anti-human immunodeficiency virus agents)

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ABSTRACT **Lipid analyses of the human immunodeficiency virus (HIV) propagated in Hut 78 cells indicated a low total lipid/protein ratio, a high cholesterol/phospholipid molar ratio, and major phospholipids consisting of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and phosphatidylserine; comparable lipid profiles were noted for human erythrocytes and other RNA viruses. Electron spin resonance (ESR) studies of HIV labeled with 5-nitroxide stearate (*N*-oxy-4′,4′-dimethyloxazolidine derivative of ketostearate) showed a low "fluidity" at 37°C, similar to other enveloped RNA viruses and erythrocytes and probably due to the high cholesterol/phospholipid ratio. Ethanol (50%) completely disrupts the envelope, contributing to the rapid inactivation of HIV by ethanol. Contrarily, heating to 57°C causes much less fluidization, and this heating may play a role in the slower viral inactivation at high temperatures. Should a critical minimum ordering in the HIV envelope be necessary for viral stability and infectivity, manipulating the lipid composition or fluidizing the HIV membrane, or both, may provide an untried therapeutic approach.**

Epidemiological studies show that the human immunodeficiency virus [HIV or human T-cell lymphotropic virus type III (HTLV-III)/lymphadenopathy-AIDS-virus (LAV)] responsible for the acquired immunodeficiency syndrome (AIDS) is spreading among the general populations of North and South America, Europe, Africa, Australia, and Asia (1). There is an urgent need to develop therapeutic agents for prophylactic use by those at risk of infection, as well as for those who either suffer from AIDS or have been exposed to the virus but do not yet have any symptoms of the disease. An innovative approach suggested by Reimund (2) centers on inactivating HIV by perturbing the viral lipid envelope. A liposome mixture (AL 721) reduces *in vitro* HIV infection of lymphocytes at doses that fluidize membranes by removing cholesterol (3). Moreover, amphotericin B, a fluidizing agent known to complex membrane cholesterol (4), inhibited HIV replication (5). Although their precise mode of action remains unknown, these drugs may reduce HIV infectivity by disturbing the lipid envelope, either by altering the lipid composition, or increasing the fluidity, or both. Thus, it is important to establish the HIV lipid composition with analytical techniques and to study the "fluidity" of intact virions using physical methods such as electron spin resonance (ESR) spectroscopy.

MATERIALS AND METHODS

Materials. Spin probes were purchased from Aldrich and checked for purity with two-dimensional thin-layer chromatography (6).

Preparation of HIV. HIV-producing OKT4$^+$ leukemic cells (Hut 78) were cultured using either a defined serum-free medium (HB101 from DuPont) or RPMI 1640 medium/10% fetal calf serum in roller bottles, or in stationary cultures at 37°C in a humidified incubator (5% CO_2) at a concentration of 2×10^6 cells per ml. *Mycoplasma* contamination was monitored every second week using two methods: Mycotrim-TC (New England Nuclear) and DNA fluorochrome staining using Hoechst 33258 (7). To prepare purified HIV, the cells were centrifuged at 400 × *g* for 10 min, and the clarified supernatant was filtered through a 0.45 μm Millipore filter. The filtered material was concentrated with a Pellicon membrane filter with a 100,000 M_r cutoff, and the concentrate was centrifuged in a Beckman model L8-55M ultracentrifuge at 100,000 × *g* for 1 hr at 5°C. The virus was resuspended in 0.15 M NaCl/0.02 M sodium phosphate, pH 7.2 (PBS), and layered on a preformed 15–50% continuous Renografin gradient and centrifuged at 100,000 × *g* for 18 hr. The gradient was fractionated, and the viral band was resuspended in PBS and pelleted at 100,000 × *g* for 1 hr. For lipid analyses and ESR experiments, pelleted virus was suspended at 9 mg of protein per ml in 137 mM NaCl/15 mM Na_2HPO_4/1.5 mM KH_2PO_4/2.7 KCl/0.5 mM $MgCl_2$, pH 7.5.

Three isolates of HIV were used in the following experiments: (*i*) LK013 from the peripheral blood lymphocytes of a promiscuous male homosexual who subsequently died of AIDS (December 1985); (*ii*) COO1SE from the ejaculate of the above male homosexual; and (*iii*) F7529 from the lymph nodes of a female sex partner [i.e., AIDS-related complex (ARC) patient] of an intravenous drug abuser.

HIV Infectivity Assays. HIV infectivity was assayed by inoculating 2×10^6 H-9 cells (OKT4$^+$ leukemic cells) per ml with 1.0 ml of filtered supernate in the presence of 2 μg of Polybrene per ml (Aldrich). After adsorption of virus at 37°C, cells were resuspended in T-25 (25 cm^2) flasks. Aliquots were removed for infectivity assays on days 7, 14, 21, and 28. Three different infectivity assays were used: (*i*) magnesium-dependent, particulate reverse transcriptase activity with a template primer [poly(rA)·p(dT)$_{12-18}$ from Pharmacia P-L Biochemicals] and [*methyl*-^{3}H]dTTP (8); (*ii*) antigen-trapping assays using mouse monoclonal antibodies to

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Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; 5-NS, 5-nitroxide stearate (*N*-oxy-4′,4′-dimethyloxazolidine derivative of 5-ketostearate); 12-NS, 12-nitroxide stearate (*N*-oxy-4′,4′-dimethyloxazolidine derivative of 12-ketostearate); 16-NS, 16-nitroxide stearate (*N*-oxy-4′,4′-dimethyloxazolidine derivative of 16-ketostearate); P/L, probe/total lipid ratio; AL 721, cholesterol-poor liposome; BHT, butylated hydroxytoluene; $a_{N'}$, isotropic hyperfine coupling constant (G); *S*, polarity-corrected order parameter; $S(T_{\|})$, polarity-uncorrected order parameter; C/P, cholesterol/phospholipid molar ratio; ARC, AIDS-related complex; RBC, erythrocytes.

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p24 and p55 precursors (Cytotech, San Diego, CA); and (*iii*) immunofluorescence assays on acetone-fixed slides.

Lipid Analyses of HIV. Viral lipids were extracted by incubating intact HIV with chloroform/methanol (2:1), followed by the addition of 5% NH_4OH, using the procedures of Rouser and Fleischer (9) as modified by Aloia and coworkers (10–13). The lipid extract was concentrated by low-temperature, low-pressure rotary evaporation, passed through a Sephadex G-25 column, and the eluent, free of nonlipid contaminants, was collected. The extracts were chromatographed, and lipid spots were identified from standards run under identical conditions. The first-dimensional solvent was chloroform/methanol/saturated, aqueous ammonia (28%), 65:25:5, whereas the second was acetone/chloroform/methanol/acetic acid/water, 4:3:1:1:0.5. Lipid phosphorus was quantified according to Aloia (10–13).

The cholesterol level in the total-lipid extracts was assayed using the Boehringer Mannheim High-Performance K-Kit (692905) (14). The protein content of the intact virions was measured according to the Folin method of Lowry *et al.* (15).

HIV Spin-Labeling and Spectral Recording. HIV (9 mg of protein per ml) was spin-labeled as described for human erythrocytes (16). Spectra were recorded with a Varian E-109 ESR spectrometer fitted with a Deltron (Sydney, Australia) model DCM 20 temperature-control accessory, digitized with a Hewlett–Packard 7470A plotter and 9816 computer, and stored on floppy disc for data manipulations (16). Probe/total lipid (P/L) ratios were calculated from double integration of ESR spectra (16).

Evaluation of the Flexibility and Polarity of the HIV-Incorporated Fatty-Acid Spin Probes. The order parameters [$S(T_{\parallel})$ and S] may be used to assess the flexibility of the probe:

$$S(T_{\parallel}) = \frac{1}{2} \times \left[\frac{3(T_{\parallel} - 6.1)}{26.3} - 1\right] \quad [1]$$

and

$$S = \frac{(T_{\parallel} - T_{\perp})}{(1/3\ T_{\parallel} + 2/3\ T_{\perp})} \times 0.5653, \quad [2]$$

where $T_{\parallel}$ and $T_{\perp}$ for the HIV-incorporated probe are the hyperfine splittings parallel and perpendicular to the symmetry axis of the effective Hamiltonian function. S and $S(T_{\parallel})$ are sensitive to membrane fluidity and lie between 0 and 1, with the extremes indicating that the probe samples, respectively, fluid and immobilized environments. S includes a correction for small polarity differences between the membrane and reference crystal (6, 17).

Polarity of the environment of the probe may be estimated from the isotropic hyperfine coupling constant a_N' (6),

$$a_N' = 1/3(T_{\parallel} + 2T_{\perp}). \quad [3]$$

Increases in a_N' reflect a more polar environment.

Experimentally determined low P/L ratios were used to insure that the order parameters and a_N' values were not affected by probe–probe interactions (18, 19).

RESULTS AND DISCUSSION

Analysis of HIV membrane lipids by two-dimensional thin-layer chromatography shows a clear separation of all phospholipid classes and a high recovery (Fig. 1*A* and Table 1). Similar to other enveloped RNA viruses (21, 22), HIV has a low lipid/protein (wt/wt) ratio and a high molar cholesterol/phospholipid (C/P) ratio. Both the elevated C/P and high levels of lipids normally found in surface membranes (e.g., sphingomyelin, phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine) (Table 1) suggest that HIV lipids are derived from the host-cell plasma membrane. These results support earlier electron microscopy studies showing that HIV "buds" from the surface membrane of host lymphocytes after membrane insertion of virus-encoded proteins (e.g., gp120 and gp41) (23, 24). Apparently, the surface membrane is used as a "template" for the production of HIV virions, which include lipids derived from the host cell. Interestingly, both the high C/P ratio and the phospholipid class distribution are comparable with those of erythrocytes (Fig. 1*B* and Table 1) and other RNA viruses such as Newcastle disease virus and vesicular stomatitis virus (21, 22).

The dynamic, or "fluid", properties of the HIV lipids were studied by incorporating fatty-acid spin probes into the envelope of intact virions and recording ESR spectra (Fig. 2). With HIV at 37°C, the ESR spectra show that 5-nitroxide stearate (*N*-oxy-4′,4′-dimethyloxazolidine derivative of 5-ketostearate; 5-NS) readily inserts into the membrane, because no probe partitions into the aqueous buffer (Fig. 2). The spectra indicate that 5-NS rapidly rotates about its long

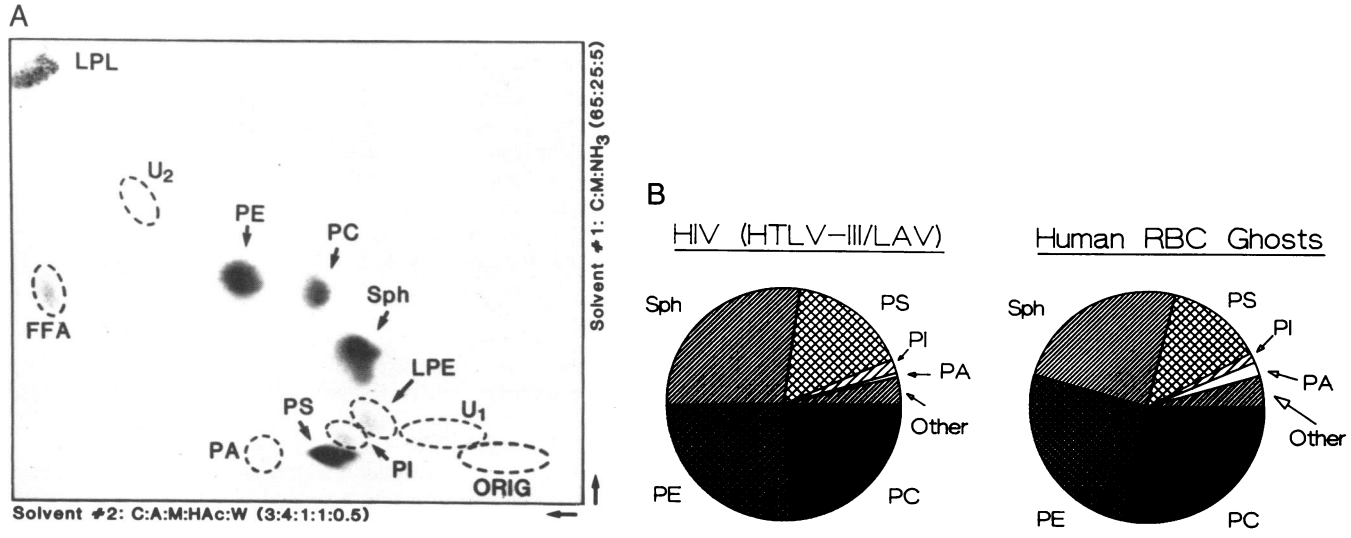

FIG. 1. Phospholipid composition of the HIV envelope and erythrocyte (RBC) ghosts. (*A*) Two-dimensional thin-layer chromatography of HIV membrane lipids. Sph, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; LPE, lysophosphatidylethanolamine; Orig, origin; FFA, free fatty acids; LPL, less polar lipid (including cholesterol); U_1 and U_2, unknowns; PA, phosphatidic acid. (*B*) Percent distributions of major phospholipids in HIV and human RBC ghosts (Table 1).

Table 1. Lipid compositions of HIV and human erythrocyte (RBC) ghosts

Membrane	Total lipid/protein, wt/wt*	C/P†	Major phospholipids, mol % of total phospholipids							
			PC	PE	Sph	PS	PI	PA	Other	Rec, %‡
HIV§	0.28 ± 0.05	0.88 ±0.05	23.8 ±1.7	24.6 ±1.8	28.3 ±0.7	15.1 ±0.9	2.1 ±0.6	0.9 ±0.4	5.0¶ ±1.0	97.6 ±0.9
RBC‖	1.00	0.91	28.3 ±0.6	26.0 ±0.6	24.6 ±0.4	13.4 ±0.8	1.1 ±0.4	2.1 ±0.2	4.6**	98.7

See Fig. 1 for phospholipid abbreviations.
*Total lipid/protein is (mg of cholesterol plus mg of phospholipid) per mg of protein.
†C/P is cholesterol/phospholipid molar ratio.
‡Rec, percent recovered phospholipid (determined by comparing total quantity of phospholipid recovered from chromatography with quantity of phosphorus recovered from an unchromatographed aliquot).
§Present study. Mean ± SD from three determinations on each of isolates LK013 and F7529 (see text for definition of isolates).
¶Includes U_1 and U_2 (see Fig. 1), material remaining at origin during chromatography and lysoglycerophosphatides.
‖Data from Turner and Rouser (20). For phospholipids, values are mean ± SD for eight samples.
**Includes 13 minor components plus lysophosphatidylcholine.

molecular axis (≈10^8 revolutions per sec), with movement of this axis away from the preferred orientation severely restricted. This rapid anisotropic motion creates an effective symmetry axis. $2T_{\parallel}$ and $2T_{\perp}$ are the hyperfine splittings due to the interaction of the spin of the unpaired electron with the nuclear spin of the ^{14}N and are measured from the ESR spectra parallel and perpendicular to the unique symmetry axis (Fig. 2B). Increasing the distance of the reporter group from the carboxyl terminus of the incorporated probe [i.e., 5-NS to the similarly conjugated 12-ketostearate (12-NS) to the conjugated 16-ketostearate (16-NS)] decreases $2T_{\parallel}$ and increases $2T_{\perp}$, indicating greater movement of the nitroxide group (Fig. 2A). Consequently, the carboxyl group of the fatty-acid spin probe is relatively anchored to the polar surface, whereas the more mobile methyl terminus lies inside the membrane interior. Because the respective a_N' values for 5-, 12-, and 16-NS-labeled HIV are 15.58, 14.80, and 13.60 G, the polarity of the environment of the oxazolidine ring decreases as it approaches the envelope interior. This vertical flexibility and polarity gradient, with the interior more fluid and less polar than the surface, is a ubiquitous feature of other enveloped viruses (25) and biomembranes (26). The most likely interpretation is that the long molecular axis of each NS probe is perpendicular to a lipid bilayer.

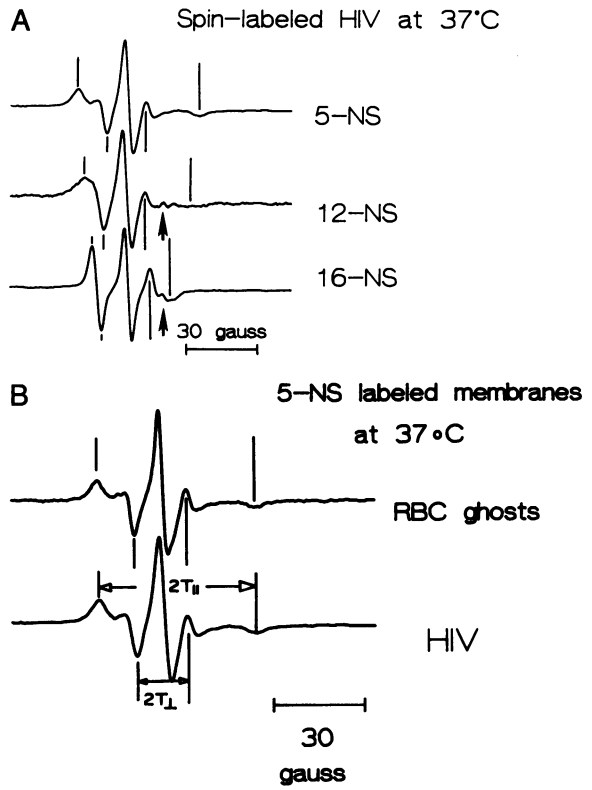

FIG. 2. ESR spectra of nitroxide-stearate-labeled intact HIV and human erythrocyte ghosts. (A): HIV-labeled with 5-NS, 12-NS, and 16-NS at 37°C. ESR spectrometer conditions were as follows: 8-min scan time, 3.2-G modulation amplitude, 10-mW microwave power, and 1-sec time constant. Peak heights of the central band were normalized. For each spectrum, the outer vertical hatch marks indicate $2T_{\parallel}$, whereas the inner ones denote $2T_{\perp}$ (see Fig. 2B). The horizontal axis represents varying magnetic field, whereas the vertical axis reflects absorption of microwaves. Probe/total lipid (P/L) ratios for the 5-NS, 12-NS, and 16-NS spectra were 1/139, 1/697, and 1/159. Arrows in the 12-NS and 16-NS spectra denote a "liquid-line" component, due to a small amount of probe (<2% total spin probe) partitioning into the aqueous buffer. (B) ESR spectra of 5-NS-labeled human RBC ghosts (P/L = 1/4600) (16) and intact HIV (P/L = 1/139) at 37°C. Measurement of $2T_{\parallel}$ and $2T_{\perp}$ are indicated in the HIV spectrum; $2T_{\perp}$ is corrected by addition of 1.6 G (16). Horizontal bar, 30 G.

The fluidity of 5-NS-labeled HIV indicates that the viral envelope is among the most rigid membranes studied to date. To quantitate membrane fluidity, either a polarity-corrected order parameter [S = 0.652 ± 0.001 (mean ± 1 SD) for the LK013, COO1SE, and F7529 isolates] or polarity-uncorrected order parameters [$S(T_{\parallel})$ = 0.723 ± 0.009 for the three isolates] may be calculated from the ESR spectra of 5-NS-labeled virus at 37°C (Fig. 2B). These elevated order parameters show that the HIV lipid envelope is highly ordered. For example, the high $S(T_{\parallel})$ for HIV is similar to those of other 5-NS-labeled viruses, such as equine infectious anemia virus, bovine leukemia virus, Friend murine leukemia virus, and avian myeloblastosis virus (Fig. 3) (28). That equine infectious anemia virus and HIV share a rigid envelope supports the genetic study of Stephens et al. (29) showing that these two retroviruses are closely related. Another interesting near-identity is that of the ESR spectra and order parameters of 5-NS-labeled HIV and of another rigid membrane, human erythrocytes (Figs. 2B and 3) (16); similar fluidities are most likely due to their comparable lipid compositions (Table 1 and Fig. 1B).

The structural property or properties forming the highly ordered envelope of 5-NS-labeled HIV need clarification. Previous ESR studies show the ordering properties of 5-NS-labeled membranes to be sensitive to (i) the protein/lipid ratio, (ii) the phospholipid unsaturated fatty acid/saturated fatty acids ratio, (iii) the C/P ratio, (iv) the glycolipid distribution, (v) anesthetics and drugs, and (vi) temperature (27). Increases in ordering properties i.e., membrane rigidity) are produced by raising the protein/lipid ratio; augmenting saturated fatty acids, glycolipids, or cholesterol; or cooling. In contrast, heating, removing cholesterol, or most local anesthetics lowers order parameters (i.e., membrane fluidization).

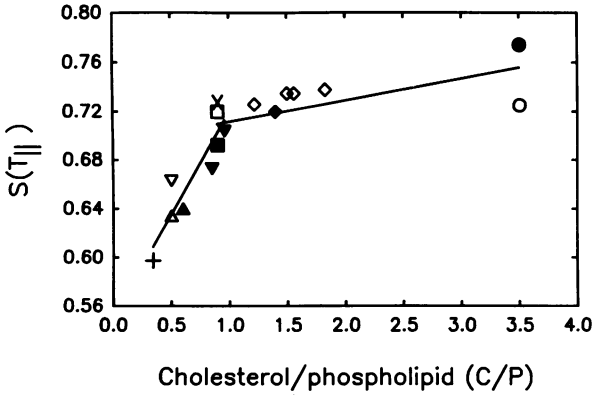

FIG. 3. $S(T_{\parallel})$ vs. C/P for 5-NS-labeled HIV (×), rat heart plasma membranes (+), human platelets (△) and human platelet plasma membranes (▽), rat liver plasma membranes (▲) and cholesterol-enriched rat liver plasma membranes (▼), whole human erythrocytes (■) and human erythrocyte ghosts (□), chicken intestinal brush border membranes (♦), human lens cortex (●) and lens cortex lipid extracts (○) (27), and (◇, left to right) equine infectious anemia virus, avian myeloblastosis virus, Friend murine leukemia virus, and bovine leukemia virus (28), at 37°C.

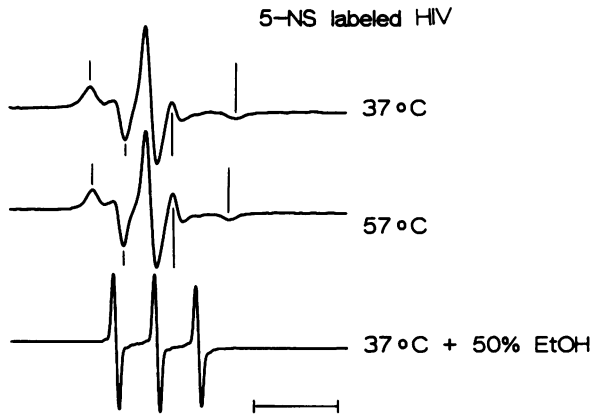

FIG. 4. ESR spectra of 5-NS-labeled intact HIV at 37°C, 57°C, and 37°C with 50% ethanol. At 37°C and 57°C, the splittings ($2T_{\parallel}$) are denoted by the outermost vertical hatch marks, whereas $2T_{\perp}$ are indicated by the innermost pair of vertical hatch marks. The P/L was 1/139. The HIV isolate used was LK013. Horizontal bar, 30 G.

The ordered HIV envelope is most likely due to its high C/P (Fig. 3). In earlier ESR studies of mammalian plasma membranes, we determined that the C/P is one of the single most important regulators of bilayer fluidity (27, 30, 31). For example, treatment with high-cholesterol liposomes artificially enriched native rat liver plasma membranes from a C/P of 0.70 to 0.95 and elevated $S(T_{\parallel})$ by ≈10% at 37°C (Fig. 3) (30); this is equivalent to cooling native membranes by 10°C. Here, $S(T_{\parallel})$ was plotted against the C/P ratio for various 5-NS-labeled surface membranes and viruses at 37°C (Fig. 3). High-cholesterol contents are associated with decreased probe flexibility. Fig. 3 illustrates that $S(T_{\parallel})$ increases with cholesterol content for C/P <≈1.0 and plateaus for higher C/P ratios, suggesting that the elevated lipid ordering seen with HIV and other enveloped viruses is due to increased cholesterol content.

HIV infectivity may be maintained by a combination of lipid ordering and enriched levels of C/P. Recall that the fluidity and C/P of human erythrocytes resemble those of HIV (Figs. 2*B* and 3). Long-term stability of both HIV and erythrocytes at low temperatures (32, 33) may in part be due to their rigid membranes. Incubation above 42°C inactivated HIV (32, 33), with the virus losing infectivity by a logarithmic factor of three after 1 hr at 56°C (34). We have examined the effects of high temperature on HIV fluidity. Fig. 4 shows the ESR spectra of 5-NS-labeled HIV at 37°C and 57°C; at the higher temperature the outer splitting ($2T_{\parallel}$) is narrowed, while the inner splitting ($2T_{\perp}$) is greater. Heating from 37° to 57°C decreases the order parameter $S(T_{\parallel})$ of HIV (COO1SE isolate) from 0.714 to 0.610, for a $\Delta S(T_{\parallel}) = -14\%$.

Although other explanations cannot presently be excluded, heating may decrease HIV viability by increasing the envelope fluidity above a critical minimum. These heating effects can be contrasted with the actions of 50% ethanol, an agent known to abolish HIV infectivity within 1 min (34). As opposed to the moderate fluidizing actions of 57°C, Fig. 4 shows that 50% ethanol acts as a chaotropic agent to destroy the lipid bilayer; the sharp three-line spectrum is due to the 5-NS probe freely tumbling in solution. Although 50% ethanol is likely to immediately abolish HIV infectivity by disrupting the viral envelope, other mechanism(s) must be invoked to explain why the more modest fluidization at 57°C slowly inactivates HIV. One possibility is that fluidization promotes a time-dependent, thermal denaturation or aggregation of viral envelope proteins. In this context, increased fluidity produced by local anesthetics decreased (35), whereas elevated lipid ordering accomplished by cholesterol enrichment increased (30) the thermostability of membrane-bound enzymes.

The above findings suggest that pharmacological interventions that selectively raise the fluidity of HIV may also destabilize the virus. Although the dramatic dissolution of the HIV envelope produced by 50% ethanol seen in Fig. 4 cannot be attained in a therapeutic setting, a more modest fluidization, such as that seen with 57°C, may well be achieved with drug therapies. Using Fig. 3 to calibrate the sensitivity of HIV fluidity to cholesterol content (as a first approximation), removal of ≈30–40% cholesterol would be predicted to lower the order parameter similarly to that observed by heating native HIV to 57°C (i.e., $S(T_{\parallel})$ = ≈0.61). If membrane fluidity is a critical regulator of HIV stability, then cholesterol depletions of this magnitude may well inactivate virus. We note, particularly, the report of Sarin *et al.* (3) that the cholesterol-poor liposome AL 721 at doses that fluidize human erythrocytes by removing cell surface cholesterol (36) inhibited HIV infectivity. For erythrocyte ghosts, AL 721 treatment increased membrane fluidity by an amount similar to that produced by heating native membranes by ≈10°C (36). Given similarities in the C/P ratios and lipid compositions of HIV and human erythrocyte ghosts (Fig. 1*B* and Table 1), speculation is not untoward that AL 721 will also directly extract cholesterol from HIV and raise the "effective" viral membrane temperature to >50°C. Then, the inhibited HIV replication induced by AL 721 (3) would simply be due to a heating-like effect at 37°C that promotes viral inactivation. A similar mechanism may be invoked to account for the ability of amphotericin B, an agent that fluidizes membranes by complexing cholesterol (4), to inhibit HIV replication in lymphocytes (5).

The hypothesis that the cholesterol content and fluidity of the HIV envelope play critical roles in viral infectivity agrees with other studies on lipid-enveloped viruses. For example, the infectivity of vesicular stomatitis virus is reduced by incubating this virus with cholesterol-poor liposomes (37), a treatment that removed cholesterol and also fluidized the viral membrane as shown by a fluorescent probe. Another fluidizing drug, butylated hydroxytoluene (BHT) (38), inactivates *in vitro* both lipid-enveloped DNA and RNA viruses (39–41). Should high C/P and lipid ordering prove an absolute requirement for the viability of enveloped viruses, then therapeutic regimens that reduce these properties may be effective not only for HIV-1, but also for another viral type (HIV-2) that also causes AIDS (42). In future studies, lipid

composition and fluidity of HIV strains that differ by at least 20% in nucleotide sequence of their envelope genes should be examined. For healthy seropositives and patients with ARC or AIDS, combination-drug therapies may use reverse transcriptase inhibitors, envelope-perturbing agents, and immunostimulants to restore helper T cell/suppressor T cell ratios (2).

Also intriguing is the possibility that a dietary supplement of an envelope-perturbing agent may be "chemoprophylactic" for those at risk for HIV infection. The fatal infection of chickens caused by Newcastle disease virus is prevented by diets containing BHT levels normally used for antioxidant purposes (100–200 ppm of diet) (39). BHT is of particular interest to test as a chemoprophylactic agent for HIV infection, because HIV and Newcastle disease virus are similarly enriched in cholesterol and phospholipids found in surface membranes (Table 1) (22), and BHT has limited toxicity and attains high tissue levels due to its fat solubility (43). Indeed, preliminary results indicate that incubation of HIV with 320 μg of BHT per ml for 30 min at 37°C reduces infectious viral activity in H9 lymphocyte cultures by a logarithmic factor of 4 (F.C.J., unpublished data).

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