# Transbilayer Phospholipid Asymmetry and Its Maintenance in the Membrane of Influenza Virus†

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ABSTRACT: Two phospholipid exchange proteins and two phospholipases C have been employed to determine the phospholipid composition of the outer surface of the membrane of influenza virus. These four protein probes have defined the same accessible and inaccessible pool for each viral phospholipid. Phospholipids which are exchangeable or hydrolyzable are located on the outer surface, whereas the inaccessible pool is located at the inner surface of the viral bilayer. The two pools are unequal in size, with ca. 30% of the total phospholipid accessible to the four proteins, and ca. 70% inaccessible. The membrane is thus highly asymmetric with regard to the amount of phospholipid on each side of the membrane. There is also a marked asymmetry of phospholipid composition. Phosphatidylcholine and phosphatidylinositol are enriched in the outer surface, and sphingomyelin is enriched in the inner surface, whereas phosphatidylethanolamine and phosphatidylserine are present in similar proportions in each surface. This distribution is qualitatively different from that previously reported for the human erythrocyte. The close agreement between results obtained with exchange proteins and phospholipases C demonstrates that the hydrolytic action of these enzymes does not alter phospholipid asymmetry. The nonperturbing nature of the exchange proteins has permitted the rate of transmembrane movement of phospholipids (flip-flop) in the intact virion to be studied. This process could not be detected after 2 days at 37 °C. It was estimated that the half-time for flip-flop is indeterminately in excess of 30 days for sphingomyelin and 10 days for phosphatidylcholine at 37 °C. These extremely long times provide a simple explanation for the maintenance of transbilayer asymmetry in influenza virions and, possibly, other membranes. Since the viral membrane is acquired by budding through the host cell plasma membrane, the transbilayer distribution of phospholipids observed in the virions presumably reflects a similar asymmetric distribution of phospholipids in the host cell surface membrane. Because animal cells in culture do not incorporate extracellular phospholipid, our results demonstrate that individual cells have the capacity to generate asymmetric membranes.

Compelling evidence now exists that phospholipids are distributed in a highly asymmetric manner between the two sides of the plasma membrane of red blood cells (Bretscher, 1972a,b; Verkleij et al., 1973; Gordesky and Marinetti, 1973; Whiteley and Berg, 1974). It is of considerable interest to extend these studies to other biological membranes in order to determine whether they also exhibit phospholipid asymmetry, whether this asymmetry is similar to that of red blood cells, and how such an asymmetry could be maintained over biologically relevant periods of time. Enveloped viruses such as influenza offer several advantages in this regard. Lipids are incorporated into the viral particles by budding through the plasma membrane of the host cell, and the viral lipid composition bears a close resemblance to that of the host cell plasma membrane (Lenard and Compans, 1974; Klenk, 1973). The sidedness of the host cell plasma membrane lipids is, therefore, presumably preserved in the viral particles. Spin label (Landsberger et al., 1971, 1973) and x-ray diffraction studies (Harrison et al., 1971) have shown that the viral lipid is arranged in a bilayer in the virion, and that the fluidity of the viral bilayer is a property conferred by the host cell (Landsberger et al., 1973). Previous work employing phospholipases indicated that the individual phospholipids of influenza virus are asymmetrically distributed between the two sides of the membrane (Tsai and Lenard, 1975). Here we describe a novel technique which has been applied to investigate phospholipid bilayer asymmetry in an influenza virus with a different lipid composition.

Phospholipid exchange proteins (Wirtz, 1974) are uniquely suitable as nonperturbing probes for studying the asymmetry of membrane structure. These water-soluble proteins catalyze the exchange of individual molecules of phospholipid (Johnson and Zilversmit, 1975; Demel et al., 1973) between populations of biological membranes and phospholipid bilayer vesicles (Kamp et al., 1973; Enholm and Zilversmit, 1973; Hellings et al., 1974) without effecting a net transfer of phospholipids (Wirtz and Zilversmit, 1968) and with no detectable fatty acyl chain preference (Wirtz et al., 1970). Furthermore it has been demonstrated that only phospholipids which are in the outer monolayer of single-walled bilayer vesicles are available for exchange (Rothman and Dawidowicz, 1975; Johnson et al., 1975). Application of the exchange proteins thus permits the identification of phospholipid molecules on the outer surface of a membrane without degrading or covalently modifying that membrane. This property makes it possible to measure the rate of transbilayer exchange of phospholipid [flip-flop (Kornberg and McConnell, 1971a)], a parameter of fundamental importance for understanding how biological membrane asymmetry is created and maintained.

In this study we demonstrate that the distribution of individual phospholipids between the inner and outer surfaces of the influenza membrane bilayer is highly asymmetric, and that phospholipid exchange between the two surfaces occurs extremely slowly, or not at all.

## Materials and Methods

*Growth and Purification of Virus.* The WSN ($H_0N_1$) strain of influenza A virus was grown in Maden Darby bovine kidney

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(MDBK)[1] cells as described by Choppin (1969). The cells were inoculated at a multiplicity of ca. 5 PFU/cell and harvested 16–24 h after inoculation. For $^{32}P$-labeled virus, cells were grown and virus infection was carried out in Dulbecco's modified Eagle's medium containing 0.1 mM instead of the usual 1 mM phosphate. Total inorganic phosphate concentration in the medium was determined by the method of Bartlett (1959) after addition of the appropriate amount of serum. [$^{32}P$]Orthophosphate (New England Nuclear) was added to the medium at a specific activity of 1 $\mu$Ci/$\mu$mol P during both cell growth and virus infection. Since each cell passage involves four to five cell doublings, the presence of $^{32}P$ at a constant specific activity during the entire cell growth period and during virus infection assures that all phospholipids are uniformly labeled in the progeny virus, as has been demonstrated by Quigley et al. (1972) for a different cell–virus system. Uniform labeling in the present experiments was demonstrated as follows. MDBK cell cultures grown in parallel were individually infected with influenza virus on each of 5 successive days, starting 3 days after cell passage. The distribution of $^{32}P$ among individual phospholipids in the purified virions did not change.

For labeling with [$^3H$]leucine or [$^3H$]uridine (New England Nuclear), the isotope was added to the medium after infection at about 1 $\mu$Ci/ml. [$^3H$]Leucine was used for all phospholipid exchange and phospholipase hydrolysis experiments, and [$^3H$]uridine was used for the ribonuclease digestion experiments. Virus was precipitated with polyethylene glycol and sodium chloride (Landsberger et al., 1971), purified by centrifugation on a 15–60% sucrose gradient (Lenard and Compans, 1975), and dialyzed against a buffer containing 0.1 M KCl–1 mM $Na_2EDTA$–0.01 M Tris-HCl, pH 7.4. The disaggregated virus band was used for all experiments. On one occasion the sucrose-purified virus was further purified on a 5–40% potassium tartrate gradient (Compans et al., 1970) before use in exchange and phospholipase experiments. The results obtained were identical with those for virus on which the prior tartrate purification was omitted. Virus was purified on tartrate gradients after each exchange or phospholipase experiment prior to analysis in order to remove any disrupted virions. [$^3H$]Uridine-labeled virions prepared for ribonuclease digestion experiments were purified on both sucrose and tartrate gradients before use. The concentration of viral phospholipid in $^{32}P$-labeled preparations was determined from the radioactivity of the virus and the specific activity of the growth medium, assuming a typical value (0.85) for the fraction of viral phosphorus which is present as phospholipid. For preparations without $^{32}P$ label, phospholipid was estimated from the $A_{280}$ of a sample diluted with 9 volumes of 1% sodium dodecyl sulfate. An $A_{280}$ of 0.0042 in the diluted sample corresponded to 1 nmol/ml phospholipid in the undiluted viral suspension, using a $^{32}P$-labeled virus preparation as a standard.

*Phospholipid Vesicles.* Dioleoylphosphatidylcholine was synthesized according to the procedure of Robles and van den Bergh (1969). Sphingomyelin was obtained from Applied Science Laboratories. Cholesterol was recrystallized two times from ethanol before use. To prepare vesicles (Huang, 1969), 1.5 $\mu$mol of dioleoyl-PC, 1.5 $\mu$mol of SM, and 3.0 $\mu$mol of cholesterol were lyophilized from benzene solution, dispersed in 1.0 ml of 0.1 M KCl–1 mM $Na_2EDTA$–0.01 M Tris-HCl, pH 7.4, and sonicated at 4 °C under $N_2$ as described previously (Rothman and Dawidowicz, 1975). Vesicles were stored at 4 °C under $N_2$ and remained stable for use in these experiments for up to a month.

*Assays of Exchange Proteins.* Two assays were used for purification and monitoring of exchange activity. The microsome–vesicle assay system, originally developed by Kamp et al. (1973), was modified as described earlier (Rothman and Dawidowicz, 1975). A novel assay utilizing exchange between erythrocyte ghosts and vesicles was also employed. Vesicles were made from [$^3H$]dioleoyl-PC and a trace of [$^{14}C$]cholesteryl oleate, a nonexchangeable marker, as previously described (Rothman and Dawidowicz, 1975). Ghosts were prepared according to a standard procedure (Hanahan and Ekholm, 1974) and were stored at −20 °C at a concentration of 1.5 $\mu$mol/ml lipid P until use. Under these conditions, ghosts could be used for at least 6 months. Exchange activity was determined by measuring the protein dependent transfer of [$^3H$]PC from vesicles to ghosts. Protein fractions were incubated with 0.15 $\mu$mol/ml lipid P ghosts, 2 nmol/ml PC vesicles (with 0.05 $\mu$Ci/ml $^3H$ and 0.005 $\mu$Ci/ml $^{14}C$) in a final volume of 0.5 ml of 0.1 M KCl–0.01 M Tris-HCl, pH 7.4, for 10 min at 37 °C. The samples were chilled on ice (the reaction rate at 0 °C is less than 10% of the rate at 37 °C) and centrifuged in an Eppendorf 3200 centrifuge for 2 min. The supernatant was aspirated and the pellet was suspended in 1 ml of water and counted with 10 ml of a Triton–toluene scintillation fluid (Patterson and Greene, 1965). The amount of [$^3H$]PC in the pellet was corrected for vesicle contamination as reflected by pellet $^{14}C$, and the percent of vesicle [$^3H$]PC transferred to ghosts was calculated (Rothman and Dawidowicz, 1975). One unit of exchange activity is defined as a rate of transfer of [$^3H$]PC of 1% per min, and is approximately equivalent to 0.2 unit (Kamp et al., 1973) of activity in the microsome–vesicle assay. The measurement is linear up to at least 1.5 units, with high reproducibility. This assay affords greater sensitivity (ca. tenfold) and is more rapid than the microsome–vesicle assay. Exchange activity throughout this paper is expressed in the units of the ghost–vesicle assay.

*Phospholipid Exchange Proteins.* A PC-specific exchange protein was purified from calf liver by DEAE-cellulose, CM-cellulose, and Sephadex G-50 column chromatography according to the procedure of Kamp et al. (1973) using the microsome–vesicle assay. A 2000-fold purification was achieved, and the preparation had a specific activity of 7000 units/mg in the ghost–vesicle assay. The exchange protein was stored at −20 °C in 50% glycerol and was dialyzed before use against 0.1 M KCl–10 mM 2-mercaptoethanol–10 mM Tris-HCl, pH 7.4, in the presence of 0.1 mg/ml bovine serum albumin.

Phospholipid exchange protein from bovine heart was purified by Sephadex G-75 and CM-cellulose column chromatography as described by Johnson and Zilversmit (1975) except that the ghost–vesicle assay was used. The preparation used in this work was 390-fold purified when compared with the pH 5.1 supernatant by the microsome–vesicle assay, a degree of purification by which a different exchange assay corresponds to a nearly homogeneous preparation (Enholm and Zilversmit, 1973). When our preparation was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, however, several major bands were observed. The purified exchange protein had a specific activity of 200 units/mg in the ghost assay and was stored in small aliquots at a protein concentration of 1 mg/ml at −70 °C in 40 mM Tris-acetate, pH 7.4, 5 mM 2-mercaptoethanol, 0.02% $NaN_3$. Under these conditions, full activity

[1] Abbreviations used are: PC, phosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; MDBK, Maden Darby bovine kidney; $Na_2EDTA$, disodium ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; CM, carboxymethyl; PFU, plaque forming unit.

was retained for at least 4 months. Both liver and heart exchange proteins retail full activity after 24 h at 37 °C under conditions similar to those employed here (Rothman and Dawidowicz, 1975; Johnson et al., 1975).

*Phospholipases C.* Phospholipase C from *Bacillus cereus* was prepared according to Haverkate and van Deenen (1965) and stored at 4 °C at a protein concentration of 1 mg/ml. A crude preparation of phospholipase C from *Clostridium welchii* was purchased from Sigma Chemical Co. (Type 1) and had a specific activity of 3.6 units/mg. It was dissolved in 0.02 M $CaCl_2$–0.05 M Tris-HCl, pH 7.4.

*Phospholipid Exchange between Vesicles and Virus.* Exchange reactions were carried out at 37 °C in 1 ml of 0.1 M KCl–1 mM $Na_2EDTA$–5 mM 2-mercaptoethanol–0.02% $NaN_3$–10 mM Tris-HCl, pH 7.4, and contained (unless otherwise specified): 5 nmol/ml phospholipid P of virus labeled with [$^{32}$P]- and [$^{3}$H]leucine, 30 nmol of P/ml of phospholipid vesicles, 0.1 mg/ml bovine serum albumin, and 20 units/ml of either liver exchange protein or heart exchange protein. In control incubations, either the exchange protein or the vesicles and the exchange protein were omitted. After an appropriate time of incubation, the reaction was chilled on ice and the viral particles were separated from the vesicles by isopycnic centrifugation in a linear 5–40% (w/v) potassium tartrate gradient for 45 min at 35 000 rpm at 4 °C in the SW 50.1 rotor. In early experiments, fractions were collected and aliquots counted for $^{3}$H and $^{32}$P to locate the virus band. The peak fractions were then pooled for further analysis. In most experiments, the virus band was located visually and removed in a minimum volume with a Pasteur pipette. Equivalent results were obtained.

*Phospholipase Digestions.* Phospholipase C digestions proceeded at 37 °C in 1 ml of 0.1 M KCl–1 mM $Na_2EDTA$–5 mM $MgCl_2$–5 mM $CaCl_2$–10 mM Tris-HCl, pH 7.4, containing 5 nmol/ml phospholipid P of virus labeled with [$^{32}$P]- and [$^{3}$H]leucine and either 20 μg/ml *B. cereus* phospholipase C or 10 μg/ml *Cl. welchii* phospholipase C. After an appropriate time, the treated virus was repurified by tartrate gradient centrifugation as described above.

*Analysis of Phospholipids after Phospholipase and Exchange Reactions.* The virions, suspended in potassium tartrate, were extracted by the method of Folch et al. (1957) after the addition of about 10 μg of carrier lipid (previously extracted from canine erythrocytes). The lower organic phase was evaporated under a stream of nitrogen, and the residue was dissolved in benzene–ethanol (1:1, v/v) and lyophilized. The extract was dissolved in a minimum volume of chloroform–methanol (2:1, v/v) and spotted on a freshly activated (1 h at 110 °C) glass-backed silica gel G 1500 thin-layer plate (Schleicher and Schuell, Keene, N.H.) in a dry nitrogen atmosphere. The plates were developed using the solvent system chloroform–methanol–acetic acid–water (25:15:4:2, v/v/v/v) (Skipski et al., 1968; Skipski and Barclay, 1969). Fractions were scraped from the plates into vials and radioactivity was counted with a toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene scintillation fluid. Recovery of counts from the plates routinely exceeded 95% of those applied.

When the vesicles were to be analyzed after exchange reactions, the sample layer above the tartrate gradients was removed following centrifugation, extracted, and chromatographed in a similar manner.

*Measurement of Phospholipid Exchange.* In reactions catalyzed by the liver exchange protein, which is specific for PC (Kamp et al., 1973), the fraction of viral [$^{32}$P]PC transferred to vesicles was calculated from the radioactivity in the virions after the incubation as $1 - [([^{32}P]PC/[^{32}P]SM)_E/([^{32}P]PC/[^{32}P]SM)_C]$, where the subscripts E and C denote exchange and control reactions, respectively. This quantity could also be calculated by normalization to [$^{32}$P]PE instead of [$^{32}$P]SM, with equivalent results. For the heart protein, which does not catalyze PE exchange (Enholm and Zilversmit, 1973), the fraction of individual viral $^{32}$P-labeled phospholipids transferred to vesicles was calculated as $1 - [([^{32}P]PX/[^{32}P]PE)_E/([^{32}P]PX/[^{32}P]PE)_C]$, where PX is either PC, SM, PI, or PS.

All the data for phospholipid exchange presented in this paper represent reaction end points, where all available phospholipid has been equilibrated between virions and vesicles. That is, further incubation produced no detectable increment in the transfer of individual $^{32}$P-labeled phospholipids to vesicles. The time required to attain equilibrium was determined for each viral preparation used; although some variation from one preparation to another was observed, in general end points were reached by about 5 h of incubation. Heart-protein-catalyzed SM exchange consistently lagged behind PC exchange, as has been reported previously (Enholm and Zilversmit, 1973).

The total amount of $^{32}$P-labeled phospholipids transferred was measured directly in a few instances from the $^{32}$P/$^{3}$H ratio of the virions as described below.

*Measurement of Phospholipase C Hydrolysis.* The amount of total phospholipid hydrolyzed was determined independently in either of two ways. First, an aliquot of the virus band from the tartrate gradient was counted and the [$^{32}$P]/[$^{3}$H]leucine ratio was measured. The percent decrement in this ratio as compared with the untreated virus after tartrate purification is the percent of total $^{32}$P released by the enzyme. The percent of $^{32}$P-labeled phospholipid hydrolyzed was calculated by correcting the total $^{32}$P for the amount which was not extractable into chloroform–methanol (15%). Alternatively, an aliquot of the digestion mix was taken immediately prior to gradient centrifugation, precipitated with trichloroacetic acid, and the percent of total $^{32}$P rendered acid soluble was determined as described below. This figure was also corrected for nonlipid $^{32}$P to yield the percent of phospholipid $^{32}$P hydrolyzed. The two methods were in excellent agreement.

The individual $^{32}$P-labeled phospholipids of the treated virus were analyzed by thin-layer chromatography. For *B. cereus* phospholipase C, which does not hydrolyze SM in red cell ghosts (Verkleij et al., 1973), the amount of PC, PS, PI, and PE hydrolyzed was ascertained by normalizing these phospholipids to SM and comparing these values with the corresponding values for untreated tartrate-purified controls in exactly the same way as for the exchange proteins. Alternatively, the amount of each phospholipid hydrolyzed was determined from the phospholipid composition of the digested virus as compared with an untreated tartrate-purified control, and the independently measured percent of total phospholipid hydrolyzed (see above). The two measurements gave similar results, confirming the lack of SM hydrolysis for influenza virus. For the *Cl. welchii* enzyme, hydrolysis of individual phospholipids could only be assessed by the latter method. This was so because all the viral phospholipids were hydrolyzed by the crude enzyme preparation used here. In contrast, similar preparations did not hydrolyze PS or PI in red cell ghosts (Zwaal et al., 1973; Coleman et al., 1970). We have no explanation for this difference.

The data for phospholipid hydrolysis reported here are end points; detectable increments in hydrolysis did not occur upon prolonged incubation. End points were reached in less than 1 h.

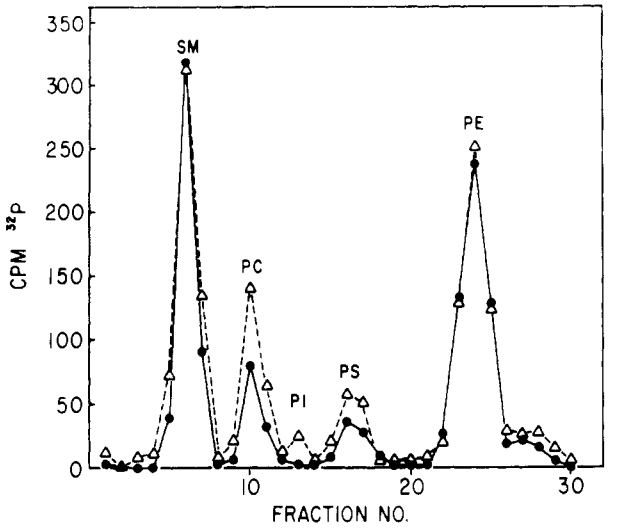


FIGURE 1: Thin-layer chromatographic analysis of virion phospholipids after incubation with phospholipid vesicles in the presence (●—●) or in the absence of (△- - -△) heart exchange protein (10 units/ml). Incubation was for 6 h. One chromatogram was scaled slightly in order to make the total counts in the PE peak of both chromatograms the same. Each vial was counted for 10 min.

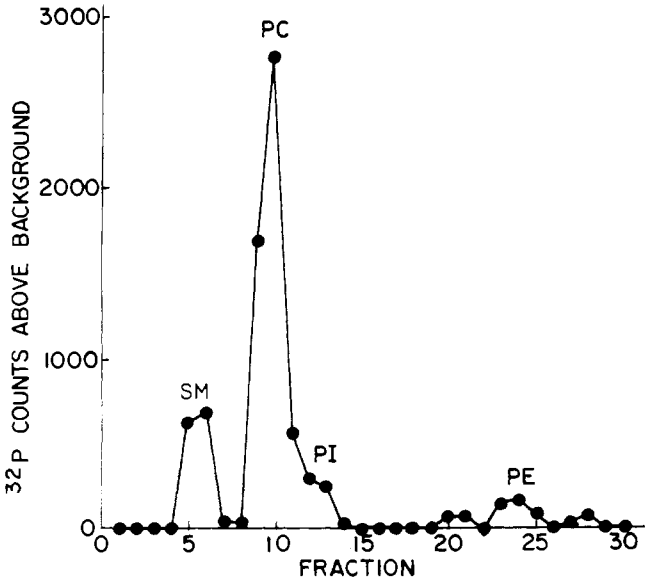


FIGURE 2: Thin-layer chromatographic analysis of phospholipid vesicles after an exchange reaction different from that of Figure 1. Vesicles were collected after tartrate gradient centrifugation from four 1-ml exchange reactions which contained the heart exchange protein. The vesicles were pooled, extracted, and chromatographed. Counts were accumulated over a 20-min period. Background was 32 cpm.

*Determination of Trichloroacetic Acid Soluble Radioactivity.* To 100 μl of a sample containing radioactive virus were added 0.1 ml of 10 mg/ml bovine serum albumin, 1 ml of water, and 0.3 ml of 25% trichloroacetic acid. After about 5 min at 0 °C, the precipitate was centrifuged. One milliliter of the supernatant was neutralized with 50 μl of 5 N NaOH and counted with 10 ml of a Triton-containing scintillation fluid (Patterson and Greene, 1965).

## Results

*Exchange of Phospholipid between Virus and Vesicles.* When $^{32}$P-labeled virions are incubated with heart exchange protein and unlabeled phospholipid vesicles, a redistribution of $^{32}$P-labeled phospholipids takes place. Analysis of the subsequently isolated virions by thin-layer chromatography (Figure 1) shows a selective decrease in the radioactivity of individual phospholipids. Vesicles examined after the exchange reaction (Figure 2) contain a complementary pattern of $^{32}$P-labeled phospholipids (with the exception of PS, as noted below). Thus, significant amounts of labeled SM, PC, and PI are transferred to the vesicles, whereas less than 2% of the virion [$^{32}$P]PE is detected, in confirmation of the previously reported inability of heart exchange protein to catalyze PE exchange (Enholm and Zilversmit, 1973). The $^{32}$P-labeled phospholipids transferred to the vesicles (Figure 2) by heart exchange protein are a highly nonrepresentative sample of the total virion phospholipids. Whereas PC is a minor component in the viral membrane, it is the major $^{32}$P-labeled phospholipid in the vesicles; the converse is true for SM.

When the liver exchange protein is incubated with labeled virus and vesicles, [$^{32}$P]PC is the only virion phospholipid which is decreased and is the only labeled phospholipid present in the vesicles (not shown). This protein has been previously shown to be specific for PC (Kamp et al., 1973).

The ratio of individual virion $^{32}$P-labeled phospholipids to [$^{32}$P]PE was indistinguishable for virions incubated alone or in the presence of phospholipid vesicles (Table I). This fact, plus the absence of significant amounts (less than 2%) of [$^{32}$P]PE in the vesicles after protein-catalyzed exchange (Figure 2) demonstrates that transfer does not occur in the absence of exchange protein.

The exchange of individual phospholipids by exchange proteins was routinely measured from the phospholipid composition of the repurified virions as described under Materials and Methods. To confirm the reliability of this procedure, the predicted phospholipid composition of the vesicles after an exchange reaction was calculated from the virion phospholipid composition and found to be in close agreement with that determined directly from the vesicles of the same reaction.

Table II presents the maximum percent of viral phospholipid which could be transferred to vesicles by the heart and liver exchange proteins. A relatively large amount, 43 ± 4% and 48 ± 2%, of the viral PC could be exchanged by the heart and liver proteins respectively, as compared with only 15 ± 2% of the SM by the heart protein. The figure for PI transfer, 78 ± 15%, is less accurate because the number of [$^{32}$P]PI counts which remain in the virion after exchange is not much above instrumental background. The identity of this phospholipid and its accessibility were confirmed in preliminary experiments using

TABLE I: Effect of Incubation with Phospholipid Vesicles on the Phospholipid Composition of Influenza Virions.[a]

| Phospholipid | Incubation | |
|---|---|---|
| | Virus Alone | Virus with Vesicles |
| Sphingomyelin | 0.98[b] | 0.99 |
| Phosphatidylcholine | 0.44 | 0.44 |
| Phosphatidylserine | 0.34 | 0.35 |
| Phosphatidylethanolamine | [1] | [1] |

[a] $^{32}$P-Labeled virions were incubated with or without vesicles for 7 h under the conditions described in Materials and Methods, except that either exchange protein and vesicles (virus alone) or only exchange protein (virus with vesicles) were omitted. After the incubation, the virus was repurified on a tartrate gradient and analyzed by thin-layer chromatography. [b] The ratio of the stated $^{32}$P-labeled phospholipid to [$^{32}$P]PE in the virions after the incubation.

TABLE II: Accessibility of Influenza Virion Phospholipids to Exchange Proteins and Phospholipases C.

| Protein | % of Phospholipid Exchangeable or Hydrolyzable[a] | | | | | |
|---|---|---|---|---|---|---|
| | PC | SM | PI | PS | PE | Total Phospholipid |
| Liver exchange protein | 48 ± 2 (3) | | | | | 6[b] |
| Heart exchange protein | 43 ± 4 (10) | 15 ± 2 (7) | 78 ± 15 (5) | 14 ± 5 (5)[d] | | 12[b], 13 ± 2 (2)[c] |
| *Cl. welchii* P'lipase C | 45 ± 10 (2) | 23 ± 11 (2) | 90 (1) | 23 ± 2 (2) | 30 ± 1 (2) | 29[b], 25 ± 4 (9)[c] |
| *B. cereus* P'lipase C | 34 ± 8 (4) | | 71 ± 21 (3) | 25 ± 6 (2) | 28 ± 6 (3) | 20[b], 18 ± 3 (4)[c] |

[a] The numbers given are the mean percentages of individual or total phospholipids lost from the repurified virion either by hydrolysis by phospholipase or transfer by exchange protein, as appropriate. The standard deviation follows the mean, and the number of determinations is given in parentheses. All figures are end points, beyond which further exchange or hydrolysis did not occur. The differing number of determinations for different lipids with the same protein probe arises because end points for all lipids were not always reached in all experiments. [b] Values were calculated from the fraction of each phospholipid exchanged or hydrolyzed and the phospholipid composition as given in Table VII. [c] Values were measured directly, either from the $^3H/^{32}P$ ratio of the virions or by acid precipitation, as described in Materials and Methods. [d] PS was not found in vesicles (see text).

TABLE III: Effect of Exchange Reactions and Phospholipase C Digestions on the Accessibility of Virion Ribonucleic Acid to Ribonuclease.

| Reaction | Additional Components[a] | RNA Hydrolyzed[b] (%) |
|---|---|---|
| 1. Disrupted virus | 1% Triton-X-100 | [100] |
| 2. Disrupted virus | 1% Triton-X-100, 30 nmol/ml P vesicles | 102 |
| 3. Control | None | 8.7 |
| 4. *Cl. welchii* | 10 μg/ml phospholipase, 5 mM $CaCl_2$, 5 mM $MgCl_2$ | 8.4 |
| 5. *B. cereus* | 20 μg/ml phospholipase, 5 mM $CaCl_2$, 5 mM $MgCl_2$ | 6.8 |
| 6. Heart exchange protein | 30 nmol/ml P phospholipid vesicles, 10 units/ml heart exchange protein, 0.1 mg/ml albumin, 5 mM 2-mercaptoethanol, 0.02% $NaN_3$ | 8.7 |
| 7. Liver exchange protein[c] | 15 nmol/ml P phospholipid vesicles, 20 units/ml liver exchange protein, 0.03 mg/ml albumin, 3 mM 2-mercaptoethanol | 4.4 |

[a] Reactions were in a final volume of 0.1 ml of 0.1 M KCl–1 mM $Na_2EDTA$–10 mM Tris-HCl pH 7.4 buffer and contained 0.5 mg/ml ribonuclease A (Worthington), 5 nmol/ml phospholipid P of influenza virus labeled with [$^3$H]uridine, and any additional components as indicated. The mixtures were incubated for 2 h at 37 °C and then the trichloroacetic acid soluble radioactivity was determined as described under Materials and Methods. [b] The numbers presented are the amounts of trichloroacetic acid soluble [$^3$H]uridine normalized to that found with detergent-disrupted virus (reaction 1). Approximately 60% of the total $^3H$ label was rendered acid soluble in reactions 1 and 2. About 5% of the $^3H$ was acid soluble before RNAse digestions, and this amount of radioactivity was subtracted from acid-soluble counts of each reaction before normalization. [c] A different virus preparation was used in this experiment.

virions which were labeled with [$^3$H]inositol in addition to $^{32}P$. All of the tritium in the lipid extract co-chromatographed with [$^{32}$P]PI, and the majority of this was accessible both to the *Clostridium welchii* phospholipase C and to the heart exchange protein.

Data are presented for a decrease in virion [$^{32}$P]PS which was dependent on heart protein and vesicles, as exemplified by Figure 1. Surprisingly, attempts to identify [$^{32}$P]PS in the vesicles have been unsuccessful (Figure 2). We have no explanation for this anomaly.

The amount of PC, SM, or PI exchanged did not increase detectably if, after equilibration had occurred, fresh vesicles and/or exchange protein were added and incubation was prolonged. Therefore, the data in Table II represent the total amount of each phospholipid which is accessible to the exchange proteins. It can be concluded that both PC and SM exist in two pools in the membrane, one of which is readily exchangeable, the other being nonexchangeable or only very slowly exchangeable. The amount of PC in the exchangeable pool greatly exceeds that of SM. The exchangeable pool corresponds to the outer surface of the viral membrane and the nonexchangeable pool to the inner surface (see Discussion).

For one experiment, the glycoprotein spikes were removed by chymotrypsin digestion. This treatment does not alter the lipid composition (Schulze, 1970) or affect the ESR spectra of fatty acid spin labels (Landsberger et al., 1971). The amount of PC and SM available for exchange from spikeless particles was similar to that reported in Table II for intact virions. A notable difference, however, was that spontaneous phospholipid exchange in the absence of exchange protein occurred in a matter of a few hours. It seems likely that removal of glycoproteins from the intact virion facilitates spontaneous exchange of phospholipids between the viral bilayer and the vesicles by allowing direct contact. A similar effect of the viral glycoproteins in preventing cholesterol exchange has been reported (Lenard and Rothman, 1976).

Several observations suggest that the exchange reaction does not measurably perturb the structure of the viral membrane. When virus was analyzed by isopycnic centrifugation through a gradient of potassium tartrate after a heart protein exchange reaction, a single peak containing both protein and phospholipid was observed. The position of this band was indistinguishable from that of virus incubated without vesicles and exchange protein. Material from disrupted virions or virions fused with phospholipid vesicles would be expected to have banded elsewhere in the gradient. In addition, the accessibility of virion RNA to pancreatic ribonuclease A in incubations of virus with ribonuclease was not affected by the inclusion of vesicles and either of the exchange proteins (reactions 3, 6, and 7 in Table III). Since ribonuclease A has a molecular weight of 13 700 (Hirs et al., 1956), while both liver (Kamp et al., 1973) and heart (Enholm and Zilversmit, 1973) exchange

TABLE IV: Comparison of the Accessible Pools of Virion Phosphatidylcholine Defined by the Heart and Liver Phospholipid Exchange Proteins.

| Reaction[a] | Exchange Protein | % of Virion Phosphatidylcholine Transferred to Vesicles |
|---|---|---|
| 1 | Heart | 45 |
| 2 | Liver | 47 |
| 3 | Liver, then heart | 47 |

[a] Reaction 1 was for 12 h as described in Materials and Methods except that 10 units/ml heart exchange protein was used. Reaction 2 was identical with reaction 1, except it has 20 units/ml liver exchange protein in place of the heart protein. Reaction 3 was incubated for 12 h with 20 units/ml liver exchange protein, at which point 10 units/ml heart exchange protein was added, and the incubation was continued for another 12 h.

TABLE V: Comparison of the Accessible Pools of Virion Phospholipid Defined by *Bacillus cereus* Phospholipase C, *Clostridium welchii* Phospholipase C, and the Heart Phospholipid Exchange Protein.[a]

| Time (h) | Phospholipase C | Ratio:[b] Hydro. with Ex Protein / Hydro. without Ex Protein |
|---|---|---|
| 1 | *Cl. welchii* | 0.99 |
| 2 | *Cl. welchii* | 0.94 |
| 1 | *B. cereus* | 1.15 |
| 2 | *B. cereus* | 1.16 |

[a] Each of two parallel incubations (A and B) contained 5 nmol/ml P $^{32}$P-labeled virions, 30 nmol/ml P phospholipid vesicles, 0.1 mg/ml bovine serum albumin, and 0.02% $NaN_3$ in a final volume of 0.5 ml of 0.1 M KCl–1 mM $Na_2EDTA$–5 mM 2-mercaptoethanol–10 mM Tris-HCl, pH 7.4. Incubation A had, in addition, 30 units/ml of heart exchange protein. After 4 h at 37 °C, A and B were each divided into two equal parts, each of which was made 4 mM in $MgCl_2$ and 4 mM in $CaCl_2$. *Cl. welchii* phospholipase C (40 μg/ml) was added to one part from each of the two incubations A and B, and 80 μg/ml of *B. cereus* phospholipase C was added to the other part from each incubation. These four mixtures were then incubated at 37°C to allow the phospholipase to digest the vesicles and virions. After 1 and 2 h, 0.1-ml aliquots of each of the four digests were precipitated with trichloroacetic acid and the acid-soluble $^{32}$P was determined as described under Materials and Methods. The acid-soluble $^{32}$P in all four digestions did not increase from 1 to 2 h, demonstrating that the digestion was complete by 1 h. [b] The ratio [(hydrolysis with exchange protein)/(hydrolysis without exchange protein)] shown is the acid-soluble $^{32}$P released by phospholipase digestion of reaction A divided by the $^{32}$P released by digestion of reaction B with the stated phospholipase C for the indicated time. Approximately 200 cpm of $^{32}$P per 0.1 ml was released in each digestion, and about 3000 total counts was accumulated before the ratios were calculated. A ratio greater than 1 indicates that the exchange protein makes available to the phospholipase phospholipid which is inaccessible to the phospholipase alone.

proteins have molecular weights in excess of 20 000, the low accessibility of RNA to ribonuclease (Table III) indicates that the membranes of almost all virions remain sealed to both exchange proteins during incubations. Finally, the close agreement between the amount of PC, SM, and PI exchangeable with vesicles and the amount hydrolyzable by phospholipase C in the absence of vesicles (Table II) argues that neither reaction perturbs the distribution of phospholipid between accessible and inaccessible pools in the viral membrane. Indeed, incubation of virus with vesicles followed by digestion with phospholipase C gave, for two determinations, 30 ± 3% of the virion phospholipid hydrolyzed by the *Cl. welchii* enzyme and 16 ± 1% hydrolysis by the *B. cereus* enzyme, values which are not distinguishable from those obtained in the absence of vesicles (Table II).

*Accessibility of Virion Phospholipids to Phospholipases C.* Digestion of intact virions with phospholipase C caused hydrolysis of 25 ± 4% of the total phospholipid in the case of the *Cl. welchii* enzyme and 18 ± 3% with the *B. cereus* enzyme (Table II). The difference between these two values is mainly accounted for by SM which is not a proper substrate for the *B. cereus* enzyme. When the remaining viral phospholipids were analyzed by thin-layer chromatography (Table II), it was found that 45 ± 10% of the PC, 23 ± 11% of SM, about 90% of PI, 23 ± 2% of PS, and 30 ± 1% of PE were accessible to the *Cl. welchii* enzyme. Very similar results were obtained for the *B. cereus* digestions, with 71 ± 21% of the PI, 25 ± 6% of PS, and 28 ± 6% of PE accessible to the enzyme; only the amount of PC hydrolyzed, 34 ± 8%, was noticeably different, being slightly smaller than that for the *Cl. welchii* phospholipase.

Thus, each phospholipid exists in two pools in the viral bilayer, one of which is readily accessible to phospholipases C, the other of which is either totally inaccessible or only very slowly accessible to these enzymes. It is quite striking that the size of the accessible pools of individual phospholipids as defined by two phospholipases and two exchange proteins are virtually identical (Table II).

In one experiment spikeless particles were digested with both phospholipases C simultaneously, a treatment which resulted in 20 ± 1% hydrolysis of total phospholipid (two determinations), as compared with 22 ± 1% for the digestion of intact virions from the same preparation with both enzymes simultaneously (two determinations). Thus, removal of glycoprotein did not affect the accessibility of virion phospholipid.

The viral membrane excluded both phospholipases during the digestion since the accessibility of RNA to ribonuclease included in phospholipase digestions was as limited as for control ribonuclease incubations run without phospholipase (Table III, reactions 3, 4, and 5).

*Comparison of the Phospholipid Pools Defined by Exchange Proteins and Phospholipases.* Although the numerical amounts of individual phospholipids in the accessible pools defined by the four viral treatments discussed above are quite similar, these pools are not necessarily physically identical, representing the same molecules.

Table IV shows the size of the pool of viral PC which is accessible to heart exchange protein, liver exchange protein, and to both proteins together. The amount of exchangeable PC is the same in all three cases, showing that the PC pools defined by the heart and liver exchange proteins are physically identical.

A more general test for nonoverlapping physical pools was devised according to the following rationale: if there exist any viral phospholipids which are accessible to the exchange protein but not to a phospholipase, these phospholipids should be rendered accessible to the phospholipase by prior transfer to a vesicle by the exchange protein, where they should remain externally localized (Rothman and Dawidowicz, 1975). In confirmation of this, it was found that phospholipase C quantitatively hydrolyzed the $^{32}$P-labeled phospholipids contained in vesicles isolated from an exchange reaction with the heart protein. Thus, exchange reactions with the heart protein and control reactions in which only the exchange protein was

omitted were run in parallel. Then, both reaction mixtures, which still contained the vesicles, were digested with phospholipase C and the amount of total $^{32}P$-labeled phospholipids hydrolyzed was determined (Table V). Any viral phospholipids accessible to the heart exchange protein but not to the phospholipase C should be reflected by a larger amount of $^{32}P$-labeled phospholipid hydrolyzed from the exchange reaction than from the control reaction. Table V shows that, when *Cl. welchii* phospholipase C was tested against the heart protein, no increment in the amount of hydrolysis was observed, suggesting that the phospholipid pools accessible to these two probes are physically the same. In contrast, a 15% increment in hydrolysis was observed when the heart protein was tested against the *B. cereus* phospholipase C. It is noteworthy that the size of PC pool accessible to the *B. cereus* enzyme is consistently somewhat smaller than that for the heart exchange protein (Table II). These experiments, therefore, indicate that, with the possible exception of the PC pool accessible to *B. cereus* phospholipase C, the pool of individual phospholipids accessible to each protein is physically identical with that accessible to all the other proteins.

*Stability of Inaccessible Phospholipid Pools.* It has been shown that the influenza virus membrane contains a pool of PC and of SM which is nonexchangeable or only very slowly exchangeable by the heart exchange protein. When the duration of these exchange incubations was extended to as long as 2 days, no increase in the amount of [$^{32}P$]PC or [$^{32}P$]SM transferred to vesicles was detected (Table VI). Thus, the PC and SM in the nonexchangeable pool do not equilibrate with phospholipid in the exchangeable pool to a detectable extent during a 51-h incubation.

The limits of detectability and, therefore, the experimental lower limit for the interpool exchange times, are set by the standard deviations listed in Table II. By analogy with an earlier calculation (Rothman and Dawidowicz, 1975), it can be estimated that the half-time of equilibration of SM between the accessible and inaccessible pools is in excess of 30 days. Similarly, the half-time for PC was calculated to be in excess of 10 days at 37 °C.

TABLE VI: Lack of Equilibration between Exchangeable and Nonexchangeable Pools of Virion Phosphatidylcholine and Sphingomyelin.[a]

| Time (h) | % of Individual Virion Phospholipid Transferred SM | PC |
|---|---|---|
| 7 | 10 | 44 |
| 33 | 15 | 45 |
| 51 | 16 | 44 |

[a] The exchange reaction was as described under Materials and Methods for the heart exchange protein, except the volume was 3 ml and 3 units/ml of exchange protein was used. One-milliliter aliquots were taken for time points and analyzed. For the 33- and 51-h points, the aliquot was taken 2 h before the stated time and 30 nmol of P/ml of fresh phospholipid vesicles and 5 units/ml fresh heart exchange protein were added. The aliquot was then incubated for 2 h at 37 °C and analyzed. This procedure ensured that the results obtained could not be attributed to inactivated exchange protein or vesicles. Parallel control incubations in which only the exchange protein was omitted showed no exchange even after 51 h.

## Discussion

*Transbilayer Asymmetry in Influenza Virus.* The results presented here demonstrate that the phospholipids in the membrane of influenza virus exist in two pools, one exchangeable by exchange proteins, and the other nonexchangeable. Studies with bilayer vesicles have shown that both the liver and heart phospholipid exchange proteins can utilize as substrates only those phospholipids that are in the outer monolayer (Rothman and Dawidowicz, 1975; Johnson et al., 1975). In order to enter the exchangeable pool, inner bilayer phospholipid would have to undergo transbilayer flip-flop.

We propose that the exchangeable pool contains all of the phospholipid of the outer surface and none of the phospholipid of the inner surface. The following arguments indicate that this is the case.

(1) Four independent protein probes of the outer viral surface, two of which modify membrane components and two of which do not, have defined accessible and inaccessible pools of individual phospholipids which are physically as well as numerically identical.

(2) It is somewhat surprising that less than 50% of the total phospholipid is accessible to these probes. However, this result can be predicted from the high glycolipid content of virus grown in MDBK cells. Klenk and Choppin (1970) have studied the glycolipids of the paramyxovirus SV5, a virus whose structure is closely related to influenza. When SV5 was grown in MDBK cells, about 5 mg of glycolipid per 100 mg of protein was present in the virions. If these results were applicable to influenza virus grown in the same cell line, the resulting lipid composition of the virions would be approximately 15 mol % glycolipid, 45 mol % phospholipid, and 40 mol % cholesterol, as calculated from available data (Lenard and Compans, 1974). Preliminary analyses of our influenza virus preparations indicate a similarly high glycolipid content. If the total virion lipid is assigned equally[2] to the inner and outer membrane surfaces, with 55% of the cholesterol (Lenard and Rothman, 1976) and all of the glycolipids externally localized (Steck, 1972; Gahmberg and Hakamori, 1973; Nicolson and Singer, 1971, 1974; Eylar et al., 1962), then the above lipid composition would place about 30% of the phospholipid in the external monolayer. This figure is in agreement with the amount of total phospholipid actually found in the accessible pool (Table II)

(3) It has been shown that lipid mixtures can segregate laterally into domains of different structure and/or composition. This segregation can occur spontaneously in certain lipid mixtures (Shimshick and McConnell, 1973) and can also result from interaction of lipids with membrane proteins (Jost et al., 1973; Warren et al., 1974). Although there is no evidence for such laterally segregated phases in virus membranes, it is noteworthy that, if such domains were to exist on the outer surface, only one of them need be directly accessible to the exchange proteins in order for all the external phospholipid to be exchangeable. This is the case because of the rapid lateral equilibration which would be expected to occur between domains on the same surface (Kornberg and McConnell, 1971b; Scandella et al., 1972; Wu and McConnell, 1975; Jost et al., 1973; Warren et al., 1974).

Therefore, the sizes of the accessible vs. inaccessible phospholipid pools reflect the transmembrane phospholipid distribution. The influenza virus used here thus has an asymmetric distribution of total phospholipid, about 30% being in the outer monolayer and 70% in the inner monolayer of the bilayer. In

[2] Geometric considerations indicate that the ratio of outer to inner surface area in the membrane is about 55:45. However, the area of each surface occupied by protein is not known, so the distribution of total lipid between the two surfaces cannot be assigned with certainty.

TABLE VII: Comparison of the Phospholipid Composition of the Influenza Virus Used in These Experiments with That Used in Previous Experiments.

| Phospholipid | Virus Used in Present Expts | Virus Used in Previous Expts |
|---|---|---|
| SM | 31 ± 4 mol %[a] | 16 ± 1 mol %[a] |
| PC | 12 ± 2 | 26 ± 4 |
| PE | 38 ± 3 | 37 ± 3 |
| PS | 16 ± 3 | 21 ± 2[b] |
| PI | 3 ± 1 | |

[a] Mean ± standard deviation. Ten independent batches of the virus used in the present experiments were prepared and analyzed over a 6-month period. Four independent preparations of the virus used in the previous experiments were also analyzed over a different 6-month period. [b] This value represents PS plus PI. Reproducible separation of these phospholipids was not achieved in the thin-layer chromatographic analysis used earlier.

addition, the phospholipid composition of the outer monolayer is markedly different from that of the inner monolayer. Thus, the outer surface is characterized by a high mole percent of PC and PI, while the inner surface composition is relatively enriched in SM. In contrast, PE and PS are present in more nearly equal proportions in each monolayer.

Phospholipase C has previously been used to study transbilayer asymmetry in influenza virus (Tsai and Lenard, 1975) with apparently different results from those reported here. It must be emphasized that, although the influenza virus preparations used in the experiments reported here and in the previous work are nominally the same, the membranes of the two viruses are quite different. Table VII shows the phospholipid composition of the viruses used in the present and in the past work. The difference in PC and SM content is striking. Each of these compositions was highly reproducible over at least a 6-month period. During a 1-month period there was an abrupt transition in the phospholipid composition of the virus grown in this laboratory from the previous high PC to the present low PC values. Despite considerable effort, no explanation for this phenomenon has been forthcoming. The two types of influenza virus have identical protein compositions, as revealed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and are not distinguishable by electron microscopy with negative staining.

The shift in composition from high PC to low PC virus, although unexplained, permits an examination of the effect of lipid composition on transbilayer asymmetry in two viruses which are in other respects indistinguishable. Two phospholipases C were used in the study of high PC virus (Tsai and Lenard, 1975). The excellent agreement between the phospholipases and the far less perturbing exchange proteins in the present work on low PC virus indicates that the phospholipases are reliable probes for the outer surface of influenza virions and probably other membranes as well. About 45% of the total phospholipid of high PC virus was accessible to phospholipase C (Tsai and Lenard, 1975) as compared with 30% in the low PC virus. The difference suggests that total phospholipid is more nearly symmetrically distributed in high PC than low PC virus.

Although the actual amount of each phospholipid accessible in the high and low PC virions is quite different, this is largely a reflection of the differences in the outside to inside ratio of *total* phospholipid between the two viral types. This becomes evident when the molar composition of phospholipids in each monolayer is compared. Thus, PE and PS are present in similar molar proportions in the outer and inner phospholipid pools of low PC virions (Table II). This is also true for high PC virions (cf. Table 1 of Tsai and Lenard, 1975). In both types of particles, PC is enriched in the outer phospholipid pool (Figure 2). The exception is SM, which is enriched in the outer phospholipid pool of high PC virions, and in the inner pool of low PC virions.

*Comparison of Transbilayer Phospholipid Distribution in Influenza Virus with Erythrocyte Membranes.* The sidedness of all the major phospholipids has also been quantitatively determined for the human erythrocyte (Bretscher, 1972a,b; Gordesky and Marinetti, 1973; Verkleij et al., 1973) and the rat erythrocyte (Renooij et al., 1976; Bloj and Zilversmit, 1976). Although the viral and erythrocyte membranes are all asymmetric, the nature of the asymmetry in the two erythrocytes differs qualitatively from the influenza virions as regards the aminophosphatides PE and PS. Whereas these molecules are nearly symmetrically distributed in the virions, they are almost exclusively found in the internal surface of the red cell membranes.

The differences in phospholipid distribution between the viral system and the red cell may be related to differences in the mode of origin of their respective phospholipids. Once formed, the mature erythrocyte exists for long periods of time (ca. 120 days in humans) in the vasculature without de novo synthesis of phospholipids (van Deenen and de Gier, 1974). During this time, exchange of the phospholipids (Reed, 1968) of the outer but not the inner membrane surface (Renooij et al., 1976) with a quantitatively comparable (Skipski, 1972), but much more rapidly turning over (Margolis and Capuzzi, 1972) pool of serum phospholipids takes place. Because aminophosphatides comprise only a very small fraction of the plasma phospholipids (Rouser et al., 1968), the phospholipid composition of the external but not the internal monolayer of the erythrocyte membrane could, by this exchange process, gradually be modified to more nearly approximate that of plasma, provided that flip–flop is either very slow or highly specific (see below). Thus, a transmembrane distribution such as that found in the virions could be transformed into one in which the remaining aminophosphatides would be primarily internal, and in which the outer monolayer would have mainly choline phosphatides of serum origin.

Such a modification of cellular phospholipid distribution by extracellular phospholipid is avoided in the in vitro cell–virus system used here. Cells in culture do not incorporate either intact phospholipid molecules or lipid phosphorus from serum into cellular or viral phospholipids (Quigley et al., 1971, 1972). The cells multiply and their phospholipids turn over while the serum does not, in contrast to the case of the red cell. In our experiments, the composition and accessibility of influenza virus phospholipids did not change in response to twofold variations in the level of serum during cell growth and was independent of cell age.

Therefore, the asymmetric bilayer described for influenza virions is the product of cellular processes exclusively. Our results indicate that individual cells have the capacity to generate asymmetric bilayers and suggest that asymmetry is a general feature of animal cell membranes. However, there is no reason to believe that all membranes possess a similar type of asymmetry.

*Transbilayer Movement of Phospholipid.* The half-time for equilibration of phospholipid molecules between exchangeable and nonexchangeable pools is the half-time for transbilayer equilibration of phospholipid by flip–flop. We have determined

that these half-times are extremely long, being in excess of 30 days for SM and 10 days for PC at 37 °C. Flip-flop times of this magnitude have now been reported for PC (Rothman and Dawidowicz, 1975; Johnson et al., 1975) and cholesterol (Poznansky and Lange, 1976) in artificial bilayer vesicles and for cholesterol in influenza virus membranes (Lenard and Rothman, 1976).

The lower limits for the half-time of the flip-flop process that we report here are long compared with the turnover times of phospholipid in most biological membranes and the generation times of most animal cells. They therefore provide a straightforward explanation for the maintenance of transmembrane asymmetry.

It has recently been demonstrated by two independent methods that transmembrane equilibration of PC occurs in rat red blood cell membranes with a half-time of 2 to 5 h (Renooij et al., 1976; Bloj and Zilversmit, 1976). Because asymmetry (Renooij et al., 1976) is maintained in this membrane during the lifespan of the erythrocyte, a period of time much longer than the PC flip-flop time, it is evident that the observed PC flip-flop process is such as to conserve the differing phospholipid compositions of the inner and outer cell surfaces. That is, a specific mechanism apparently exists in the rat erythrocyte for the transmembrane exchange of PC. The markedly higher rate for this process in the rat erythrocyte as compared with PC vesicles or influenza virus, taken together with the apparent specificity of the process, is commensurate with classical criteria for the presence of a biological (protein) catalyst (Bretscher, 1974). If proteins which catalyze transmembrane phospholipid exchange were present in the plasma membrane of the host cell in our experiments, they would almost certainly have been excluded from the membrane of the formed influenza virion since the viral particle contains only viral-coded proteins (Lenard and Compans, 1974).

Alternatively, it is conceivable that flip-flop in the virion may be specifically prevented by interaction of the lipid with viral protein. However, the similarity of the present results with those previously described for protein-free model membranes (Rothman and Dawidowicz, 1975; Johnson et al., 1975) make this explanation unlikely.

Rapid flip-flop has also been reported for spin-labeled phospholipids in biological membranes (Grant and McConnell, 1973; McNamee and McConnell, 1973). These results, in our opinion, are not necessarily reliable because of artifacts that can be associated with the method of introduction of the spin label, and because head-group spin labeled PC flips at least two orders of magnitude faster than authentic PC when the two molecules are compared in similar model membranes (Kornberg and McConnell, 1971a; Rothman and Dawidowicz, 1975).

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# Isolation and Properties of Platelet Myosin Light Chain Kinase†

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ABSTRACT: A protein kinase which phosphorylates the 20 000-dalton light chain of platelet myosin has been isolated from human blood platelets and purified approximately 600-fold. Elution of a 7.5% polyacrylamide gel following electrophoresis of the partially purified enzyme yielded a single peak of kinase activity which could be aligned with a protein band on a stained gel. Assuming a globular shape, a native molecular weight of 83 000 (±10%) was determined by gel filtration on Bio-Gel P-200. The kinase requires $Mg^{2+}$ for activity and is not sensitive to the removal of trace $Ca^{2+}$. The enzyme purified from human platelets phosphorylates the 20 000-dalton light chain of mouse fibroblast and chicken gizzard myosin, but does not phosphorylate human skeletal and cardiac myosin.

Human blood platelets contain actin and myosin which are similar in structure and function to the proteins found in smooth muscle cells (Adelstein and Conti, 1974). Platelet myosin is composed of two heavy chains (200 000 daltons) and two different light chains (20 000 and 15 000 daltons).

Previous work in this laboratory demonstrated that the 20 000-dalton platelet myosin light chain could be phosphorylated by incubating a crude extract of platelet actomyosin with $\gamma$-labeled $AT^{32}P$ (Adelstein et al., 1973). It was surmised that the platelet extract contained a kinase which phosphorylated the 20 000-dalton light chain. Moreover, phosphorylated platelet myosin differed from nonphosphorylated myosin in its ability to be activated by actin (Adelstein and Conti, 1975a).

Studying phosphorylation in rabbit skeletal muscle, Perrie et al. (1973) isolated a single chymotryptic peptide from the 18 500-dalton light chain of rabbit skeletal muscle myosin which contained the radioactive phosphorus. Recently, Pires et al. (1974) partially purified an enzyme from rabbit skeletal muscle responsible for this phosphorylation.

In this paper, we report the isolation and purification from

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