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Association of the Polioviral RNA Polymerase Complex with Phospholipid Membranes[1]

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Polioviral RNA polymerase complex, which consists of enzyme, template, and nascent RNA, is membrane bound in vivo. The solubilized RNA polymerase complex associated spontaneously in vitro with phospholipid bilayer membranes (liposomes) of defined composition. The degree of association at 37°C was greater for those membranes that were more fluid, suggesting that the binding involves the interaction of the RNA polymerase complex with the hydrocarbon chains in the interior of the lipid bilayer. The polymerase activity was not enhanced by addition of the lipid; in fact, the addition of some of the longer-chain lipids resulted in up to a 40% inhibition of the polymerase activity. Spin-label electron paramagnetic resonance experiments, which measured the membrane fluidity, and kinetic experiments on the rate of incorporation of tritiated UTP into RNA by the polymerase were performed as a function of temperature. The results indicated that the activity of the polymerase was not affected by the physical state of the phospholipid membrane and that its active site was not intimately associated with the membrane. Analysis of both the viral and host polypeptides associated with the smooth membrane-bound polymerase indicated that X was the primary viral polypeptide present. In addition, host polypeptides of molecular weight 86,000, 62,000, 54,000, and 46,000 were also present. If the membrane was disrupted with detergent, polypeptide X was released from the polymerase activity, suggesting that X may play a role in binding the polymerase to the membrane. In an analogous manner, polypeptide X associated spontaneously with phospholipid membranes to a greater extent than the capsid polypeptides. Analysis of both the host and viral polypeptides associated with the viral RNA polymerase purified by precipitation in 2 M LiCl indicated that host polypeptides of molecular weight 106,000, 38,000, 33,000, and 14,000 were the major constituents, whereas relatively small amounts of the viral polypeptides were present. It was confirmed that of the viral polypeptides found, polypeptide 4 was present in the largest amount.

Biological membranes are believed to consist of lipid bilayers with proteins attached to the bilayer surface, penetrating it, or spanning the bilayer completely (30). One approach to the study of the properties of membrane-bound proteins is to remove the protein from its native membrane and incorporate it into chemically defined phospholipid bilayer vesicles called liposomes. The bilayer structure of liposomes is believed to be analogous to that found in biomembranes. Liposomes are readily formed from a variety of natural and synthetic phospholipids by dispersing the lipids in water or by dialyzing away a detergent in which the lipid is dissolved. Because of their simplicity and the ability to specify their composition, liposomes lend themselves to biochemical investigations as model membrane systems (1). Several proteins that are membrane associated in vivo have been found to undergo an association with liposomes in vitro. These include rhodopsin (12), glycophorin (10), cytochrome oxidase (14), ATPase (35), and the acetylcholine receptor (21).

The polioviral RNA polymerase complex, consisting of enzyme, template, and nascent RNA, has been shown to be associated with a specific cellular membrane fraction (5, 6, 8, 9). In this communication we report the spontaneous in vitro association of the polioviral RNA polymerase complex with liposomes of defined composition. It is possible that this enzyme-lipid interaction is analogous to that found in vivo. Spin-label electron paramagnetic reso-

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nance (EPR) experiments, which measure the fluidity of the membrane, compared with parallel kinetics experiments of enzyme activity as a function of temperature for both the free enzyme and the lipid-associated enzyme, revealed that the enzyme was only slightly affected by the physical state of the membrane. Comparison of both the host- and viral-specific polypeptides that spontaneously associated with the liposomes, found in the polymerase-rich membrane fraction of the cell and in the purified polymerase, suggested that the viral polypeptide X may be responsible for organizing the polymerase on the membrane and revealed several host polypeptides that may be components of the polymerase.

MATERIALS AND METHODS

Cells and virus. The source and propagation of HeLa cells and poliovirus type 2 have been described (2).

Materials. The components of the dialysis buffer, the 10× SDS solubilizing solution, medium AL, and the scintillation solvent tT21 have been described (3). Reticulocyte standard buffer is 0.01 M Tris-hydrochloride (pH 7.2)–0.01 M NaCl–0.0015 M $MgCl_2$. TKM buffer is 0.01 M Tris-hydrochloride (pH 7.8)–0.01 M KCl–0.0015 M $MgCl_2$. Tris-salt buffer is 0.01 M Tris-hydrochloride (pH 8.0)–0.01 M NaCl (19). The ^{3}H-labeled amino mixture, [^{35}S]methionine, [^{3}H]acetic anhydride, and [^{3}H]uridine were obtained from New England Nuclear Corp. Cholesterol was purchased from Sigma. L-α-Dimyristoyl phosphatidylcholine (DMPC), L-α-dipalmitoyl phosphatidylcholine (DPPC), L-α-distearoyl phosphatidylcholine (DSPC), and L-α-dipalmitoyl phosphatidylethanolamine (DPPE) were obtained from Calbiochem. All were used without further purification. The egg yolk lecithin was prepared by the method of Singleton et al. (31) and chromatographed on silica; elution was done with chloroform-methanol. The spin label 2,2,6,6-tetramethyl piperidine-1-oxyl (TEMPO) was synthesized by the method of Rozantsev (27) and purified by sublimation.

Infection. Unless otherwise stated, HeLa cells were infected with 200 PFU of virus per cell and cultured at 4×10^6 cells/ml at 37°C in medium AL containing 5 μg of actinomycin D per ml as described (3). The infection procedure used in the experiment described in Fig. 6 was that of Lundquist et al. (19).

Preparation of the polymerase. The LiCl-precipitated polymerase complex was prepared by the method of Lundquist et al. (19). The smooth membrane-bound polymerase was prepared by the method of Caliguiri and Tamm (5).

Polymerase assay. Samples were assayed for RNA polymerase activity by the addition of an equal amount of a 2× cocktail concentrate so that the final concentrations in the reaction mixture were: 10 μCi of [^{3}H]UTP per ml, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 1.0 mM phosphoenolpyruvate, 50 mM Tris-hydrochloride (pH 8.2), 5 mM $MgCl_2$, 40 mM KCl, 20 μg of pyruvate kinase per ml, 5 mM Cleland reagent, and 5 μg of actinomycin D per ml. Unless otherwise noted, the incubation temperature was 37°C. One hundred-microliter samples were withdrawn into 0.5 ml of 1% SDS-0.15 M sodium pyrophosphate to stop the reaction and dissolve any membranes. The RNA was precipitated by the addition of 3 ml of ice-cold 10% trichloroacetic acid. Samples were collected and washed with cold 5% trichloroacetic acid on a 0.45-μm membrane filter (Millipore Corp.) and counted in Hay scintillation cocktail in a liquid scintillation spectrometer.

Labeling with [^{3}H]acetic anhydride. The procedures for labeling proteins with [^{3}H]acetic anhydride have been described (18, 22).

EPR studies. The EPR experiments were done on a Varian 4502 spectrometer equipped with a variable temperature accessory. Temperature was measured with a copper-constantan thermocouple and a Doric thermocouple indicator. The EPR sample cell was a sealed 50-μl capillary tube mounted in a polypropylene holder.

Liposome formation. The phospholipids were dispersed in TKM buffer to give a final lipid concentration of 1% (wt/vol) by alternately warming the lipids above their transition temperature and blending in a Vortex mixer. The liposomes were then lightly sonicated for approximately 5 min in a sonicating bath (Heat Systems Ultrasonic Inc.). Another method of preparing liposomes was to dissolve the lipids in deoxycholate (DDC) and remove the detergent by dialysis against frequent changes of buffer in the cold for several days (12).

Polyacrylamide gel electrophoresis. The procedures for solubilizing cells, SDS-polyacrylamide gel electrophoresis and fractionation, and computer analysis of the data have been described (2).

RESULTS

Association of the polymerase complex with liposomes. Ehrenfeld et al. (8) and Lundquist et al. (19) developed a technique for purifying the polioviral RNA polymerase complex in which the enzyme was extracted with 1% NP40-0.05% DOC followed by specific precipitation of the complex in 2 M LiCl. It was found that this RNA polymerase complex would spontaneously associate with preformed, sonicated liposomes by simple incubation at 37°C. The LiCl precipitate of the polymerase was dissolved and incubated for 30 min at 37°C with each of various lipid suspensions, which were then sedimented, and the activities of the supernatants and precipitates were measured. Table 1 shows that in most cases there was a strong spontaneous association of the polymerase with the liposomes. In general, the partition coefficients were greater for the shorter-chain lipids, which were fluid at 37°C (Table 1). Column 3 of Table 1 shows the effect on the enzymatic activity of adding the different sonicated lipid suspensions to solutions of the LiCl-precipitated enzyme

TABLE 1. *Association of the polioviral RNA polymerase complex with liposomes*

Lipid	Enzymatic act found in liposome precipitate (% of total)[a]	Partition coefficient[b]	Act of enzyme to which sonicated lipid had been added (% of control)[c]
Control			100
EYL[d]	92	430	95
DMPC	93	560	93
DPPC	97	390	70
DSPC	35	8	63
DPPE	70	35	58
66% DMPC[e] 34% DPPE	78	120	85
70% DMPC 30% Cholesterol	90	320	88

[a] The polioviral RNA polymerase complex was prepared by the LiCl preparation procedure (19). The precipitate from 2.4 × 10^8 cells was dissolved in 0.6 ml of Tris-salt buffer and heated to 37°C for 3 min, and the undissolved precipitate was sedimented at 2,000 × *g* for 10 min. Two hundred-microliter portions of the polymerase were incubated for 30 min at 37°C with 1.0 ml of each of the various lipid suspensions at 10 μmol/ml, which were then sedimented for 10 min at 2,000 × *g*, and the activities of the supernatants and precipitates were measured.

[b] The partition coefficients were calculated as the concentration of the enzyme (activity) in the precipitate divided by the concentration of the enzyme in the supernatant.

[c] In a parallel experiment, 100 μl of the enzyme was added to 300 μl of each of the different sonicated lipid suspensions (10 μmol/ml) and incubated for 30 min at 37°C, and the activities were measured.

[d] EYL, Egg yolk lecithin; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine.

[e] Moles percent.

and allowing the complex to form. In every case, the enzyme was less active with the lipid present, demonstrating that lipids were not essential for activity. In general, the longer-chain, higher-melting lipids inhibited the elongation reaction more than did the shorter-chain lipids.

The interaction of the polymerase complex and lipid was fairly strong and irreversible. A sucrose gradient was formed in which a small amount of liposome suspension, which would float to the top during centrifugation, had been placed in the bottom of the tube. When the polymerase complex was sedimented into such a gradient, it became associated with the liposomes, reversed its direction of migration, and ended up on the top of the gradient (data not shown).

The LiCl precipitation step in the polymerase purification yields a visible pellet. Figure 1 shows the absorption spectra of the dissolved precipitate and supernatant, respectively. The spectrum of the dissolved precipitate shows a sharp peak with a maximum at 256 nm, indicating that it is primarily single-stranded, cellular RNA. It was calculated from the optical density at 260 nm of the dissolved precipitate that approximately 10% by weight of the RNA polymerase-DMPC complexes formed in the kinetic and fluidity experiments was RNA. Possibly, this copurifying RNA plays a role in the formation of the polymerase-lipid complex.

Effect of the physical state of the mem-

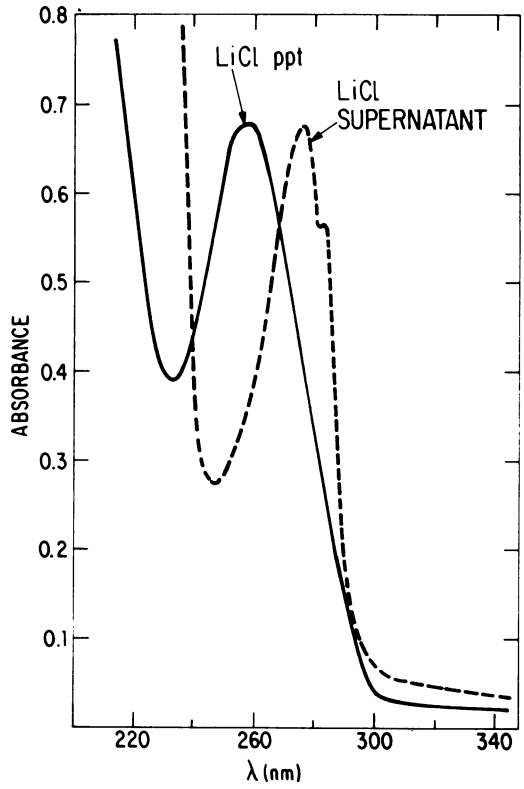

FIG. 1. *Absorption spectra of the LiCl supernatant (-----) and the LiCl precipitate (——) recorded on a Cary 15 spectrophotometer. The LiCl precipitate from 2.4 × 10^8 cells dissolved in 0.6 ml of TKM was diluted by a factor of 40 and the supernatant, 3.0-ml total volume, was diluted by a factor of 5 to give these spectra. The estimated amounts of RNA in the precipitate and supernatant are 0.6 and 0.2 mg, respectively. In the kinetic and fluidity experiments, 0.5 ml of the LiCl precipitate (1.1 mg/ml in RNA) was incubated with 0.5 ml of 1% DMPC. Thus, 9.7% (wt/wt) of the RNA polymerase-DMPC complex was RNA.*

brane. Phospholipids undergo thermal-phase transitions in which the hydrocarbon chains go from a rigid and well-ordered configuration to a fluid and less well-ordered one (V. Luzzati, A. Tardieu, T. Gulik, L. Mateu, J. L. Ranck, E. Schecter, M. Chabre, and F. Carom, Proc. 8th FEBS Meet., Amsterdam, 1972, p. 2). The activity of a membrane-bound enzyme or transport protein can be affected by these phase transformations, especially if the active site is intimately associated with the interior of the membrane (16, 35).

We have measured both the activity of the RNA polymerase-liposome complex and the fluidity of the membrane as a function of temperature to determine if the fluidity of the membrane is a determining factor in the polymerase activity and also if the polymerase perturbs the structure of the membrane. One portion of the polymerase-liposome complex was used to measure the initial reaction rate of the polymerase as a function of temperature. In Fig. 2, an Arrhenius plot of the polymerase-DMPC liposome complex activity exhibited a slight change in slope near the transition temperature of DMPC (Fig. 2, open circles). This change in slope is not necessarily the result of the phase transition of DMPC because the Arrhenius plots of the polymerase without added lipid (Fig. 2, closed circles), or incorporated into an equimolar mixture of DMPC and DPPC (data not shown), showed similar parallel curves. This indicates that the activity of the polymerase does not greatly depend on the composition and fluidity of the membrane to which it is bound, suggesting that the active site is not intimately associated with the hydrocarbon portion of the lipids. One implication of this is that the polymerase itself is a possible extrinsic membrane protein rather than an intrinsic

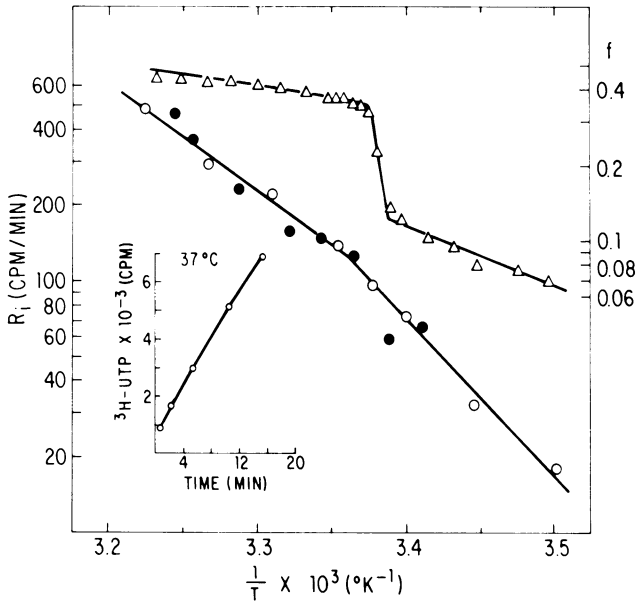

FIG. 2. *Melting curve of the polymerase-DMPC liposome complex and Arrhenius plot of the lipid-bound and free enzyme. Several preparations of the LiCl-precipitated polymerase were prepared and stored at −9°C in 2 M LiCl. The samples were sedimented for 10 min at 2,000 × g, dissolved in Tris-salt buffer (polymerase from 60 ml of infected-cell suspension was dissolved in 0.6 ml of buffer), pooled, heated to 37°C for 1 min, and clarified by centrifugation for 5 min at 2,000 × g. This polymerase solution was then mixed with an equal volume of sonicated 1% suspension of DMPC. The mixture was incubated at 37°C for 20 min to allow the polymerase-lipid complex to form (Table 1). The mixture was kept in the reaction constant-temperature bath. Samples, 0.4 ml, were removed and assayed for polymerase activity starting at 37°C and working down to 13°C. The initial reaction rates at each temperature were measured as the slopes of the [^{3}H]UTP incorporation reaction as computed by regression analysis, fitting the data to either a linear or second-degree equation (see insert). The resulting Arrhenius plot is shown by the solid line connecting the open circles. The experiment was repeated without the DMPC. The points obtained are shown as solid circles, but the connecting line has been omitted for clarity. A 1-ml sample of the original 0.5% DMPC-polymerase complex was centrifuged, and the pellet was suspended to a total volume of 100 μl, and 5 μl of 5 mM TEMPO in TKM was added. The TEMPO solubility in the lipid, f (29), was calculated from the EPR spectra in the range from 37°C to 13°C. The resulting curve (△) shows the typical sharp melting at about 23°C.*

membrane-bound protein (29). However, the fact that detergent solubilization is needed to remove the enzyme from the membrane suggests that at least one component of the polymerase is tightly bound to the membrane.

The spin-label EPR technique was used to monitor the fluidity of a second portion of the polymerase-lipid complex. The small spin label, TEMPO, partitions between the membrane and the aqueous phase. The phospholipid phase transition is reflected in an abrupt change in the spin-label solubility in the membrane. As the lipids freeze, the TEMPO solubility in the phospholipid bilayer is reduced, the spin label is excluded from the membrane, and the resulting change in signal can be measured (28).

A plot of TEMPO solubility, f, proportional to the mole fraction of spin label dissolved in the membrane, versus $1/T$ for the RNA polymerase-DMPC complex is illustrated in Fig. 2. A sharp decrease in TEMPO solubility occurred at approximately 23°C, the DMPC phase transition temperature (11). This indicates that the RNA polymerase had very little effect on the DMPC phase transition.

Polypeptides associated with the polymerase-membrane complex. Caliguiri and Tamm (5, 6) showed that the viral RNA polymerase was associated with a particular smooth membrane fraction that could be isolated by isopycnic centrifugaton in a discontinuous sucrose gradient. An infected-cell suspension was exposed to [^{35}S]methionine under conditions such that the isotope was specifically incorporated into the polioviral proteins. The smooth membrane fraction containing the polymerase activity was then isolated. This fraction was disrupted with 1% SDS and labeled with [^{3}H]acetic anhydride to tag all components, both host and viral. Figure 3 shows the polypeptide profile of this double-labeled fraction. The capsid polypeptides are usually present in equal amounts (2, 20). Since the VP3 and VPO peaks in Fig. 3 are relatively low, it can be assumed that the major portion of the peak running at 37,000 daltons (which contains both X and VP1) is polypeptide X. Thus, the ^{35}S-labeled pattern clearly shows that there is a strong enrichment of polypeptide X relative to the other viral polypeptides in this membrane fraction.

Treatment of the infected-cell suspension with 3 mM guanidine results in the specific loss of the capsid polypeptides from this fraction of the gradient. After such guanidine treatment, the molar ratio of X + VP1 to VP3 increases from the 2.5 shown in Fig. 3 to 16 to 1, confirming that X is the predominant viral polypeptide in this smooth membrane fraction (F. Yin, in preparation).

The pattern of labeled polypeptides (Fig. 3)

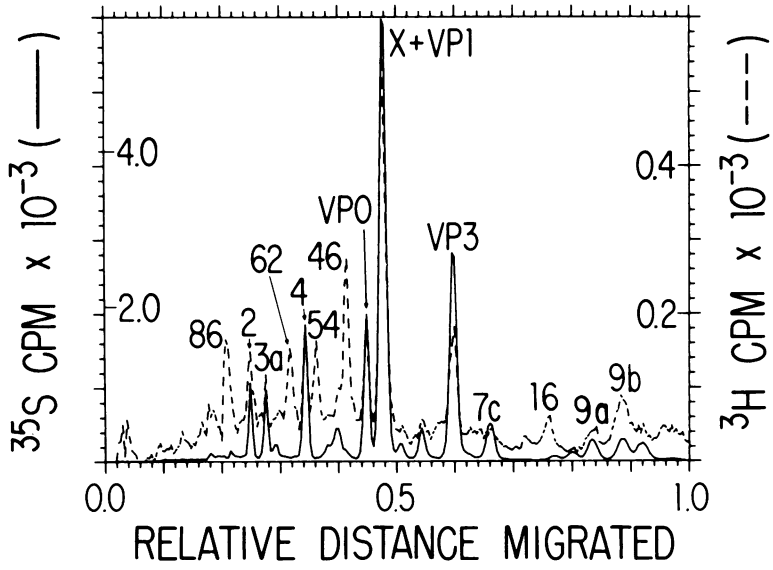

FIG. 3. *Viral and host proteins associated with the smooth membrane. A poliovirus-infected cell suspension was labeled with [^{35}S]methionine as described in Materials and Methods. The smooth membrane fraction number 2 containing the viral RNA polymerase was then isolated as described by Caliguiri and Tamm (5). This was solubilized in 1% SDS and reacted with [^{3}H]acetic anhydride to label all components, both host and viral. After dialysis, this double-labeled extract was analyzed electrophoretically as described in Materials and Methods. Direction of migration is from left to right. [^{35}S]methionine-labeled viral proteins (——); [^{3}H]acetic anhydride-labeled proteins (-----).*

shows that whenever there is a peak of ^{35}S-labeled viral protein there is also a comigrating peak of [^{3}H]acetic anhydride-labeled protein, indicating that the viral proteins make up a significant portion of this fraction. In addition, there are several host polypeptides (indicated as their relative molecular weight in thousands) in the molecular-weight range from 86,000 to 27,000 that could be membrane proteins or components of the polymerase.

Polypeptides associated with the liposomes. The observation that polypeptide X is enriched in the smooth membrane fraction presents the possibility that polypeptide X may also selectively associate with liposomes. Infected, labeled cells were extracted with DOC to yield a solubilized extract in which all labeled viral proteins were present. DMPC was dissolved in the mixture, and liposomes were allowed to form by dialyzing away the detergent. The polypeptide profiles of the supernatant and the liposome precipitate show that there are quantitative differences in the viral polypeptides present (Fig. 4). Assuming that VP0, VP1, and VP3 are present in equal amounts, Fig. 4 shows that these capsid polypeptides are the most prominent polypeptides in the supernatant, whereas polypeptide X is the major constituent in the liposomes. There is also far more VP2 present in the supernatant pattern than in the liposome pattern, indicating that the mature virions do not prefer the liposomes. More detailed studies on the viral and subviral particle interactions with the liposomes have been done (18a).

Although the partitioning shown in Fig. 4 is not as dramatic as that with the polymerase activity shown in Table 1, the observation that both polypeptide X and the viral RNA polymerase associate with liposomes parallels the observation that both components are bound to the same smooth membrane fraction in vivo (Fig. 3).

An ^{35}S-labeled polymerase preparation was prepared by precipitation in 2 M LiCl as described by Lundquist et al. (19) and allowed to associate with DMPC liposomes as described in Table 1. The polymerase activity partitioned strongly to the liposomes. However, analysis of the host polypeptides by labeling with [^{3}H]acetic anhydride and gel electrophoresis showed five times as much radioactivity in the liposome pellet compared with the clear supernatant, with most host polypeptides ending up in the liposome fraction (data not shown). This was not surprising, as an early step in this procedure involves detergent extraction, which would be expected to select for lipophilic proteins. Therefore, this technique was not as useful as sucrose gradient centrifugation (see following section) in selecting those polypeptides associated with the polymerase.

Polypeptides associated with the LiCl-precipitated polymerase. Inhibition of host protein synthesis by the virus facilitates the specific labeling of the viral polypeptides by incubation of the infected cells with radioactive amino acids such as [^{35}S]methionine. Precipitation of the RNA polymerase complex in 2 M

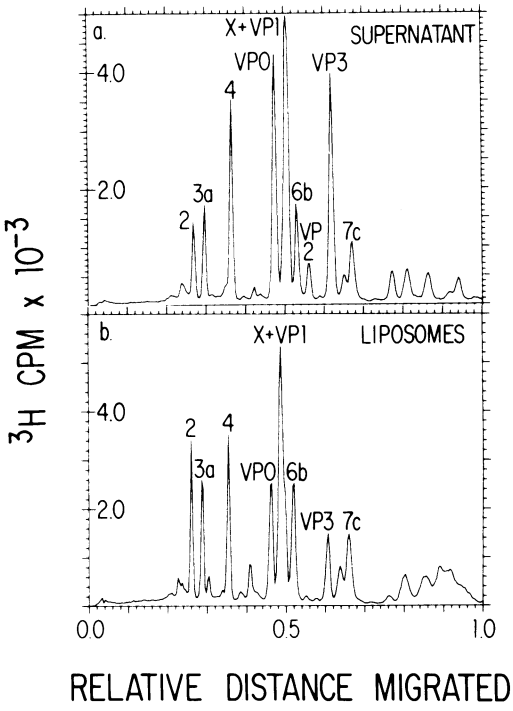

FIG. 4. *Viral proteins associated with the liposomes. Twenty microliters of a polioviral-infected cell suspension was labeled from 3.5 to 4.0 h postinfection with 50 μCi of a ^{3}H-labeled amino acid mixture per ml. The cells were then poured over 10 ml of frozen reticulocyte standard buffer, pelleted in a clinical centrifuge, and resuspended in 2 ml of cold reticulocyte standard buffer. After the cells were allowed to swell for 3 min, the suspension was homogenized with 10 strokes of a Dounce homogenizer, and the nuclei were sedimented by centrifugation. From the supernatant cytoplasm, 1.3 ml, which contained all the various ^{3}H-labeled viral proteins, was clarified by the addition of 0.5 ml of 10% DOC. Sixth-tenths milliliter of a 3% DMPC suspension was then added and the suspension was clarified by the addition of 70 μl of 10% DOC. The solution was dialyzed in the cold against frequent changes of TKM buffer for several days until the white liposome precipitate formed. The liposomes were pelleted by centrifugation at 2,000 × g for 10 min. Samples of the supernatant or the liposome precipitate were solubilized in 1% SDS and subjected to gel electrophoresis.*

LiCl effects a substantial purification, because most proteins are soluble in 2 M LiCl and single-stranded RNA (which carries the attached polymerase) is precipitated (Fig. 1). Sucrose gradient centrifugation of this precipitate yields a peak of activity at about 25S. Analysis of this region of the gradient revealed polypeptide 4 as the major viral constituent (19).

This experiment was repeated so that the viral proteins in the final gradient had been specifically labeled with [^{35}S]methionine (Fig. 5a). Figure 5 shows the polypeptide profiles of the initial whole-cell extract, the LiCl-precipitated material, and the various regions of the gradient. Figure 5g confirms that the ^{35}S-labeled polypeptide 4 is enriched relative to the other viral polypeptides in the region of the gradient corresponding to the peak of enzyme activity. However, other viral polypeptides were still present. In addition, a faster-sedimenting peak of polymerase activity was observed in which 4 was not the major viral polypeptide present (Fig. 5h).

Some samples from the final gradient were removed, disrupted with 1% SDS, and labeled with [^{3}H]acetic anhydride to tag all constituents, both host and viral (18, 22). Since the [^{3}H]acetic anhydride reacts with free amino

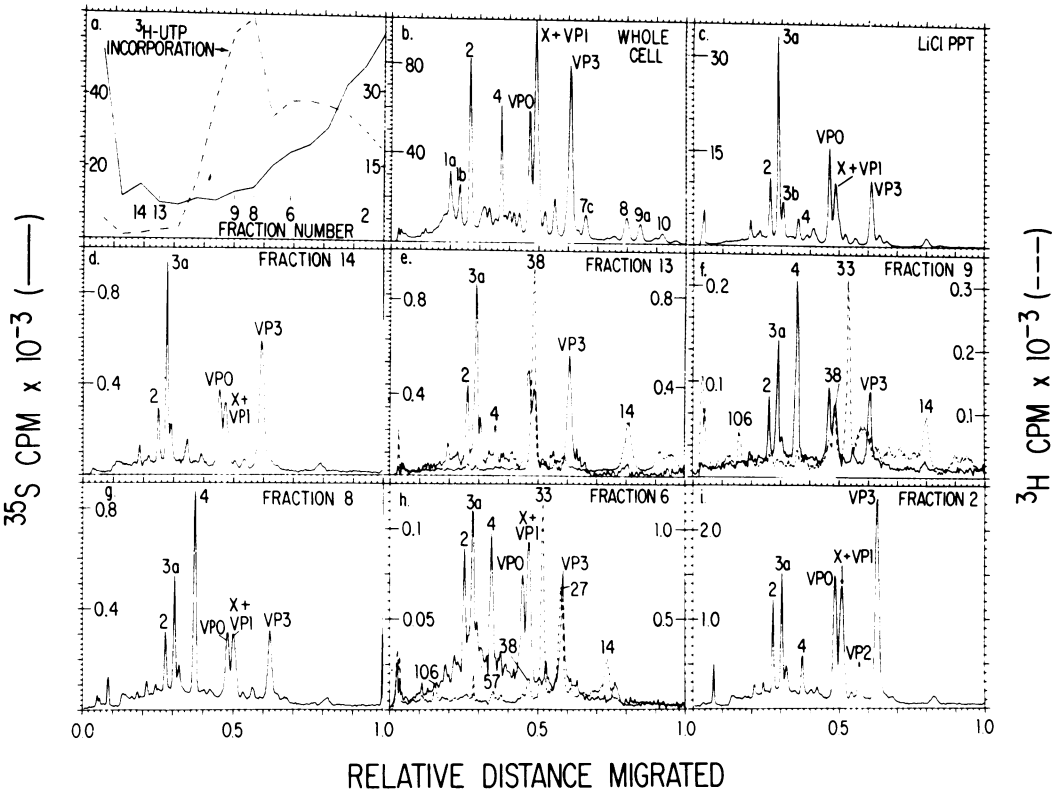

FIG. 5. *Viral and host polypeptides associated with the poliovirus polymerase. Three 20-ml portions of a HeLa cell suspension were infected with poliovirus, labeled with 125 μCi of [^{35}S]methionine per ml, from 0.5 to 3.0 h postinfection, and the polymerase was prepared by 2 M LiCl precipitation as described by Lundquist et al. (19). Samples from the whole-cell suspension and the LiCl precipitate were solubilized in 1% SDS in a boiling water bath for 3 min. These samples were then dialyzed and subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods (b,c). Direction of migration is from left to right. Five-tenths milliliter of the dissolved precipitate was layered on a 15 to 35% sucrose gradient and sedimented for 4 h at 48,000 rpm in an SW50.1 rotor. The gradient was fractionated into 16 5-drop fractions from the bottom of the tube (a). Direction of migration is from left to right. (a) One hundred-microliter portions were removed from each fraction and assayed for polymerase activity ([^{3}H]UTP incorporation [-----]), and 35S-labeled viral polypeptides (——). The remainder of each fraction was solubilized with 1% SDS and analyzed on SDS-polyacrylamide gels as described above. Portions were taken from solubilized fractions and reacted with [^{3}H]acetic anhydride to label all proteins present, both host and viral. After dialysis, these double-labeled samples were also analyzed electrophoretically: (d-i) ^{35}S viral protein (——), [^{3}H]acetic anhydride-labeled protein (-----).*

groups that are common in most proteins, the relative amount of any one protein present can be expected to be roughly proportional to the amount of ^{3}H label bound to that protein. The polypeptide profiles of the samples double-labeled in this manner show that in the peak region of enzyme activity there is far more host than viral protein present. For example, there is only a trace of ^{3}H-labeled protein moving at the position of polypeptide 4 compared with the ^{3}H peak moving with an apparent molecular weight of 33,000 (Fig. 5f). Since polypeptide 4 is the major viral constituent (as determined by the ^{35}S label), then all other viral peaks must be assumed to be present in even smaller amounts than 4. The major host peaks present have been designated as their relative molecular weight in thousands, i.e., 106, 38, 33, and 14 (Fig. 5f).

DISCUSSION

Polymerase-phospholipid interaction. The polioviral RNA polymerase complex, which consists of enzyme, template, and nascent RNA, is membrane associated in vivo (5, 6, 8, 9). Infection of HeLa cells with poliovirus results in proliferation of smooth membranes of higher phospholipid/protein ratio to which the polymerase is bound (23, 24). Table 1 shows that the partially purified RNA polymerase complex associated spontaneously in an analogous manner with phospholipid membranes. The polymerase complex associated with egg yolk lecithin, DMPC, DPPC, DSPC, DPPE, and mixtures of DMPC-DPPE and DMPC-cholesterol. In general, at 37°C, the binding was more extensive for those membranes that were above their phase transition temperatures and thus exhibited greater fluidity, suggesting that the binding involves interaction of the protein or RNA with the hydrocarbon chains of the lipid bilayer. At 37°C, egg yolk lecithin, which is a mixture of lipids, and DMPC are fluid and exhibit the highest partition coefficients. DSPC and DPPE are well below their phase transition temperatures at 37°C and exhibit correspondingly low partition coefficients. Lipid mixtures of both fluid and solid phases also showed reduced partition coefficients.

It is not known whether the polymerase-lipid interaction found is directly analogous to that found in vivo. However, it has been suggested that viral RNA does not bind to DMPC liposomes (18a), suggesting that the protein-lipid interaction is responsible for the binding. However, we did see some binding of 18*S* ribosomal RNA with DMPC liposomes (data not shown).

Some membrane-bound enzymes require association with specific lipids for activity (26). With the polioviral RNA polymerase complex, activity was not increased by the addition of lipids shown in Table 1. In fact, some of the higher-melting lipids inhibited the polymerase reaction as much as 40%.

It has been demonstrated for several enzymes that are intimately associated with membranes that their activity is greatly affected by the fluidity of the membrane. For example, the sarcoplasmic reticulum ATPase is completely inhibited in DMPC below the phase transition temperature of the lipid (35). To determine if the phospholipid phase transformation influences the polymerase activity, spin-label EPR experiments measuring the membrane fluidity and kinetic experiments measuring the rate of incorporation of tritiated UTP into RNA by the polymease were performed as a function of temperature. The TEMPO solubility curve for the polymerase-liposome complex shows a sharp phase transition at approximately 23°C (Fig. 2). There was only a slight change in slope in the Arrhenius plot of the polymerase activity at this point (Fig. 2). Further, the Arrhenius plots of the lipid-free complex and a polymerase DMPC-DPPC liposome complex also showed similar parallel curves, suggesting that the active site is not associated with the hydrocarbon portion of the membrane.

Polypeptides associated with the polymerase. At least three primary gene products (polypeptides 1a, X, 1b) are synthesized during translation of the polioviral RNA. Polypeptides 1a and 1b are subsequently processed by a series of proteolytic cleavages. Most, if not all, of the viral-specific proteins have been identified and many of the precursor-product relationships have been established (2, 13, 33).

Other than the structural aspect of the capsid polypeptides, little progress has been made on the assignment of functions to the various viral polypeptides. Typical is the difficult problem of identifying the viral and host components of the viral RNA polymerase. Although in vivo both plus and minus RNA strands are initiated, elongated, and released in a regulated manner, the purification schemes thus far developed yield an enzyme that does not initiate new chains, does not respond to added viral RNA, and possesses only the ability to complete nascent chains. Although many different polypeptides have been proposed as constitutents of the picornaviral polymerase or as involved in RNA synthesis (4, 7, 15, 17, 19, 25, 34; G. F. Vande Woude, R. Ascione, J. Card, K. M. Cowan, and J. Polatnick, Abstr. Annu. Meet. Am. Soc. Microbiol. 1972, V340, p.242), little agreement remains among the various reports.

Analysis of the smooth membrane fraction to which the polymerase is bound shows that polypeptide X is the major viral polypeptide present (Fig. 3). A similar polypeptide pattern for this fraction was also seen by Caliguiri and Mosser (4). Treatment of the infected cells with 3 mM guanidine specifically releases the capsid polypeptides from this fraction, whereas X is retained as the major viral constituent (Yin, in preparation). [^{3}H]acetic anhydride, which was used to label all components, both host and viral, yielded a pattern in which the major ^{3}H-labeled peak did comigrate with the ^{35}S-labeled X, indicating that X was the major polypeptide present. The possibility does exist, however, that this is a host polypeptide with the exact same mobility as X. Treatment of this membrane fraction with detergent and high salt releases the polymerase activity from both the membrane and from polypeptide X (data not shown). This suggests that X may play the important role of organizing the polymerase on the membrane, but is not essential for the in vitro activity recovered. Consistent with this is the observation that polypeptide X would spontaneously associate with DMPC liposomes to a greater extent than would the capsid polypeptides (compare X and VP3 in Fig. 4).

Polypeptides 2 and 4 are also present in the polymerase-enriched smooth membrane fraction. The host polypeptides 86, 62, 54, and 46 (Fig. 3) may either be components of the membrane or the polymerase. Isolation of the encephalomyocarditis viral RNA polymerase showed four major polypeptides with molecular weights of 72,000, 65,000, 57,000, and 45,000 (25). These may correspond to polypeptides 2, 62, 4, and 46 in Fig. 3, respectively.

Lundquist et al. (19) demonstrated that the polioviral RNA polymerase complex had enough single-stranded RNA character to precipitate in 2 M LiCl. Using this as a purification step, they showed that the sucrose gradient profile of this material had a peak of enzymatic activity at about 25S, with considerable activity sedimenting as a leading shoulder. The main viral polypeptide present in this region of the sucrose gradient was 4. Repeating this experiment confirmed that polypeptide 4 was enriched relative to the other viral polypeptides (Fig. 5g). In addition, a leading shoulder of activity was observed (Fig. 5a) in which 4 was not the major viral polypeptide present (Fig. 5h). Using [^{3}H]acetic anhydride to label all the polypeptides present revealed several major host polypeptides (Fig. 5f). There was little ^{3}H labeling of the viral components relative to these host polypeptides, indicating that the polymerase complex isolated may consist, in part, of host proteins with polypeptides 106, 38, 33, and 14 as prime candidates for constituents of the enzyme.

It is not understood why there seems to be no obvious correlation between the ^{3}H-labeled polypeptides in Fig. 3 and 5, unless 38 and 27 in Fig. 5 correspond to X(37) and VP3(26) in Fig. 3 and for some reason have a slightly altered mobility in the two systems. This discrepancy, as well as several other considerations, necessitates that restraint be used in elucidating the components of the polymerase. Other polypeptides, present in small amounts in Fig. 5f, could be part of the enzyme. In addition, there are other enzyme activities such as a polyadenylic acid polymerase (32) and a ligase-like activity (Yin, in preparation) that are associated with or copurify with the enzyme. Furthermore, this technique precipitates a visible amount of material with an absorbance maximum of 256 nm (Fig. 1), most of which is probably cellular single-stranded RNA. If ribosomal RNA is a component of the precipitate, then there is a strong possibility that ribosomal proteins that specifically interact with this structural RNA may be carried along in this purification scheme. The polypeptide patterns in Fig. 5 are from cells that had been labeled from 0.5 to 3 h postinfection. Repeating the experiment, except for labeling from 2.5 to 3.5 h postinfection, we found that qualitatively the same host and viral polypeptides were found throughout the gradient. However, in this case, 4 was no longer enriched relative to the other viral polypeptides in the polymerase peak in the gradient. Also, the small peak of ^{35}S radioactivity at about fraction 14 was predominantly a polypeptide moving in the position of VP3. Thus, it is not possible at this time to state which polypeptides constitute the viral polymerase.

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LITERATURE CITED

1. **Bangham, A. D., M. W. Hill, and N. G. A. Miller.** 1974. Preparation and use of liposomes as models of biological membranes. Methods Membr. Biol. 1:1-68.
2. **Butterworth, B. E.** 1973. A comparison of the virus-specific polypeptides of encephalomyocarditis virus, human rhinovirus-1A, and poliovirus. Virology 56:439-453.
3. **Butterworth, B. E., L. Hall, C. M. Stoltzfus, and R. R. Rueckert.** 1971. Virus-specific proteins synthesized in encephalomyocarditis virus-infected HeLa cells. Proc. Natl. Acad. Sci. U.S.A. 68:3083-3087.
4. **Caliguiri, L. A., and A. G. Mosser.** 1971. Proteins associated with the poliovirus RNA replication complex. Virology 46:375-386.
5. **Caliguiri, L. A., and I. Tamm.** 1970. The role of cyto-

plasmic membranes in poliovirus biosynthesis. Virology 42:100–111.

6. **Caliguiri, L. A., and I. Tamm.** 1970. Characterization of poliovirus-specific structures associated with cytoplasmic membranes. Virology 42:111–122.
7. **Cooper, P. D., E. Geissler, P. D. Scotti, and G. A. Tannock.** 1971. Further characterization of the genetic map of poliovirus temperature-sensitive mutants, p. 75–100. *In* G. E. W. Wolstenholme and M. O'Connor (ed.), Ciba Foundation symposium on strategy of the viral genome. Livingstone, New York.
8. **Ehrenfeld, E., J. V. Maizel, and D. F. Summers.** 1970. Soluble RNA polymerase complex from poliovirus-infected HeLa cells. Virology 40:840–846.
9. **Girard, M., D. Baltimore, and J. E. Darnell.** 1967. The poliovirus replication complex: site for synthesis of poliovirus RNA. J. Mol. Biol. 24:59–74.
10. **Grant, C. W. M., and H. M. McConnell.** 1974. Glycophorin in lipid bilayers. Proc. Natl. Acad. Sci. U.S.A. 71:4653–4657.
11. **Hinz, H. J., and J. M. Sturtevant.** 1972. Calorimetric studies of dilute aqueous suspensions of bilayers formed from synthetic L-α-lecithins. J. Biol. Chem. 247:6071–6075.
12. **Hong, K., and W. L. Hubbell.** 1973. Lipid requirement for rhodopsin regenerability. Biochemistry 12:4517–4523.
13. **Jacobson, M. F., J. Asso, and D. Baltimore.** 1970. Further evidence on the formation of poliovirus proteins. J. Mol. Biol. 49:657–669.
14. **Jost, P. C., R. A. Capaldi, G. Vanderkooi, and O. H. Griffith.** 1973. Lipid-protein and lipid-lipid interactions in cytochrome oxidase model membranes. J. Supramol. Struct. 1:269–280.
15. **Korant, B. D.** 1975. Regulation of animal virus replication by protein cleavage, p. 621–644. *In* Proteases and biological control. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Linden, C. D., K. L. Wright, H. M. McConnell, and C. F. Fox.** 1973. Lateral phase separations in membrane lipids and the mechanism of sugar transport in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 70:2271–2275.
17. **Loesch, W. T., Jr., and R. B. Arlinghouse.** 1975. Stable polypeptides associated with the 250s mengovirus-induced RNA polymerase structure. Arch. Virol. 47:201–215.
18. **Lonberg-Holm, K., and B. E. Butterworth.** 1976. Investigation of the structure of polio- and human rhinovirions through the use of selective chemical reactivity. Virology 71:207–216.

18a. **Lonberg-Holm, K., L. B. Gosser, and E. J. Shimshick.** 1976. Interaction of liposomes with subviral particles of poliovirus type 2 and rhinovirus type 2. J. Virol. 19:746–749.

19. **Lundquist, R. E., E. Ehrenfeld, and J. V. Maizel, Jr.** 1974. Isolation of a viral polypeptide associated with poliovirus RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 71:4773–4777.
20. **McGregor, S., L. Hall, and R. R. Rueckert.** 1975. Evidence for the existence of protomers in the assembly of encephalomyocarditis virus. J. Virol. 15:1107–1120.
21. **Michaelson, D. M., and M. A. Raftery.** 1974. Purified acetycholine reception: its reconstitution to a chemically excitable membrane. Proc. Natl. Acad. Sci. U.S.A. 71:4768–4772.
22. **Montelaro, R. C., and R. R. Rueckert.** 1975. Radiolabeling of proteins and viruses *in vitro* by acetylation with radioactive acetic anhydride. J. Biol. Chem. 250:1413–1421.
23. **Mosser, A. G., L. A. Caliguiri, A. S. Scheid, and I. Tamm.** 1972. Chemical and enzymatic characteristics of cytoplasmic membranes of poliovirus-infected HeLa cells. Virology 47:30–38.
24. **Mosser, A. G., L. A. Caliguiri, and I. Tamm.** 1972. Incorporation of lipid precursors into cytoplasmic membranes of poliovirus-infected HeLa cells. Virology 47:39–47.
25. **Rosenberg, H., B. Diskin, L. Oron, and A. Traub.** 1972. Isolation and subunit structure of polycytidylate-dependent RNA polymerase of encephalomyocarditis virus. Proc. Natl. Acad. Sci. U.S.A. 69:3815–3819.
26. **Rothfield, L., and D. Romeo.** 1971. Enzyme reactions in biological membranes, p. 251–284. *In* L. Rothfield (ed.), Structure and function of biological membranes. Academic Press Inc., New York.
27. **Rozantsev, E. G.** 1970. Free nitroxyl radicals, p. 217. Plenum Press, New York.
28. **Shimshick, E. J., and H. M. McConnell.** 1973. Lateral phase separations in phospholipid membranes. Biochemistry 12:2351–2360.
29. **Singer, S. J.** 1974. The molecular organization of membranes. Annu. Rev. Biochem. 43:805–833.
30. **Singer, S. J., and G. L. Nicolson.** 1972. The fluid mosaic model of the structure of cell membranes. Science 175:720–731.
31. **Singleton, W. S., M. S. Gray, M. L. Brown, and J. L. White.** 1965. Chromatographically homogeneous lecithin from egg whites. J. Am. Oil Chem. Soc. 42:53–56.
32. **Spector, D. H., and D. Baltimore.** 1975. Polyadenylic acid on poliovirus RNA. III. In vitro addition of polyadenylic acid to poliovirus RNAs. J. Virol. 15:1432–1439.
33. **Summers, D. F., and J. V. Maizel, Jr.** 1968. Evidence for large precursor proteins in poliovirus synthesis. Proc. Natl. Acad. Sci. U.S.A. 59:966–971.
34. **Traub, A., B. Diskin, H. Rosenberg, and E. Kalmar.** 1976. Isolation and properties of the replicase of encephalomyocarditis virus. J. Virol. 18:375–382.
35. **Warren, G. B., P. A. Toon, N. J. M. Birdsall, A. G. Lee, and J. C. Metcalfe.** 1974. Reversible lipid titrations of the activity of pure adenosine triphosphatase-lipid complexes. Biochemistry 13:5501–5507.