Acidic phospholipids in a fluid phase dissociate ADP or ATP tightly bound to DnaA protein and, in the presence of ATP and DNA, activate DnaA in the ADP form to full activity (Sekimizu, K., and Kornberg, A. (1988) *J. Biol. Chem.* 263, 7131–7135). Further studies of the interactions between DnaA protein and lipids have used two functional assays: 1) release of ADP or ATP from DnaA and 2) DNA replication upon rejuvenation of an inactive ADP-DnaA protein complex. Among a variety of phospholipids tested were pure synthetic compounds and the mixtures from *Escherichia coli* auxotrophs (faba), which are unable to synthesize unsaturated fatty acids and can be supplemented with different acyl derivatives. Fatty acid composition was determined by gas-liquid chromatography and membrane fluidity by fluorescence spectroscopy using 1,6-diphenyl-1,3,5-hexatriene as a probe. Lipid requirements of DnaA protein were shown to be: 1) phospholipids in a fluid phase (i.e., above the transition temperature), 2) a charged polar head group, 3) a lamellar phase (i.e., hexagonal II structures were inactive), and 4) a certain degree of fluidity imparted by the fatty acids esterified to the glycerol backbone. This conclusion was based on the incorporation of: 1) cholesterol, known to increase membrane packing of lipids, or 2) a branched fatty acyl derivative, which exhibits a fluidizing effect similar to that of a cis double bond. Both agents demonstrated that membrane fluidity is required for DnaA protein function in vitro, consistent with early studies of chromosome initiation in growing cells.

DnaA, the initiator protein of chromosomal replication in *Escherichia coli*, and the sites of its action at the origin (oriC) have been identified (1–3). However, the interactions of DnaA with cell membranes, while strongly implied (4–9), have not been clarified. Two main lines of evidence have been presented for such interactions. One is based on a preferential membrane attachment of the replicated chromosomal origin, presumably in its transient hemimethylated state (7, 8). However, the DnaA protein has yet to be implicated in this attachment. The second line of evidence is the correlation between the nature of the phospholipid interactions of DnaA observed in vitro (4, 5) with the membrane composition needed for initiation of a replication cycle in vivo (9).

The DnaA protein-membrane interactions in vitro were monitored by the capacity of phospholipids in the lamellar phase to dissociate a tightly bound ADP or ATP (4, 5). By virtue of this interaction, the inert ADP form of the protein can, in the presence of ATP and oriC (10), be converted to the ATP form, active for replication. Essential features of the phospholipids in the lipid bilayer were identified as acidic head groups (e.g., phosphatidylglycerol, cardiolipin) and an unsaturated fatty acid, the contribution of which to membrane fluidity may be responsible for the dissociation of ATP or DnaA from DnaA (5).

In the present study, we have proceeded further in defining the influences of phospholipid composition and fluidity on the interactions of DnaA with cell membranes.

**MATERIALS AND METHODS**

*Reagents—Sources were as follows: ATP and dNTPs, Pharmacia LKB Biotechnology Inc.; Hepes, U.S. Biochemical Corp.; GTP, CTP, UTP and Tricine, Sigma; cholesterol, [a-32P]ATP (430 Ci/mmol) and [a-32P]dGTP (800 Ci/mmol), Amersham Corp..*  

**Bacterial Strains—**D10 (rfa-1, relA1) and CY50 (galK2, λ, fabA2, trp-45, his-68, rpsL118, malA1[λ]), obtained from the E. coli Genetic Stock Center.

**Growth Media—** *E. coli* D10 was grown in Luria broth supplemented with 0.5% glucose. *E. coli* CY50 was grown in the same medium, to an A650nm of 0.3; the culture was diluted 1:20 into a minimal medium (Na2HPO4, 0.6%; KH2PO4, 0.3%; NaCl, 0.08%; and NH4Cl, 0.1%) with or without the addition of oleic acid to 0.5% w/v. Cells were harvested after five generations.

**Enzyme—** Purified DNA replication proteins were prepared and assayed as described (11). DnaA protein (1.2 x 106 units/mg) was purified (6) to greater than 90% as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 1 unit of DnaA protein activity corresponds to 1 pmol of DNP incorporated per min.

**Phospholipids—** All phospholipids were from Avanti Polar Lipids. Purity was tested by thin-layer and gas-liquid chromatography. To prepare multilamellar vesicles, phospholipids were dried to a thin film under a flow of N2 and suspended in an appropriate volume of water. *E. coli* phospholipids were extracted by a modification of the Bligh and Dyer method (12) as previously described (13). Phospholipids were quantitated by measuring inorganic phosphate (13).

**ATP-binding Assay—** The standard reaction mixture (15 μl) contained Tricine-KOH (pH 8.25 at 1 μ), 50 μM; magnesium acetate, 2.5 mM; EDTA, 0.3 mM; glycerol, 20% (v/v); Triton X-100, 0.007%; dithiothreitol, 7 mM; [a-32P]ATP (5 μCi/mmol), 1 μM; and 2 pmol of DnaA protein. After incubation at 0°C for 15 min, the solution was filtered through a nitrocellulose membrane (Millipore HA 0.45-μm pore) presoaked in the wash buffer (Tricine-KOH (pH 8.25 at 1 μ), 50 μM; magnesium acetate, 0.5 mM; EDTA, 0.3 mM; dithiothreitol, 5 μM; (NH4)2SO4, 10 μM; glycerol, 17% (v/v); and Triton X-100, 0.005%). The filter was washed with 6 ml of cold wash buffer and dried under an infrared lamp. Retained radioactivity was measured by liquid scintillation counting.

1 The abbreviations used are: Tricine, N-tris(hydroxymethyl)methylglycine; DPP, 1,6-diphenyl-1,3,5-hexatriene; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine.

*This work was supported by National Institutes of Health Grant GM07851 and National Science Foundation Grant DMB 87-1007945. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.**

† Supported by a National Institutes of Health Fogarty International research fellowship, Member of the Career of the Investigator of CONICET, Argentina.

‡ Supported by an American Cancer Society, California Division senior postdoctoral fellowship. Present address: Dept. of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, D.C. 20007.
Activation of DnaA Protein by Phospholipids

Nucleotide Release Assay—The ATP- (or ADP-)DnaA protein complex was exposed to phospholipids at 37 °C for 20 min (5), and the samples were filtered as described above to measure the loss of nucleotide. ATP rather than ADP was usually used because of the ready availability of \(^{32}P\)ATP; results with ADP were indistinguishable from those with ATP.

Reconstitution Assay for DNA Replication—DNA synthesis with purified proteins (11) was measured as described previously (10).

Fluorescence Measurements—These were made in an SLM 8000 spectrofluorimeter equipped with polarizers as described elsewhere (14, 15). Temperature scans (10–40 °C) were performed by heating the cuvette holder at an average rate of 20 °C/h and monitored with a thermistor probe inserted into the cuvette, just above the excitation beam. For polarization measurements, the sample was excited with vertically polarized light, while vertical (I\(_v\)) and horizontal (I\(_h\)) emission intensities were recorded. The instrumental anisotropy (G) was determined by measuring vertical and horizontally polarized light. Light scattering was avoided by measuring the anisotropy as a function of vesicle concentration; no reabsorption of the emitted light occurred (i.e., sample absorbances were <0.05). Results are expressed after appropriate blank and instrumental corrections, as fluorescence anisotropy, \(r_s\),

\[
\frac{I_v - I_h}{I_v + 2I_h} = \frac{1}{G} \quad \text{(Eq. 1)}
\]

1,6-Diphenyl-1,3,5-hexatriene (DPh) was used as the lipophilic probe; light was excited at 283 nm and fluorescence collected with a 310 nm cut-off filter. The lipid:probe ratio was 500:1 (mol:mol).

Fatty Acid Analysis—Phospholipids were trans-esterified in methanol, 3% HCl (3 h at 64 °C). The resulting methyl esters were separated on a capillary column using a HP 5895 gas-liquid chromatograph. Fatty acids were identified by comparing their relative retention times with those of reference standards.

RESULTS

Release of ATP or ADP from DnaA Protein Requires Certain Features in a Phospholipid—A variety of pure phospholipids and the phospholipids extracted from E. coli were tested for their ability to release a tightly bound ADP or ATP. The bacterial phospholipids were obtained from wild-type cells and from a mutant strain (\(fabA\)) defective in the synthesis of unsaturated fatty acids (16). All the phospholipids are known to adopt a lamellar structure in an aqueous solution and are negatively charged at the pH of the release assay (17). Except for dimyristoyl-PG and dipalmitoyl-PG, the pure phospholipids contained one or two unsaturated acyl chains. Fatty acid composition determined by gas-liquid chromatography indicated that the levels of unsaturated fatty acids in the phospholipid mixtures from the \(fabA\) mutants was 15%, compared with 51% in the wild type. Among the phospholipids tested, dimyristoyl-PG, dipalmitoyl-PG, and those from the \(fabA\) mutant were all relatively inactive in effecting the release of ATP from DnaA protein, compared with wild-type E. coli phospholipids, dioleoyl-PG, dipalmitoyl-PG, and cardiolipin (Fig. 1). Comparable results were obtained when the release of ADP from DnaA protein, by phospholipids from these sources, was measured by the restoration of replication function to the inert ADP form of DnaA protein (data not shown).

A Bilayer Structure Is Necessary for the Release of ATP from DnaA Protein by Phospholipids—To confirm the requirement for a vesicular structure previously suggested (5), cardiolipin and dioleoyl-PG (in a bilayer arrangement) were induced to adopt a hexagonal II structure typical of PE by adding 30 mol % of CaCl\(_2\) (17). Under this condition, the dissociation of ATP from DnaA protein was decreased to two-thirds of the original values for both phospholipids.

A Negatively Charged Polar Head Group Is Required to Release the Nucleotide from DnaA Protein—To characterize the type of phospholipid that can effectively dissociate ATP from DnaA protein, a variety of phospholipids either negatively charged (PA, PS, and PG) or zwitterionic (PC and PE) were tested in the release assay (Table I). Some negatively charged phospholipids, such as cardiolipin, dioleoyl-PA, dioleoyl-PS, and dipalmitoyl-PE, were effective, while others, such as distearyl-PA and dipalmitoyl-PS, were not. Inasmuch as zwitterionic phospholipids (e.g., dioleoyl-PC, dipalmitoyl-PE, and dioleoyl-PE) were unable to liberate ATP, a negatively charged polar head group appears to be a necessary but insufficient feature for the effective interaction of DnaA protein with phospholipids.

A Fluid Lamellar Phase Is Needed to Dissociate ATP from DnaA Protein—Lipid-protein interactions are known to depend on both the chemical structure and the physical state of phospholipids (14). The fluorescence anisotropy of DPH-labeled vesicles, a value inversely related to the fluidity of the lipid bilayer (18), has been widely used to monitor the dynamic properties of artificial and natural membranes. Phospholipids extracted from wild-type E. coli cells and from \(fabA\) mutants supplemented and unsupplemented with oleic acid undergo a significant change of fluidity at a characteristic temperature (Fig. 2). This phase transition indicates the replacement of an ordered gel state by a more disordered fluid phase (18). The transition temperature (\(T_m\)) of phospholipids from wild-type E. coli and oleic acid-supplemented \(fabA\) mutants, both with high levels of unsaturated fatty acids, was lower (i.e. 19 °C) than that of unsupplemented \(fabA\) mutants (i.e. 24 °C), due to the disruptive effect of a cis double bond on the tightly packed

**TABLE I**

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Charge*</th>
<th>Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiolipin</td>
<td>Negative</td>
<td>97</td>
</tr>
<tr>
<td>Dioleoyl-PA</td>
<td>Negative</td>
<td>82</td>
</tr>
<tr>
<td>Dioleoyl-PS</td>
<td>Negative</td>
<td>78</td>
</tr>
<tr>
<td>Dipalmitoyl-PS</td>
<td>Negative</td>
<td>24</td>
</tr>
<tr>
<td>Distearoyl-PA</td>
<td>Negative</td>
<td>16</td>
</tr>
<tr>
<td>Dioleoyl-PE</td>
<td>Zwitterionic</td>
<td>25</td>
</tr>
<tr>
<td>Dioleoyl-PC</td>
<td>Zwitterionic</td>
<td>17</td>
</tr>
<tr>
<td>Dipalmitoyl-PE</td>
<td>Zwitterionic</td>
<td>15</td>
</tr>
</tbody>
</table>

*At pH 8.0.
The ATP-DnaA complex (see "Materials and Methods") was incubated with saturated and unsaturated pure acidic phospholipids and phospholipid mixtures from unsupplemented and oleic acid-supplemented fabA mutants. Experimental conditions were as in Table I.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristoyl oleoyl-PG</td>
<td>86</td>
</tr>
<tr>
<td>Palmitoyl oleoyl-PG</td>
<td>84</td>
</tr>
<tr>
<td>fabA mutant + oleic acid</td>
<td>84</td>
</tr>
<tr>
<td>Dioleoyl-PG</td>
<td>82</td>
</tr>
<tr>
<td>Dimyristoyl-PG</td>
<td>35</td>
</tr>
<tr>
<td>fabA mutant</td>
<td>21</td>
</tr>
<tr>
<td>Dipalmitoyl-PG</td>
<td>19</td>
</tr>
</tbody>
</table>

**TABLE IV**

Rigidity introduced by cholesterol reduced release of ATP from DnaA protein

Vesicles were prepared and their fluorescence anisotropy measured as indicated under "Materials and Methods." Cholesterol was added to the unsaturated phospholipids in amounts to match the fluidity of dimyristoyl-PG. Vesicles were added to the ATP-DnaA complex, and the loss of ATP was measured as described in Table I.

<table>
<thead>
<tr>
<th>Fluorescence anisotropy (±0.001)</th>
<th>Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimyristoyl-PG</td>
<td>0.136</td>
</tr>
<tr>
<td>Palmitoyl oleoyl-PG</td>
<td>0.110</td>
</tr>
<tr>
<td>Palmitoyl oleoyl-PG + cholesterol (28.6 mol %)</td>
<td>0.136</td>
</tr>
<tr>
<td>Dioleoyl-PG</td>
<td>0.076</td>
</tr>
<tr>
<td>Dioleoyl-PG + cholesterol (45.3 mol %)</td>
<td>0.135</td>
</tr>
</tbody>
</table>

hydrocarbon matrix (18).

These phospholipid mixtures as well as other pure phospholipids, in which \( T_t \) was measured as previously described, were tested for their ability to dissociate ATP from DnaA protein (Table II). A significant amount of release was obtained only with those phospholipids with a negative polar head group, which were also in a fluid phase, as defined by a temperature above the \( T_t \) value (under these experimental conditions). These included cardiolipin, dioleoyl-PG, wild-type \( E. coli \) phospholipids, and phospholipids from oleic acid-supplemented \( fabA \) mutants. Yet, some acidic phospholipids in a fluid phase, such as dimyristoyl-PG and \( fabA \) mutants, were ineffective in activating DnaA protein. Also, lipid vesicles below the \( T_t \) (e.g., dipalmitoyl- and distearoyl-PG) were inactive in the release of the tightly bound nucleotide. While both a fluid phase and a negatively charged head group are necessary, these two conditions are not sufficient to account for all the observations.

**An Unsatuated Fatty Acid Esterified in the Phospholipid Is Essential for Its Interaction with DnaA—Saturated and unsaturated derivatives of pure acidic phospholipids as well as phospholipid mixtures from unsupplemented and oleic acid-supplemented \( fabA \) mutants were tested for their ability to dissociate ATP from the ATP-DnaA complex (Table III). For pure acidic phospholipids, only those in the fluid phase and esterified with at least one unsaturated fatty acid (e.g. dioleoyl-, myristoyl oleoyl-, and palmitoyl oleoyl-PGs) were effective in liberating the nucleotide.

The release of ATP, as a measure of DnaA protein interactions with phospholipids, can be markedly effected by lipids from \( fabA \) mutants in which an oleic acid supplement in their growth raised their membrane level of unsaturated fatty acids to 48%. By contrast, phospholipids from unsupplemented \( fabA \) mutants, which still retain unsaturated fatty acids at a level of 18% were unable to dissociate the nucleotide. When the release assay was performed at 24 °C, phospholipids from wild type \( E. coli \) or \( fabA \) mutants supplemented with oleic acid failed to liberate ATP from DnaA (data not shown), inasmuch as their fluidities were equal to that of unsupplemented \( fabA \) mutants at 38 °C (Fig. 2). Since all the phospholipid mixtures are in a fluid phase at 38 °C (Fig. 2), their different effectiveness in the release assay could be ascribed either to the level of unsaturated fatty acids, the degree of fluidity, or to both.

**Unsaturated Fatty Acids Are Required Because of the Fluidity Level They Impart to the Bilayer**—The level of unsaturated fatty acids required might be due to the fluid state they promote in the bilayer matrix or to the particular conformation of the cis double bond directly in contact with the DnaA protein. Incorporation of cholesterol into the lipid bilayer can distinguish between these alternatives, inasmuch as the steroid can decrease the fluidity without interacting with protein surfaces (19-21). Among phospholipid vesicles prepared from dimyristoyl-, palmitoyl oleoyl-, and dioleoyl-PGs, fluorescence anisotropy measurements indicated that their fluidity increased in the order mentioned (Table IV). As previously described, dimyristoyl-PG was unable to release ATP, while the other phospholipids were effective. With cholesterol added to the mono- and di-unsaturated phospholipids in amounts that matched the fluidity of dimyristoyl-PG, their effectiveness in releasing ATP decreased. A complementary experiment was performed by...
supplementing \textit{fabA} mutants with a branched fatty acid (18:0, br), known to increase the fluidity of the bilayer because of the space requirement of its acyl chains. When the fluorescence anisotropy of the extracted phospholipids was as low as that observed with oleic acid-supplemented mutants (0.116 at 37 °C), a high ATP release (84%) was obtained in each case. Thus, the fluidity of the membrane appears to be the basis for the requirement for an unsaturated fatty acid in these studies and may apply to growing cells as well (9).

**DISCUSSION**

In this study we have confirmed and extended earlier observations (4, 5) of interactions of the DnaA protein with membrane vesicles. Phospholipids that constitute the membrane vesicle must satisfy four requirements to interact effectively with DnaA protein: 1) possess a negatively charged polar head group (Table I); 2) adopt a lamellar (lipid bilayer) structure; 3) reside in a fluid phase, above the transition temperature (Table II); and 4) be esterified with an unsaturated fatty acid in order to provide an adequate level of fluidity (Tables III and IV).

With regard to membrane vesicles prepared from phospholipids extracted from \textit{E. coli}, these must, as do synthetic phospholipids, attain a proper degree of fluidity to interact effectively with the DnaA protein. This result correlates nicely with those obtained in growing cells in which a mutant (e.g. \textit{fabA}) auxotrophic for oleic acid and possessing a low level of unsaturated fatty acids fails to initiate replication (9), presumably due to the low fluidity of its membranes.

Several lines of evidence suggest DnaA protein to be a peripheral membrane protein associated by electrostatic interaction with negative phospholipid head groups and partially included in a relatively fluid lipid bilayer: 1) the protein is basic (22); 2) interacts with acidic phospholipids (4, 5); 3) sediments (about half of the protein in the cell lysate) with the particulate fraction (6); 4) forms a stable protein-lipid vesicle complex dependent on the salt concentration (6); and 5) can be liberated from aggregates by phospholipase (23). Moreover, as with many peripheral proteins, DnaA could be in a dynamic equilibrium between membrane-bound and soluble forms of the enzyme.

Fluidity should be recognized as an operational term that includes the contributions of both the rate and range of molecular motions (18). The cis double bond, by disrupting the cooperativity of the tightly packed trans bonds of saturated fatty acids and by increasing the cross-sectional area per acyl chain, creates an environment of increased motional freedom that would favor interactions between DnaA protein and the lipid bilayer. How such interactions influence the functions of DnaA protein in various stages of the cell cycle remains to be clarified.

**REFERENCES**