DEOXYRIBONUCLEIC ACID-ENVELOPE COMPLEXES FROM *ESCHERICHIA COLI*

A Complex-Specific Protein and Its Possible Function for the Stability of the Complex

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ABSTRACT

The different *Escherichia coli* envelope fractions (cell wall, cytoplasmic membrane, and DNA-envelope complex fragments) were isolated by free-flow electrophoresis and analyzed by sodium dodecylsulfate-acrylamide gel electrophoresis. The DNA-envelope complex fragments possess a specific protein (mol wt 80,000–90,000). Upon treatment with trypsin, this protein disappears and the complex breaks down, thus releasing DNA, cell wall, and cytoplasmic membrane. Disaggregation of the complex can also be achieved by high salt concentrations. Lysozyme treatment dissolves the murein layer within the complex but does not disaggregate the complex. From these and other results on the stability of the DNA-envelope complex, conclusions can be drawn about the possible linkage within the described envelope particles.

Although most models that describe the control of bacterial replication and cell division propose the attachment of the chromosomal DNA to the membrane, the nature of the bonds and the structures involved in this attachment are completely unknown. Morphological studies with the electron microscope have shown associations of the chromosome with the bacterial cell surface or with the intracellular mesosomes of gram-positive bacteria (30) but have been unable to define the association. Membrane-DNA complexes have been isolated from bacterial lysates by density gradient centrifugation, a procedure which enriches for rapidly sedimenting complexes (RSC)[1] containing DNA in association with some large cellular membranous structure (24). Membranous-DNA complexes have also been isolated by their affinity for magnesium-sarkosyl crystals (10, 40). Some information has been obtained from experiments of these types about the specificity of the attached membrane-bound DNA. For example, in *Escherichia coli* and *Bacillus subtilis* the chromosomal origin (12, 39) and the active replicating site (15, 36) as well as many viral DNAs (35) have been found to be associated with membrane complexes. However, the cell lysis and membrane isolation

[1] *Abbreviations used in this paper:* FFE, free-flow electrophoresis according to Hannig; RSC, rapid sedimenting complex; sarkosyl, sodium lauroyl sarcosinate, NL 30; SDH, succinate dehydrogenase (E.C. 1.3.99.1), expressed in moles/minute per milligram protein; SDS, sodium dodecylsulfate, TRA, triethanolamine.

procedures used in these studies have not been specific enough to allow further analysis of the structure of the DNA-membrane complex.

The surface structure of *E. coli* is a chemically and morphologically complex multilayered envelope consisting of an outer cell wall and an underlaying cytoplasmic membrane (for review see reference 6). Although lying closely adjacent, the membrane is free from the cell wall over most areas of the cell surface but adheres to the wall at about 200 wall/membrane adhesion zones (2). The cell wall is composed of a rigid shape-maintaining peptidoglycan (murein) layer and a lipopolysaccharide-rich outer membrane. It has been suggested that the peptidoglycan is attached to this outer membrane by covalent linkage of the peptide side chains to the structural lipoprotein molecules (4, 3, 24).

Recently, a new technique has been developed for the preparative isolation of specific DNA-membranous complexes from *E. coli* (27). These complexes were shown to be small fragments of the cell envelope containing both the wall and the inner membrane which are held together at wall/membrane adhesion zones. This technique now enables us to begin the characterization of the structures involved in the attachment of DNA. In this paper, we describe the identification of a DNA-envelope complex-specific protein and demonstrate that the DNA, wall, and membrane are bound together in the adhesion zone through ionic interactions that do not require the presence of an intact peptidoglycan layer.

MATERIALS AND METHODS

Bacteria and Radioactive Labeling

E. coli KMBL42, grown in Difco antibiotic media no. 3 (Difco Laboratories, Detroit, Mich.), was used in all experiments. The bacterial cells were grown in 3–10 liters of medium at 37°C with aeration to 2–3 × 10^8 cells/ml (OD = 0.3–0.4, Beckman colorimeter C, green filter, Beckman Instruments, Fullerton, Calif.). Before harvesting, 500–1,500 ml of the culture was removed to a separate flask and labeled with 3 μCi [^{3}H]thymidine/ml for approximately two cell generations.

Isolation of the DNA-Envelope Complex

Unless otherwise described, crude envelope fractions were prepared and DNA-envelope complexes isolated by preparative free-flow electrophoresis (FFE) (16) as previously described (27). The protein concentration of the fractions was measured on an automated Technicon AutoAnalyzer (Technicon Corp., Ardsley, N.Y.). Lipopolysaccharide and succinate dehydrogenase (SHD) assays have previously been described (27).

Buffers and Reagents

The buffers used have been previously described (27). The following enzymes were used: trypsin (EC 3.4.4.4) was Trypure-Novo from Novo Industrie (Mainz) and contained no chymotrypsin activity. Lysozyme (EC 3.2.1.17) and contained no chymotrypsin activity. Lysozyme (EC 3.2.1.17) was obtained from Serva (Heidelberg). [^{3}H]Thymidine (29 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, England).

Electron Microscopy

The sections of samples from the electrophoresis were made as previously described (27). The DNA-envelope complex was also prepared for viewing in the electron microscope by the Kleinschmidt procedure (25). A JEOL 100-B electron microscope was used.

M-Band

The M-band gradients were prepared as described by Earhart et al. (10). Discontinuous gradients were formed from steps of 15% and 47% sucrose in M buffer (10 mM triethanolamine, 10 mM magnesium acetate, 0.1 M KCl, pH 7.0) over a shelf of 65% sucrose in the same buffer. To 0.1 ml of the sample, 20 μl of 5% sodium lauroyl sarcosinate (sarkosyl) and 10 μl of 1.0 M $MgCl_2$ were added and the solution was gently mixed. After incubation for 10 min at room temperature, the material was layered on the gradients and centrifuged in the Beckman SW 50.1 rotor for 10 min at 15,000 rpm. Fractions were collected from the bottom of the tube and assayed for radioactivity.

Polyacrylamide Gel Electrophoresis

Electrophoresis in acrylamide slab gels (7.5%) was performed in a vertical gel electrophoresis cell (E.C.-Apparatus Corp., St. Petersburg, Flor.) as previously described (17), with the addition of 0.1% sodium dodecylsulfate (SDS) to the gel and the electrophoresis buffer. Gels were run for 150 min at 3°C. The amperage during the run was maintained at 60 mA while the voltage increased from 200 V at the beginning to 270 V at the end of the run. Samples were prepared for electrophoresis by centrifuging the cell fragments and treating the resulting pellets for 20 min at 70°C with 0.01 M phosphate buffer, pH 7.4, containing 0.02 mg SDS, 0.05 ml mercaptoethanol, and 0.1 ml of glycerol per ml of buffer. The solubilized samples were then centrifuged at 50,000 *g* for 15 min, and 10 μl of the supernate (40–80 μg of protein) was applied to the gel. After electrophoresis, the gels were fixed for 2 h in 50% trichloroacetic acid, stained in a 1% Coomassie brillant blue solution (in 50% trichloroacetic acid) for 2 h, and then destained in 10% acetic acid overnight. The following day, the gels were restained by

passage through a staining series (11). The gel was held at each staining step for 2 h. Finally, the gels were again destained in 10% acetic acid overnight.

RESULTS

Isolation of the DNA-Envelope Complex

The DNA-envelope complex was isolated from enriched *E. coli* cell wall preparations (27) by FFE (16). Fig. 1 A shows the electrophoretic separation of the [^{3}H]DNA-envelope complex (fractions 17–20) from the cell wall fragments (fractions 21–26). The isolated membrane fragments (Fig. 1 B, fractions 29–36) which, due to a lower surface charge, migrate less strongly toward the anode do not contain ^{3}H-labeled DNA. The peak cell wall fractions have a lower specific SDH activity (0.8×10^{-8} mol/min per mg protein) than the membrane fractions (9×10^{-8}), while the complex fractions have intermediate values (2×10^{-8}). Free DNA migrates slightly farther toward the anode than does the DNA-envelope complex fragments (Fig. 1 C). That the [^{3}H]DNA in fractions 17–20 is attached to cellular fragments is also seen by sedimentation of the material through sucrose gradients and its recovery as a RSC at the top of the 65% sucrose shelf (see Fig. 12).

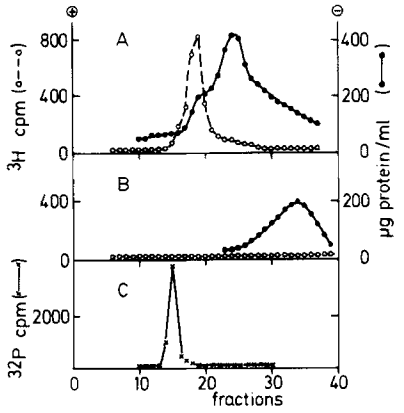


FIGURE 1 Preparative isolation of the DNA-envelope complex by FFE. *E. coli* cells were grown and labeled with [^{3}H]thymidine as described in the Materials and Methods. After harvesting, the cells were disrupted in a French pressure cell and envelope fragments were prepared (25). The envelope fragments were fractionated into enriched wall and membrane fractions by discontinuous sucrose gradient equilibrium centrifugation. The enriched wall (A) and membrane (B) preparations were then separately electrophoresed in the free-flow FF5 electrophoresis apparatus. Fractions were collected and aliquots were assayed for protein (●) and for [^{3}H]radioactivity (○). The injection port is above fraction 65. All material was deflected toward the anode, and only the first 45 of 92 total fractions are shown here. Phenol-purified *E. coli* [^{32}P]DNA (x) was added as a marker for the migration of free DNA (C).

Electron microscope observation of the DNA-envelope complex Fig. 2 shows an electron micrograph of a thin section prepared from isolated DNA-envelope complexes. The fragments contain sections of both the cell wall and inner membrane which, at zones within the complex, adhere together. These sharply defined regions, the wall/membrane adhesion zones, make up only part of the complex. The adhesion zones vary in length between 150 and 250 nm as measured from sections of about 100 particles. Therefore, it is almost impossible in the present stage of this study to speculate about their actual size, shape, or number per total surface area of the *E. coli* cell (20). For the same reason, it appears unrealistic to compare them with Bayer's contact zones (2) in plasmolyzed cells as has been proposed earlier (27).

Samples of the isolated DNA-envelope complex were also observed in the electron microscope after preparation by the Kleinschmidt procedure (25). Fig. 3 shows several representative complex fragments. Most of the envelope particles contain an attached piece of DNA. Complexes having two to six (or even more) strands of attached DNA are also seen. In most of the complex fragments, the DNA appears to originate from within the center of the curled structure.

Buoyant Densities of the E. Coli Envelope Components

The DNA-envelope complex, which contains both wall and membrane, is found during the isolation procedure after centrifugation in the discontinuous sucrose gradients (27) in the wall-enriched fractions. The determination of the actual buoyant density (ρ) of the complex relative to the wall and membrane is shown in Fig. 4. In these gradients, the envelope fragments sediment to positions corresponding to the following buoyant densities: wall ρ = 1.205, complex ρ = 1.195, membrane ρ = 1.175. There is also a small shoulder of membrane fragments which have a lighter density. Similar results for the density distributions of the envelope components have been reported for *E. coli* (19, 37) and for *Salmonella typhimurium* (28, 1).

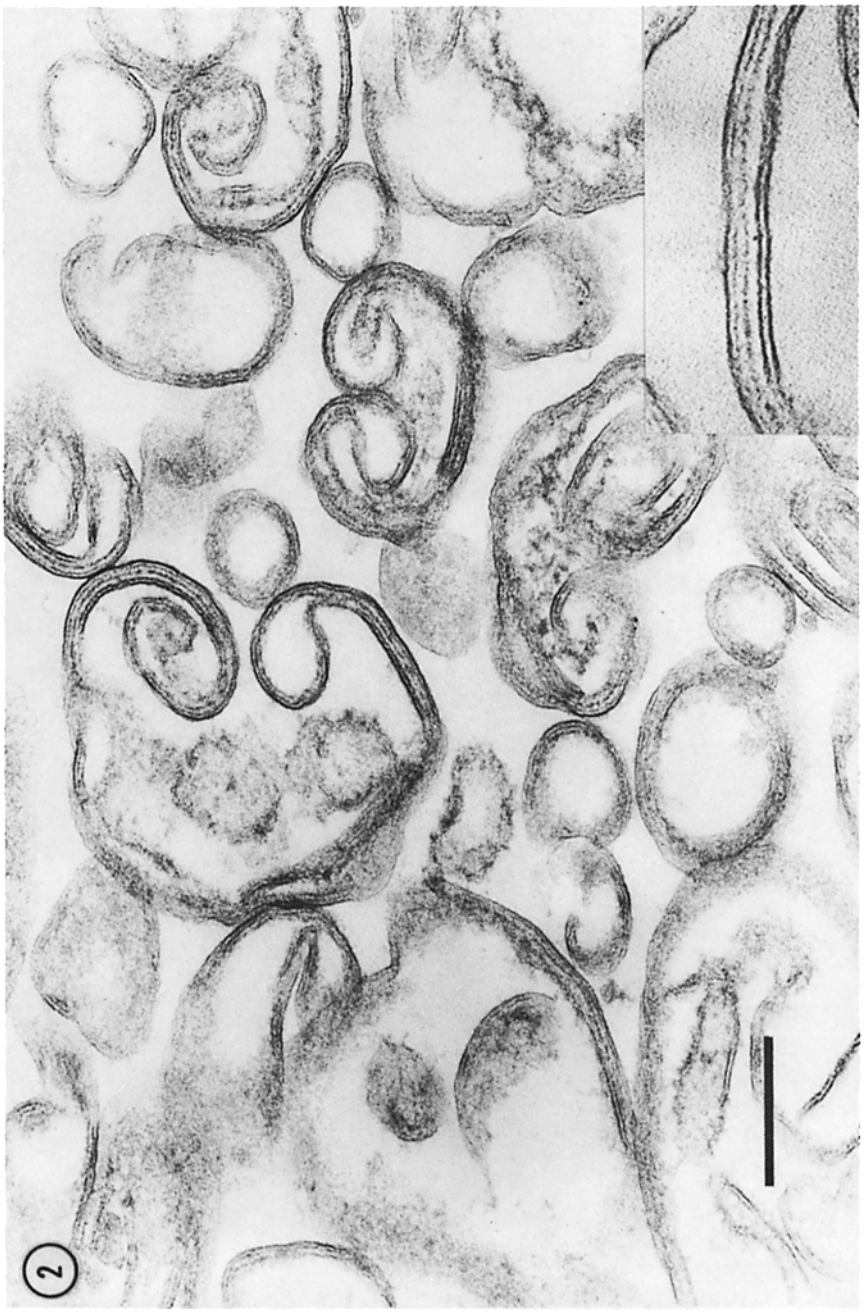


FIGURE 2 Electron micrograph (thin section) of DNA-envelope complex fragments (fractions 16–20 from Fig. 1 A). Calibration = 0.2 μm; × 100,000, insert × 200,000.

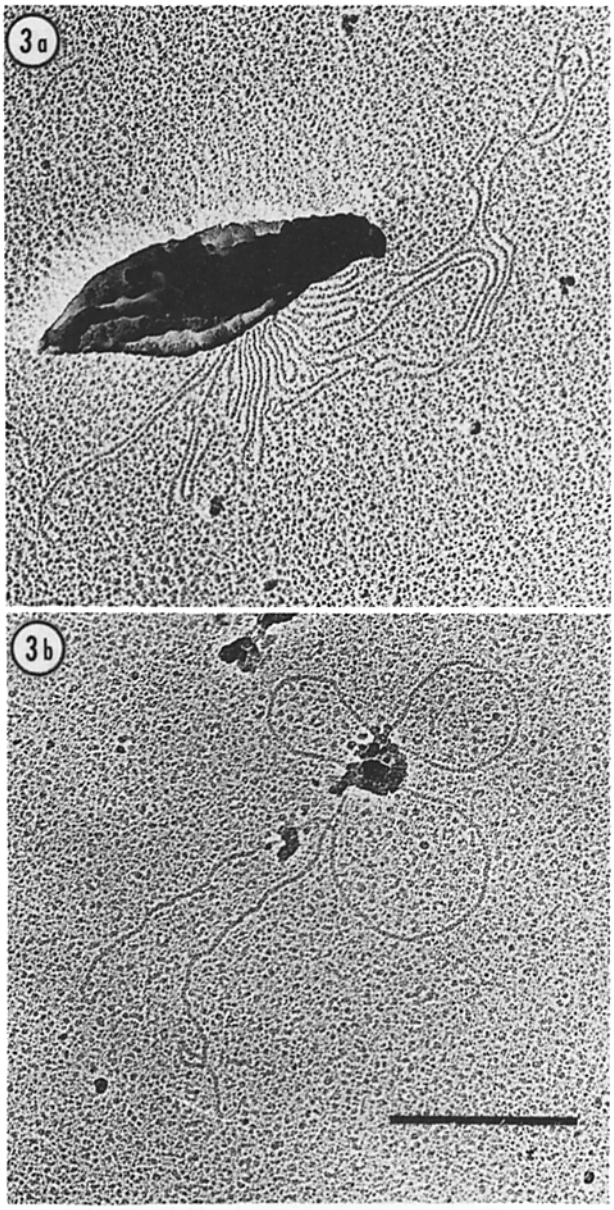
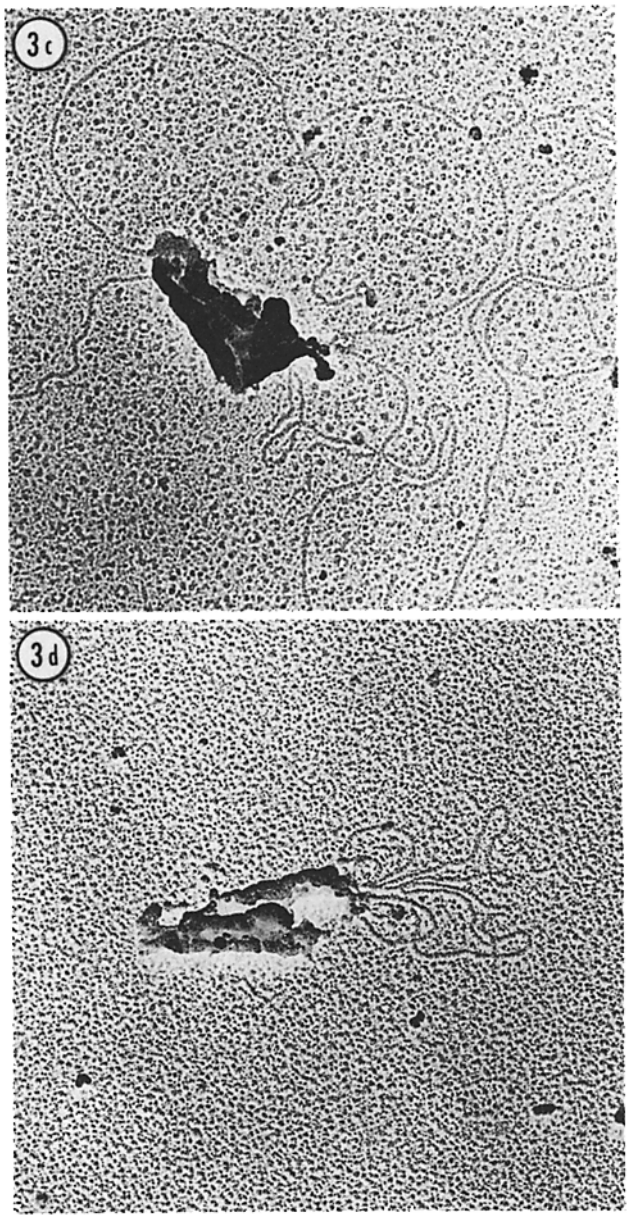


FIGURE 3 Electron micrographs (Kleinschmidt spreadings) of several representative DNA-envelope complex fragments. Calibration = 0.5 μm. × 50,000.

M-Band Formation by the DNA-Envelope Complex

A technique has been described for the isolation of bacterial membrane-DNA complexes based on the ability of the membrane to bind hydrophobically to crystals of magnesium-sarkosyl (10, 40). After a short centrifugation in a sucrose gradient, the crystals and the membranous-DNA complexes are found as a sharp band (M band) in the middle of the gradient. Since this technique has been used to demonstrate the membrane attachment of bacterial and phage DNAs (40, 35), it was of interest to determine if the DNA-envelope complex as isolated by the FFE would bind to the magnesium-sarkosyl crystals and could subsequently be found



in the M band. Fig. 5 shows that when a preparation of DNA-envelope fragments was mixed with magnesium-sarkosyl crystals, about one-half of the [^{3}H]DNA could be recovered in the M-band fractions after centrifugation. Since all the [^{3}H]DNA in the preparation was attached to envelope fragments which could be identified as RSCs, it was assumed that the DNA not recovered in the M band probably was attached to envelope fragments which were too small to bind to the detergent crystals.

Polyacrylamide Gel Electrophoresis

The DNA-envelope complex has been shown by analysis of marker enzymes, by electron microscopy, and by density equilibrium centrifugation to be composed of cell wall and inner membrane

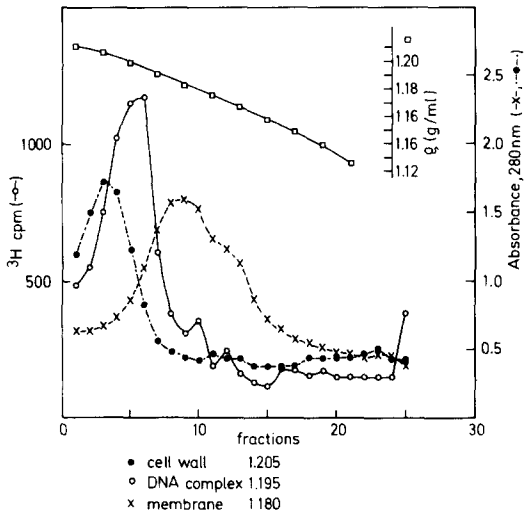


FIGURE 4 Equilibrium density gradient centrifugation of *E. coli* envelope components. Samples of a cell wall, membrane, and DNA-envelope complex isolated by FFE (Fig. 1) were layered on linear 30–55% sucrose gradients (in TRA/Mg^{++} buffer) and centrifuged in the SW 27.1 rotor for 16 h at 25,000 rpm (85,000 *g*) at 4°C. Fractions were collected from the bottom of the tubes and assayed for radioactivity or adsorbance at 280 μm. The density of the fractions was determined by refractometry. The figure is a composite of the three gradients in which the sucrose concentrations of each corresponding fractions were identical. ●, [^{3}H]DNA-envelope complex; O, A_{280} of wall; x, A_{280} of the membrane; □, density.

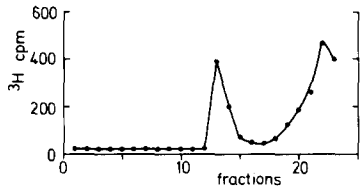


FIGURE 5 M band of the DNA-envelope complex. A sample of the DNA-envelope complex isolated by FFE was mixed with sarkosyl and $MgCl_2$ and centrifuged in the M-band gradient as described in Materials and Methods.

components (27). Polyacrylamide gel electrophoresis analysis of the DNA envelope complex should, therefore, reveal the presence of all the *E. coli* cell envelope proteins plus an enrichment for any protein specific for the complex. The results of polyacrylamide gel electrophoresis analysis of *E. coli* envelope proteins are very dependent upon the protein solubilization procedure used (23, 29), and it is therefore difficult to compare new data with other published experiments. To observe differences in the protein content of the various fractions isolated by FFE, solubilization with 2% SDS at 70°C was found to produce good resolution and to yield the most reproducible results.

In Fig. 6 the prominent bands 5 and 6 are found in the unfractionated envelope (A) and in all the envelope subfractions (B–D). These bands appear to correspond the principle envelope bands described by Henning et al. (18), and upon solubilization at 100°C they show a behavior similar to that shown in the work mentioned. These two bands 5 and 6 will not be discussed here.

In gel B of Fig. 6, several major proteins (bands 3, 4, 7, and 8) can be identified as being characteristic for the cell wall. These bands were present in all the gels run and were always reproducible, whereas some minor bands were not. Therefore, only bands 3, 4, 7, and 8 were considered to be reliable markers for the cell wall. The same is true for band 2 in gel C which was chosen as the reliable marker for the inner membrane, even though there were some minor proteins which might be characteristic for the cytoplasmic membrane. All the described proteins can also be seen in the unfractionated envelope A.

The polypeptides of the DNA-envelope complex (gel D, Fig. 6) correspond to all the polypeptides found in the total envelope A plus a band which is highly and reproducibly enriched in the complex fragments. This band was chosen as the marker protein for the complex even though there are some minor bands present. This main marker protein has a mol wt of 80,000–90,000. The migration of this band 1 was not altered upon solubilization of the complex in SDS medium at 100°C. This complex-specific protein may correspond to a protein identified by Schnaitman (33). He reported the presence of a high molecular weight Triton X-100-soluble protein (i.e., a membrane protein) which was found only in the envelope regions where the membrane is attached to the cell wall. A small amount of band-1 protein is also seen in the cell wall. This is probably due to migration in the FFE of some complex fragments which have extremely small DNA molecules together with the normal cell wall fragments. These are sometimes also seen in the electron microscope.

Effect of Lysozyme on the DNA-Envelope Complex

Since the DNA-envelope complex fragments contain all the structural layers of the cell envelope

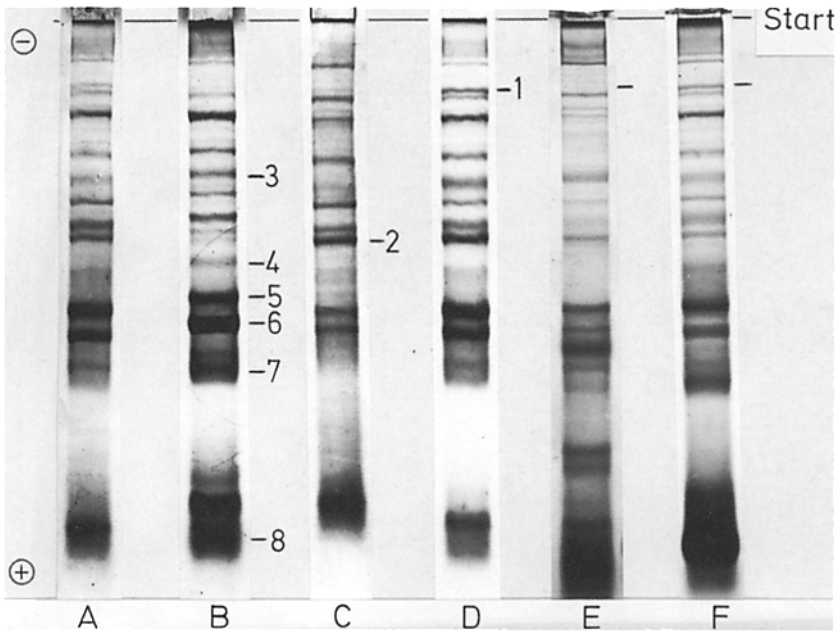


FIGURE 6 SDS-acrylamide slab gels of the different *E. coli* envelope fractions as isolated by FFE. (A) Unfractionated envelope; (B) cell wall (fractions 23–26 from Fig. 1 A); (C) cytoplasmic membrane (fractions 32–36 from Fig. 1 B); (D) DNA-envelope complex (fractions 16–20 from Fig. 1 A); (E) DNA-envelope complex after treatment with trypsin (20 μg/mg protein for 30 min at 20°C). Note here that band 1 has disappeared. (F) DNA-envelope complex after treatment with lysozyme (30 μg/mg protein for 60 min at 20°C). Band 1 is still present. Gels were run and stained as described in Materials and Methods.

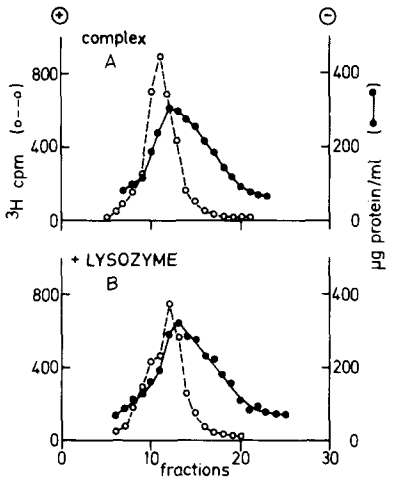


FIGURE 7 Effect of lysozyme on the electrophoretic mobility of the isolated DNA-envelope complex. A wall-enriched preparation isolated from 6 liters of [^{3}H]thymidine-labeled cells was electrophoresed as described in Fig. 1 A. The peak fractions of ^{3}H-labeled DNA-envelope fragments were pooled and the fragments concentrated by centrifugation at 183,000 *g* for 30 min. The pellets were suspended in TRA/Mg^{++} sucrose buffer at a concentration of 3 mg protein/ml. One-half of the DNA-envelope preparation was re-electrophoresed after an incubation period of 1 h at room temperature (A). To the other half, lysozyme was added (100 μg/ml) and the preparation was incubated for 1 h at 25°C before re-electrophoresis (B). Fractions were collected and aliquots were assayed for protein (●) and ^{3}H radioactivity (○).

held together at the wall/membrane adhesion zones, selective degradation of these structures might be expected to disrupt the complex. If, for example, the presence within the adhesion zone of an intact peptidoglycan layer is necessary for the integrity of the complex, then treatment of the complex with lysozyme would be expected to result in disruption of the complex. Breakdown of the complex could result in separation of the wall and membrane and/or release of the DNA from the membrane fragments. In the experiment shown in Fig. 7 isolated DNA-envelope complex fragments were treated with lysozyme and re-electrophoresed. The migration of the complex fragments was not significantly altered by the lysozyme treatment, nor was the DNA released. Free DNA migrates in the electrophoresis more strongly toward the anode than does the complex (Fig. 1 C). That the DNA is still associated with the lyso-

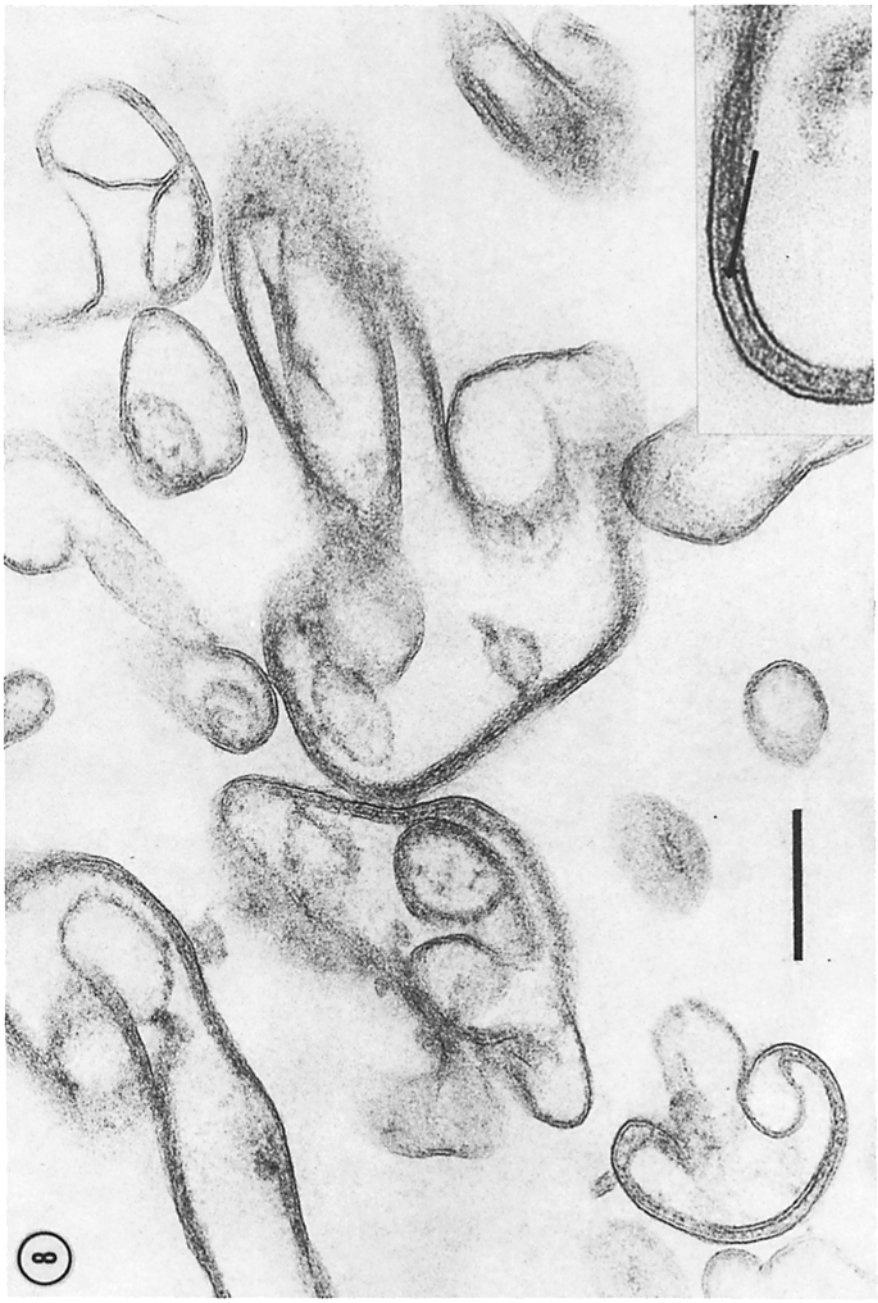


FIGURE 8 Electron micrograph of DNA-envelope complex after treatment with lysozyme. The murein layer has disappeared (arrow) but the close adhesion between wall and membrane and the basic shape of the complex fragments have been kept. Calibration = 0.2 μm; × 100,000; insert, × 200,000.

zyme-treated complex fragments is also shown by its sedimentation as a RSC in sucrose gradients (see Fig. 12). It should be noted also that after the lysozyme treatment of the DNA-envelope complex, no DNA or protein was found to have migrated into the region of the electrophoresis corresponding to membrane fragments. Electron micrographs of thin sections of the lysozyme-treated complexes (Fig. 8) show that, although the middle peptidoglycan layer of the complex has been almost completely removed in most of the fragments, the complexes have retained their basic morphology. The wall and the membrane have not separated from each other, and the typical shape has been retained.

Effect of Trypsin on the DNA-Envelope Complex

It has been suggested that trypsin cleaves specifically the lipoprotein that links the outer membrane of the cell wall (3, 18) to the peptidoglycan layer. When isolated envelopes of *S. typhimurium* or of *E. coli* are treated with trypsin, the associations between the wall and the inner membrane are also abolished (5) and the membrane no longer adheres to the wall at the wall/membrane adhesion zones. When the DNA-envelope complex is treated with trypsin, it loses most of its DNA and releases membrane fragments (Fig. 9). The [^{3}H]DNA present in fraction 18 (Fig. 9 B) was shown to be unattached to RSC by centrifugation in sucrose gradients (see Fig. 12). However, the type of membrane fragments separated by electrophoresis (Fig. 9 B, fractions 34–38) could not be identified by assaying SDH activity since it was shown in control experiments that trypsin completely inactivates this SDH in membrane preparations. Therefore, the fragments were characterized by observation of their morphology in the electron microscope. Fig. 10 *a* is an electron micrograph of the envelope fragments from the main protein peak in Fig. 9 B (i.e., fractions 24–26). In all the complex fragments, the wall/membrane adhesion zones have been destroyed. In most fragments a very diffuse membrane can be seen within the curled structure of the complex fragment but in no case is the membrane in close contact with the cell wall as is seen in the untreated complex (Fig. 2). Also, the normally heavy staining peptidoglycan layer of the wall has become diffuse after the trypsin treatment. Fig. 10 *b* is an electron micrograph of the material isolated in the right shoulder of the electrophoresis run shown in Fig. 9 B (fractions 34–38). The material is composed of double-layered membrane vesicles which were apparently released from the complex by the trypsin treatment.

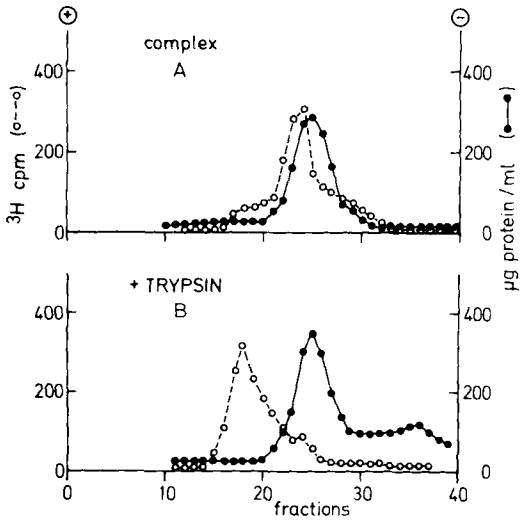


FIGURE 9 Effect of trypsin on the electrophoretic mobility of the isolated DNA-envelope complex. DNA-envelope complex was isolated as in Fig. 7 and divided into two parts. One part was re-electrophoresed without further treatment (A), while the other (B) was treated with 1 μg trypsin/50 μg of protein. After the 20-min incubation at room temperature, the preparation was cooled in an ice bath and immediately reelectrophoresed. ●, protein, O, [^{3}H]DNA.

Polyacrylamide gel electrophoresis analysis of the proteins of the DNA-envelope complex after trypsin treatment indicated clearly that the complex-specific protein in band 1 (gel D, Fig. 6) has completely disappeared (gel E, Fig. 6). The additional bands in gel E when compared with gel D are probably digestion products which stem from the trypsin treatment.

Effect of DNase on the DNA-Envelope Complex

In an earlier paper (27), it was reported that after DNase treatment of the complex, the DNA had been removed; but it could never be proven unequivocally whether the last very short pieces of DNA were completely detached from the envelope. Electron micrographs (not shown here) indicated that, even after DNase treatment, wall and membrane are still together. In electrophoresis

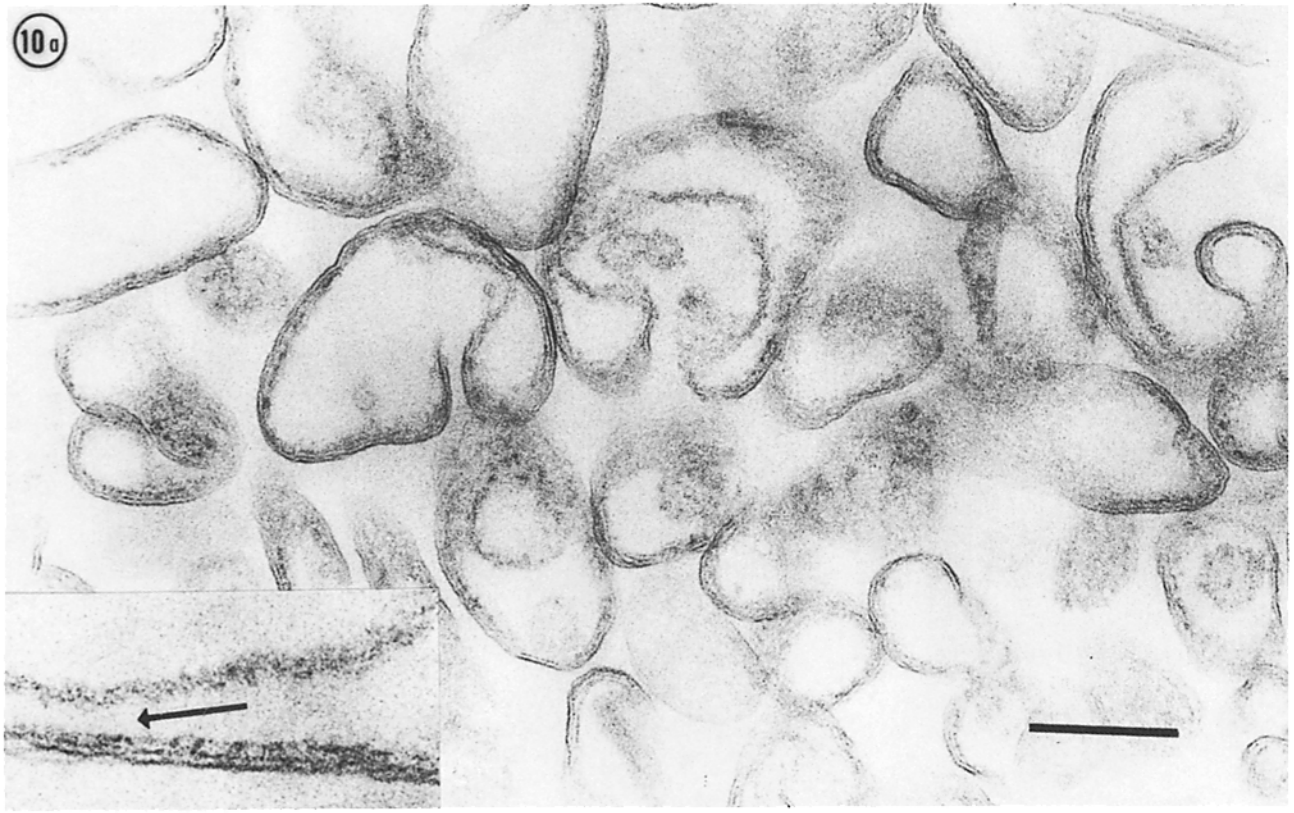


FIGURE 10 *a* Electron micrographs of DNA-envelope complex after treatment with trypsin and after FFE. Material from fractions 24–26 of Fig. 9 B. The close adhesion between wall and membrane has disappeared arrow, and the membrane is loose and very diffuse. Some complex pieces have completely lost the membrane. Calibration = 0.2 μm; × 100,000.

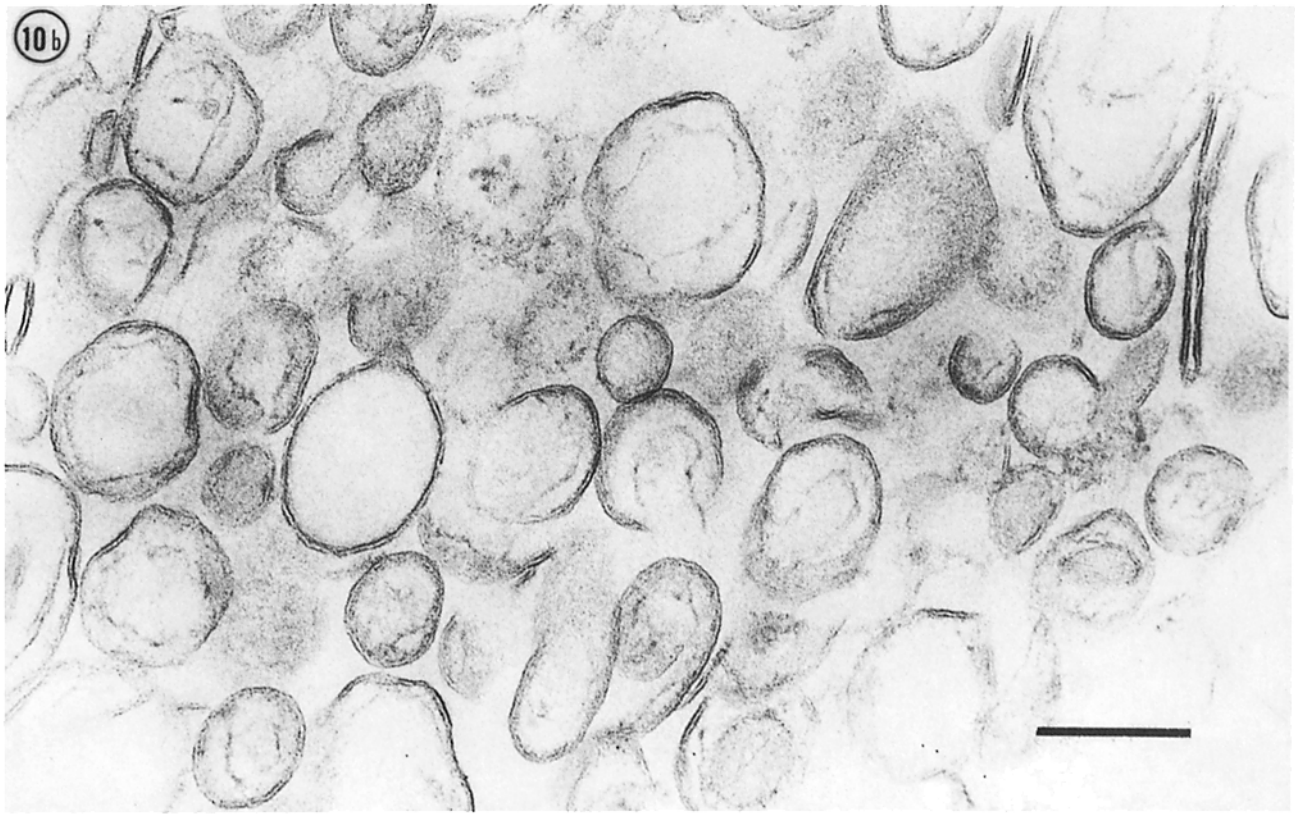


FIGURE 10 *b* Electron micrographs of DNA-envelope complex after treatment with trypsin and after FFE. Material from fractions 34–38 of Fig. 9 B. The double-layered vesicles (having the same electrophoretic mobility as membrane) are assumed to be cytoplasmic membrane which has been loosened from the complex by trypsin. Calibration = 0.2 μm; × 100,000; insert × 200,000.

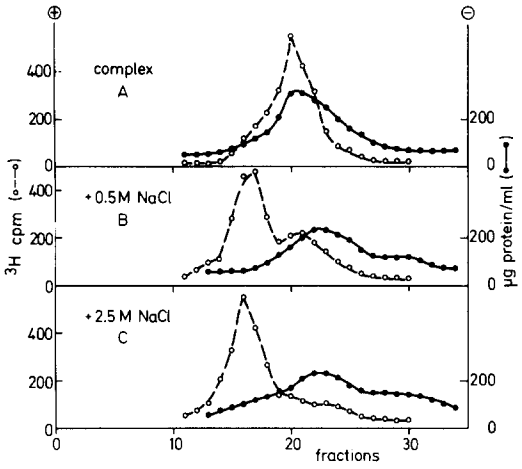


FIGURE 11 Effect of salt concentration on the electrophoretic mobility of the DNA-envelope complex. DNA-envelope complex was isolated as in Fig. 7 and divided into three parts. One part was given no further treatment (A). NaCl was added to the other two parts to final molarities of 0.5 M (B) and 2.5 M (C). After 30-min incubation on ice, all the samples were dialyzed at 4°C against several changes of TRA/Mg^{++} sucrose buffer. After dialysis, the samples were re-electrophoresed. ●, protein, O, [^{3}H]DNA.

gels, the 80,000–90,000 mol wt protein was also definitely found in the complex after DNase treatment. The results from both experiments, i.e., from electron microscopy and gel analysis of the DNase-treated complex, lead to the conclusion that DNase does not dissociate the complex.

Salt Stability

It has recently been proposed that the binding of the membrane and cell wall at the adhesion zones involves ionic interactions (6). This idea was tested by suspending the isolated DNA-envelope complex in buffer containing a high concentration of NaCl and then subjecting the samples to re-electrophoresis. Several concentrations of NaCl were tested. The results of the exposure of the complex to 0.5 M and 2.5 M NaCl and the electrophoresis runs of this material are shown in Fig. 11. Most of the DNA is separated from the complex and migrates into the position of free DNA (see Fig. 1 C). The electrophoretic migration of the fragments is also slightly altered, and some protein is found in the region corresponding to membrane fragments. Assay of the SDH activity in these fragments after exposure of the fragments to high salt showed no activities in all fractions. While the SDH protein is assumed to be firmly associated with the membrane, it may be able to be dissociated by the high ionic strength used. Therefore, to test if the loss of SDH activity was due to the exposure of the membrane to the high ionic strength buffer, purified *E. coli* inner membranes were suspended in 2.5 M NaCl for 30 min and after dialysis were assayed for SDH activity. Again, the SDH activity was

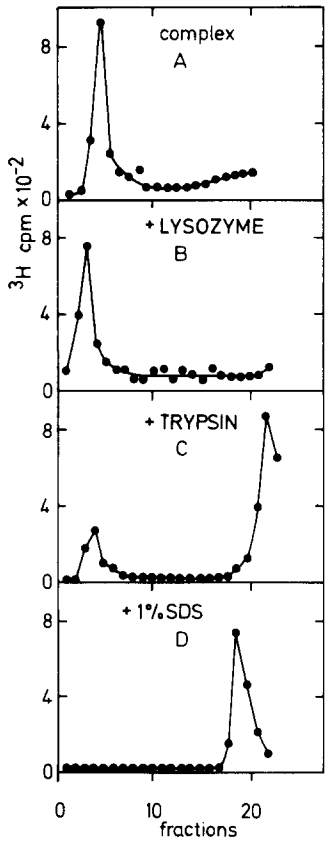


FIGURE 12 Sucrose gradient centrifugation of isolated DNA-envelope complexes. DNA-envelope complexes were isolated as described in Fig. 1. Samples of the complex preparation were exposed to various treatments and then layered on 20–35% sucrose gradients (in TRA/Mg^{++} buffer) over a 65% sucrose shelf. The gradients were centrifuged in a Beckman W SW50.1 rotor at 40,000 rpm (149,000 *g*) for 40 min at 4°C. Fractions of three drops each were collected from the bottom of the tube on filter paper disks, washed with cold 5% trichloracetic acid, and assayed for radioactivity as previously described (25). Sedimentation is from right to left. (A) No treatment. (B) DNA-envelope complex incubated with 100 µg/ml lysozyme for 1 h at room temperature. (C) DNA-envelope complex incubated with 1 µg trypsin/50 µg of protein for 20 min at RT. (D) Complex plus 1% SDS. ●, [^{3}H]radioactivity.

significantly decreased. Although the biochemical assay could not demonstrate the separation of wall and membrane (like after trypsin treatment), observation of the fragments, after exposure to the high salt, in thin sections in the EM indicate significant alteration of the structure of the complex. After the exposure to high salt, the membrane appears diffuse, is pulled into the center of the complex, and is no longer in association with the wall at the adhesion zones. This material looks like the trypsin-treated complex.

The 80–90 K protein disappeared after the breakdown of the complex with trypsin. In agreement with this result, this protein could no longer be detected by gel electrophoresis analysis after dissociation of the complex with high salt.

Stability of the DNA-Envelope Complex

The effect of several other treatments on the stability of the DNA-envelope complex was tested. An assay for the attachment of the DNA to the envelope fragments is the centrifugation of the material through sucrose gradients and the recovery of the [^{3}H]DNA as a rapidly sedimenting complex (RSC) on a shelf of high density sucrose. During the short centrifugation period used, free DNA will remain at the top of the gradient. This assay, however, can only measure cosedimentation of the DNA with large envelope structures and can not preclude the possibility of its entrapment within closed membrane vesicles. Fig. 12 is a profile of four such sucrose gradients. Treatment of the DNA-complex with 1% SDS completely releases the DNA from the complex, while trypsin digestion under the incubation conditions used freed about 70% of the DNA from the RSCs. After lysozyme treatment, the DNA still sediments through the gradient as a RSC. The data from these and other similar experiments are summarized in Table I. The complex is labile upon storage at 4°C but relatively stable when stored frozen at −20°C. The complex is unstable upon treatment with agents that disrupt ionic interactions (i.e., ionic detergents and high salt concentration), while nonionic detergents do not appreciably dissociate the complex. Triton X-100 which, in the presence of Mg^{++}, has been shown to solubilize only proteins of the inner membrane (7, 33) does not release DNA from the RSC. It appears that the 80–90 K protein is always present when the complex is intact, even after lysozyme or DNase treatment. As soon as the complex is broken down, for example by trypsin, high salt or ionic detergents, the protein cannot be found.

DISCUSSION

Previously, it has been shown that the *E. coli* chromosomal DNA is bound to the cell envelope at the wall/membrane adhesion zones (27). From the electron micrographs of several of the DNA-envelope complex fragments in Fig. 3, it can be seen that most of the DNA molecules appear to be attached to a single region within the envelope fragments and that often several strands appear to

TABLE I

Stability of the DNA-Envelope Complex

Treatment	DNA recovered as RSC
	%
None	100
1% SDS	0
1% Brij 58	90
1% sodium desoxycholate	50
1% Triton X100	100
1% sarkosyl*	20
NaCl 0.05 M	100
0.1	78
0.5	38
1.0	28
1.5	29
3.0	30
Pronase‡	0
Lysozyme§	100
Trypsin‖	30
DNase¶	0
Storage at 4°C	5–45
−20°C	95

Samples of the DNA-envelope complex were exposed to the indicated treatments and analyzed by sucrose gradient centrifugation as described in Fig. 12. The results are expressed as the percent of the total [^{3}H]DNA added to the gradient which is recovered as a RSC at the 65% sucrose shelf after centrifugation.

* The sample to be treated with sarkosyl was first dialysed against TRA/sucrose buffer to remove the Mg^{++} and then centrifuged in gradients prepared in buffer without Mg^{++}.

‡ Pronase treatment was carried out with 20 μg enzyme/ml for 30 min at room temperature.

§ Lysozyme 100 μg/ml for 30 min at room temperature.

‖ Trypsin digestion was carried out with 20 μg/ml for 20 min at room temperature.

¶ DNase 10 μg/ml for 30 min at room temperature.

originate from the same site. That there would be more than one DNA strand attached to the same binding locations is consistent with the recently proposed topology for a bi-directionally replicating circular chromosome containing several replication forks (9). It is not yet known which regions of the chromosome are represented by the DNA bound to the envelope complex. In some of the DNA-envelope complexes, however, the DNA (see Fig. 3) resembles that proposed by Dingman (9) for the membrane attachment of the origin and replication point of the bacterial chromosome.

Several experiments were performed to determine how the membranes are held together at the adhesion zones and if the integrity of the complex is necessary for the binding of DNA. It was shown that ionic interactions are involved in holding the complex together (Fig. 11 and Table I). At high salt concentration, the entire complex is unstable; both the DNA and the membrane are released from the adhesion zone. Although the membrane vesicles which then escape from the complex (to migrate as free membrane in the FFE) cannot be identified as inner membrane vesicles by SDH activity, it can be seen in the electron microscope that the adhesion zones are disrupted and that the vesicles resemble double-layered inner membrane vesicles. The observation that trypsin treatment of the complex releases membranes and all the DNA from the complex (Figs. 9 and 10) suggests that protein is also involved in the integrity of the complex and the binding of DNA. But this does not necessarily imply that covalent linkages are involved. A similar conclusion also was made by Fuchs and Hanawalt (13). Cleavage of a protein by trypsin could alter the distribution of ionic charges which could then lead to complex disruption. It has been shown that by a similarly limited trypsin treatment the lipoprotein present in the cell wall is preferentially cleaved (3, 18). It has been suggested that this molecule is involved in the attachment of the peptidoglycan to the outer membrane (4, 3, 24). However, more recently Inouye (20) has proposed a role for the lipoprotein molecules in the formation of passive diffusion pores. Braun et al. (5) have also observed that when isolated cell envelopes of *S. typhimurium* or of *E. coli* are treated with a limited concentration of trypsin, the contact associations between the wall and the membrane are abolished. A direct role of the lipoprotein in the maintenance of these associations was not implied. Instead, it was suggested that any structural transition in the wall caused by the trypsin cleavage of the lipoprotein could change the interaction of the two membranes. This interaction does not, however, require the presence of an intact peptidoglycan layer. After lysozyme treatment which cleaves the peptidoglycan and destroys the rigidity of the cell wall (8), neither the DNA nor the adhesion zones are released (Figs. 7 and 8). That lysozyme would not release DNA from the complex was expected since RSCs composed of DNA and cell membrane structures have often been isolated from *E. coli* cells lysed with lysozyme-EDTA. However, the digestion of the peptidoglycan may have been expected to dissociate the wall from the membrane within the complex. This was not observed. Although after the lysozyme treatment the peptidoglycan layer of the complex could no longer be seen by electron microscopy, the membrane and the DNA remained firmly attached to the wall. It appears that both the binding of DNA and the adhesion of the wall and membrane within the complex involve noncovalent interactions. Costerton et al. (6) have suggested that the wall/membrane adhesion involves an ionic attraction of the membrane to the peptidoglycan. But the results of the lysozyme treatment of the DNA-envelope complex indicate that the presence of an intact peptidoglycan is not necessary for the maintenance of the adhesion zone.

The existence of a defined zone within the cell envelope at which these ionic membrane interactions occur would suggest the presence within the membrane of a unique structural organization in either the lipid or the protein components. Gel electrophoresis analysis of the DNA-envelope complex revealed the presence of a high molecular weight complex-specific protein (band 1 of gel D, Fig. 6). Several previous reports have correlated the presence of different envelope proteins with DNA replication or with cell division (21, 22, 26, 34, 41). These proteins are smaller than the protein of band 1 which does not appear to be an aggregate since solubilization at 100°C did not affect its electrophoretic mobility. There is no indication yet of the physiological in vivo role of this complex-specific protein. The only evidence for its involvement in either the adhesion of wall and membrane or (and?) the binding of the DNA to the complex is the complete disappearance of this protein after trypsin treatment of the DNA-envelope complex. Further characterization of this protein is neces-

sary to elucidate its function. The presence of an ionic linkage between the wall and the membrane possibly involving a specific protein molecule is consistent with existing models for the structure of the *E. coli* cell envelope (6, 32, 14, 31). However, sufficient data are not yet available to include in these models a mechanism for the binding of DNA to the cell envelope. After this paper was finished, Sueoka and Hammers (38) published data on the isolation of DNA-membrane complexes of *B. subtilis* which also possess unique proteins.

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