RESPIRATION AND PROTEIN SYNTHESIS IN

ESCHERICHIA COLI MEMBRANE-ENVELOPE FRAGMENTS

IV. Chemical and Cytological Characterization

and Biosynthetic Capabilities of Fragments

Obtained by Mild Procedures

R. SCHARFF, R. W. HENDLER, N. NANNINGA, and
A. H. BURGESS

From the Section on Cellular Physiology, Laboratory of Biochemistry,
National Heart and Lung Institute, National Institutes of Health,
Bethesda, Maryland 20014, and the Laboratory for Electron
Microscopy, University of Amsterdam, Netherlands

ABSTRACT

Membrane-envelope fragments have been isolated from *Escherichia coli* by comparatively mild techniques. The use of DNAase, RNAase, detergents, sonication, lysozyme, and ethylenediaminetetraacetate were avoided in the belief that rather delicate, but metabolically important, associations may exist between the plasma membrane and various cytoplasmic components. The membrane-envelope fragments have been characterized in terms of their content of major chemical components as well as their electron microscope appearance. Fractions containing membrane-envelope fragments were found to possess appreciable DNA- and protein-synthesizing activities. The fragments were rich in membrane content as determined by reduced nicotinamide adenine dinucleotide (NADH) oxidase activity and deficient in soluble components as measured by NADH dehydrogenase activity. The particulate fraction obtained between 20,000 g and 105,000 g and usually considered a ribosomal fraction was rich in membrane content and had a relatively high capacity for DNA synthesis. Envelope fragments sedimenting at 20,000 g attained very high levels of incorporation of amino acids into protein.

INTRODUCTION

There is a growing appreciation of the importance of structural organization for complex metabolic activities carried out by living cells, such as respiration and the biosynthesis of macromolecules (1). The bacterial cell membrane is believed to hold the growing point for its chromosome (2). It has been proposed that RNA may be transcribed directly from the nascent DNA and that ribosomes may simultaneously proceed to translate the nascent RNA (3). Recent findings indicating that DNA polymerase may not be the major DNA-synthesizing enzyme in vivo, have redirected attention back to the bacterial cell, particularly to its membranes, to look for DNA-synthesizing activity (4, 5). Tracer studies with intact *Escherichia coli* indicate that its membranes contain sites of active RNA and protein synthesis (1).

Several membrane-envelope preparations from

E. coli have been obtained and characterized during the past five years (6–11). These preparations, however, have been subjected to various severe treatments in the belief that anything that can be removed from the membranes represents an undesirable contaminant. Therefore lysozyme, DNAase, RNAase, ethylenediaminetetraacetate (EDTA), detergents, and sonication together or in various combinations have been used to produce "clean" membrane or wall preparations.

Because rather delicate but metabolically important associations may exist in the cell between the plasma membrane and various cytoplasmic components such as DNA, RNA, ribosomes, nucleic acid synthetases, etc., we have attempted to isolate membrane-envelope fragments, using comparatively mild techniques, and to characterize them fully.

The envelope of *E. coli* consists of several contiguous layers (1, 12). The outermost layer (visible by electron microscopy) is a complex of lipoprotein and lipopolysaccharide and the innermost layer is the plasma membrane. Between these two is found a rigid layer called either mucopeptide, peptidoglycan, or murein. Lipoprotein is believed to be bound to the mucopeptide. We have analyzed acellular fractions obtained from *E. coli* for the presence of components from all three layers, utilizing both chemical and cytological techniques. In addition, we have investigated the biosynthetic capabilities of these fractions.

MATERIALS AND METHODS

Preparation of Fractions from *E. coli* Spheroplasts

Spheroplasts were produced by a penicillin procedure from *E. coli* W-6, a proline auxotroph, (ATCC No. 25377), grown on Difco Antibiotic medium No. 3 (Difco Laboratories, Detroit, Mich.) also called Penassay Broth (PAB), supplemented with proline (13). In brief, the procedure consists of placing rapidly growing cells (early log phase) into PAB containing 300 units penicillin G/ml, 0.1% $MgSO_4$, 15% (w/v) sucrose, and 250 μg L-proline/ml. After 3 hr at 37°C the cells are harvested in a Sharples continuous centrifuge (Sharples Centrifuges, Pennwalt Corporation, Warminster, Pa.) and washed once by gentle resuspension in storage medium ([13] see Table I). The washed spheroplasts are harvested by centrifugation for 15 min at 21,000 g in a Sorvall RC2B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The pellet is resuspended in a small volume of storage medium (10–15 mg of protein per ml) and stored in liquid nitrogen.

TABLE I

Preparation of Cell Fractions from Spheroplasts

Starting material	Diluting medium*	Centrifugation conditions		Fractions obtained	
		Time	Force	Pellet	Supernatant
		min	*g*		
A. Thawed sphero-plasts	Suspending medium	10	3500 Sorvall	P_1	S_1
B. S_1	None	15	20,000 Sorvall	P_2	S_2
C. S_2	None	60	105,000 (av) Spinco 40 rotor‡	P_3	S_3
D. Thawed sphero-plasts	suspending medium	15	20,000 Sorvall	P_e	S_e
E. P_e on 55/70% (w/v) sucrose-TKM	None	30	130,000 (av) Spinco SW39 or SW50.1	Gradient pellet	(1) Top (2) Interface (3) Bottom

Dilutions were always made with 1 vol of starting material plus 9 vol of diluent.

* Suspending medium is 1:1 storage: C Salts medium with a final Mg^{++} concentration of 10 mM. Storage medium is 0.05 Tris, pH 7.4, 1 mM $MgCl_2$, and 9% (w/v) glycerol. C Salts medium is 6 g Na_2HPO_4, 3 g KH_2PO_4, 3 g NaCl, 110.9 mg Na_2SO_4, and 3.86 g $MgCl_2$ per liter. TKM is 10 mM Tris, pH 7.4, 10 mM $MgCl_2$, 80 mM KCl.

‡ Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.

For the preparation of most fractions, the sphero-plasts frozen in storage medium were thawed in tepid water and diluted 1:10 as indicated in Table I. All pellets were resuspended to the concentration of the undiluted spheroplasts by use of loose-fitting glass or Teflon pestles in plastic centrifuge tubes and were stored in liquid nitrogen. Fractionation on the 55/70% (w/v) sucrose step gradients (line E, Table I) was accomplished by adding 0.5 ml of freshly made P_c (line D, Table I) in suspending medium to a tube containing 2.2 ml of cold (4°C) 55% (w/v) sucrose-TKM layered over 2.2 ml of cold 70% (w/v) sucrose-TKM. After 30 min centrifugation at 130,000 g in a swinging bucket rotor a layer of white to tan material was present at the interface of 55 and 70% sucrose and a pellet at the bottom of the tube. Starting at the top of the gradient, fractions were removed under reduced pressure through fine Teflon tubing and collected in graduated tubes. The first 2.2 ml removed, the top fraction, contains the original 0.5 ml of suspending medium from P_c as well as 1.7 ml of 55% sucrose-TKM. The interface fraction, the next 1.0 ml, consists of equal parts of 55 and 70% sucrose. The remaining 70% sucrose (1.7 ml) is the bottom fraction. The pellet was resuspended in 0.5–1 ml of storage or suspending medium.

These conditions were chosen after preliminary experiments with sucrose gradients of various concentration had shown that intact cells (examined under phase contrast) would penetrate through 70% sucrose (w/v) while, with a P_c fraction, 65–75% of the reduced nicotinamide adenine dinucleotide (NADH) oxidase, a cell envelope–associated activity (13), would penetrate 55 but not 70% sucrose. All fractions were stored in liquid nitrogen.

Visible and Viable Cell Counts

The number of visible cells was counted using a Petroff-Hausser bacterial-counting chamber in a phase-contrast microscope.

Viable cells were counted by plating appropriate dilutions on the 1% agar medium, described by Tonomura and Rabinowitz (14), supplemented with 500 μg L-proline per ml. Serial dilutions were made at 0°C in medium similar to that in which the cells were already suspended, except that proline, which is required for growth, was omitted. Mixing was accomplished with a Vortex Jr. Mixer (Scientific Industries, Inc., Springfield, Mass.). Portions were spread over the agar surface with a bent glass rod. Colonies were counted after incubation for 24 hr at 37°C.

Electron Microscopy

Electron microscopy has been carried out on the following fractions which are specified in Table I:

P_1, P_2, P_3, P_c, Interface, and Gradient Pellet. In addition, an electron micrograph of a whole spheroplast preparation is shown. All these preparations were stored in liquid nitrogen.

Apart from slightly variable pretreatments as specified below, all fractions were fixed and embedded according to the Ryter-Kellenberger procedure (15), which includes resuspension in 1 or 2% (w/v) agar, fixation in osmium tetroxide plus tryptone, posttreatment in uranyl acetate, dehydration in a graded series of acetone, and embedding in Vestopal W (Madame Martin Jaeger, Geneva, Switzerland). The Ryter-Kellenberger acetate-Veronal buffer contained 0.01 M $MgCl_2$.

Pretreatments of the fractions, which were all carried out at about 4°C, were as follows:

Spheroplasts: Prefixation for 5 hr was carried out by adding 25% (v/v) glutaraldehyde to spheroplasts in suspending medium (see Table I) to give a final concentration of 2.5% (v/v). Then a pellet was made by centrifugation for 15 min at 10,000 g in a MSE 65 ultracentrifuge (MSE, Inc., Cleveland, Ohio).

P_1 and P_2 : Resedimented at 10 min at 3,500 g and 15 min at 20,000 g, respectively, in a MSE 50 ultracentrifuge. The pellets were fixed for 3 days with 1% (v/v) glutaraldehyde. The latter was obtained by diluting a 25% (v/v) glutaraldehyde solution with S_3 (see Table I). After fixation the pellets were washed three times with S_3.

P_3 : No pretreatment.

P_c : The thawed preparation was sedimented for 30 min at 90,000 g in a MSE 65 ultracentrifuge. The pellet was fixed in 2.5% (v/v) glutaraldehyde in suspending medium (see Table I) for 4 hr.

Interface: The suspension in sucrose-TKM (see Table I) was diluted 10 times with TKM and sedimented for 3 hr at 120,000 g in a MSE 65 ultracentrifuge. The pellet was fixed for 1 hr in 2.5% (v/v) glutaraldehyde in TKM.

Gradient Pellet: Pellets were fixed for about 5 hr in 2.5% (v/v) glutaraldehyde in TKM.

Sections were cut with glass knives on an LKB Ultrotome III (LKB Instruments, Inc., Rockville, Md.). Some sections were poststained with lead citrate (16).

Electron micrographs were taken with either a Philips EM 200 or EM 300 electron microscope operating at 60 or 80 kv.

Estimation of Lipids

Cell fractions containing, in most cases, between 1 and 5 mg protein were precipitated at 0–5°C with 10% TCA. The precipitates were washed three times in 5% TCA. Lipids were extracted overnight at room temperature with 2:1 chloroform:methanol and washed by the method of Folch et al. (17), using NaCl in the wash solutions. Phases were separated by

centrifugation and interfacial precipitates, when encountered, were discarded. The washed lipid extracts were taken to dryness at 40°C under a N_2 jet and were then redissolved in chloroform.

Lipid esters were measured by the hydroxylamine method of Rapport and Alonzo with triolein as a standard (18). To convert micromoles of ester to micrograms of lipid an average lipid molecular weight of 790 per two ester bonds was assumed.

Total phosphate was measured on the lipid extracts by the procedure described by Ames and Dubin (19). A factor of 25.5 was used to convert micrograms of phosphorus to micrograms of lipid.

Estimation of DNA and RNA

Nucleic acids were precipitated from 8 ml of diluted cell fractions at 0°–4°C by the addition of 2 ml of 1 N perchloric acid (PCA) to give a final concentration of 0.2 N PCA. After 30 min the precipitates were sedimented by centrifugation and washed twice in 5 ml of 0.2 N PCA. Hydrolysis of the nucleic acids was accomplished by heating the precipitates for 15 min at 90°C in 0.5 N PCA. The precipitates were sedimented and then reextracted for 15 min at 90°C with a second portion of 0.5 N PCA. The combined supernatant fractions from the two extractions were assayed for DNA by the method of Burton (20) using 2-deoxyadenosine (Calbiochem, Los Angeles, Calif.) as a standard. 1 μmole of sugar was equated to two nucleotide residues since only purine nucleoside residues react (20) and the purine:pyrimidine ratio of the DNA is essentially 1:1 (21). An average nucleotide residue weight of 309 was calculated assuming equal molar quantities of the four bases (22). DNA was also measured after periodate oxidation and reaction with thiobarbituric acid as described below (see section on lipopolysaccharides).

RNA was measured in the nucleic acid extracts as described previously (23), except that the concentration of ferric ammonium sulfate in the original reagent was reduced to 0.1% in order to improve the sensitivity. Adenosine was used as a standard. Micrograms of adenosine was converted to micrograms of RNA by the factor 2.14. This factor was calculated on the basis that only ribose from purine residues can react, E. coli RNA contains 56.7% of the total bases as purines (24), and the average nucleotide residue in RNA has a weight of 324. Deoxyadenosine was found to give less than 5% of the color obtained from adenosine. Only a small correction was, therefore, needed for those fractions containing the highest ratio of DNA to RNA in the extracts.

Estimation of 3-Deoxy-D-Manno-Octulosonic Acid (KDO)

The lipopolysaccharide layer of E. coli contains a unique and characteristic sugar, KDO (12, 25). We have assayed for this sugar in all fractions by using a

modification of the periodate, thiobarbituric acid method (26, 27) for deoxy-sugars. The modification involves performing the periodate oxidation at both 23° and 55°C. By determining the optical absorption at both 532 nm and at 549 nm it is possible to assay simultaneously for the content of both KDO and DNA in each fraction without the necessity of first isolating the lipopolysaccharide components.[1] In some strains of E. coli (e.g., 111 and 55 [27]) the deoxy-sugar, 3-6-dideoxy-L-xylo-hexose is also present and for these strains, the lipopolysaccharide components would have to be separated from DNA before using the assay. In E. coli W-6, however, 3-6-dideoxy-L-xylo-hexose is not present[1] and no prior fractionation is required.

Estimation of Meso-2,6-Diaminopimelic Acid (DAP)

The peptidoglycan layer of E. coli consists of polysaccharide strands cross-linked by short peptide chains containing the unique basic amino acid, DAP (12). In order to assay for the presence of minute amounts of this amino acid in the presence of a great excess of amino acid coming from normal protein in fractions containing little of the peptidoglycan layer, we have used a modification of the methods of Work and Denman (28) and Gilvarg (29). The modification involves a preliminary fractionation of an acid hydrolysate on a short column of Dowex 50 (Dow Chemical Co., Midland, Mich.) in order to separate the basic amino acids from the neutral and acidic amino acids. The basic amino acids are treated with acidic ninhydrin and assayed for optical density at 410, 450, and 500 nm. A determination of the optical density of the acid hydrolysate at 275 nm is also performed in order to correct for the content of tyrosine. By solving a series of simultaneous equations using these parameters, the content of DAP is determined.[1]

Assay of d-TTP-Methyl-³H Incorporation into DNA

The incorporation of radioactive d-TTP into DNA by E. coli cell fractions at 25°C was measured in the presence and absence of denatured calf thymus DNA. With a fresh spheroplast preparation in the presence of added DNA it was found that much more rapid loss of incorporated radioactivity occurred during incubation at 37°C, than at 25°C, i.e., nuclease activity was apparently stimulated much more by the higher temperature than was synthetic activity. For this reason incubation at 25°C was chosen. The incubation conditions were approximately those described as optimal for E. coli DNA polymerase by Klenow and Henningsen (30). The final incubation mixture

[1] Hendler, R. W., and A. H. Burgess. Anal. Biochem. In press.

contained per ml: 0.015 μmoles each of d-ATP, d-GTP, d-CTP, and d-TTP; 2.5 or 5.0 μCi of d-TTP-methyl-H^3 was used (New England Nuclear Corp., Boston, Mass. or Schwarz Bio Research Inc., Orangeburg, N. Y.) to give final specific activities of 139–306 μCi/μmole; 60 μmoles of Tris, pH 7.9; 150 μmoles of KCl; 10 μmoles of $MgCl_2$; 1 μmole of 2-mercaptoethanol; 7.2 mg of glycerol; and when present, 200 μg of heat-denatured calf thymus DNA prepared as described by Momparler (31). The amount of E. coli protein used was 70–800 μg depending on the cell fraction. Because filtration through membrane filters gave variable results an alternate procedure based on the methods described by Mans and Novelli (32) and Bollum (33) was used. 0.1 ml portions were removed at various times from the incubation tubes and placed on 23 mm Whatman No. 3 MM filter paper discs pierced with a stainless steel pin. The discs were dried briefly in a stream of warm air and dropped into a beaker of ice-cold 10% trichloroacetic acid (TCA) (about 10 ml per disc). The beaker was swirled frequently and after about 0.5 hr the 10% TCA was decanted and replaced with cold 5% TCA. The discs were then washed in cold 5% TCA, twice for 15 min each time, followed by three washes for 10 min each time. The residual TCA and water were removed by a brief washing in 1:1 ether to ethanol followed by ether alone. The discs were then dried by a heat lamp on aluminum foil.

The discs were placed in counting vials and the nucleic acids solubilized by heating at 90°C for 30 min in 0.5 ml of 0.3 N perchloric acid. To reduce the quenching caused by the PCA, 0.1 ml of 1.9 M $KHCO_3$ was added to each vial to neutralize the acid. The radioactivity was then measured using 15 ml of Bray's scintillation solution (34) (with the secondary fluor omitted) and a Beckman LS-250 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The counting efficiency was 13%.[2] Incorporation was linear from about 15 min of incubation to 30 or 60 min depending on the fraction. The levels of radioactive incorporation during the first 15 min were generally quite low and subject to variation.

Incorporation rates were calculated from the slopes of lines drawn through the linear portion of the incorporation curves.

Measurement of Incorporation of Amino Acid into Protein

The incorporation of ^{14}C-labeled amino acid into protein was measured at room temperature (23°C) in

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[2] A counting efficiency of 32% can be obtained by adding 0.5 ml of NCS solubilizer (Nuclear-Chicago Corp., Des Plaines, Ill.) to the dry filter paper disc followed by 15 ml of 0.4% 2,5-diphenyloxazole (Calbiochem, Los Angeles, Calif.) in toluene.

a N_2 atmosphere. The reason for employing anaerobic conditions was so that we could study protein synthesis under conditions where it is not dependent on respiration. Tubes containing appropriate additions were flushed with N_2 and stoppered. To start incubations 0.3–1.0 ml of the cell fraction diluted in suspending medium (see Table I) was added and the tube flushed again with N_2. Portions were taken at various times and the amount of radioactivity incorporated into protein determined either by the method of Mans and Novelli (32) or by precipitation with 10% TCA, washing the precipitate with 5°C TCA, heating the precipitate for 15 min at 90°C, and dissolving the hot TCA insoluble material in NCS solubilizer (Nuclear-Chicago, Des Plaines, Ill.). Samples were counted in 15 ml of 0.4% 2,5-diphenyloxazole in toluene in a liquid scintillation counter. The final salt concentrations during incubation were those of the suspending medium with 10 mM Mg^{++}. ATP (1 mM) and an ATP-generating system (10 mM phosphoenolpyruvate and pyruvate kinase) were usually present. The ^{14}C-labeled amino acid mixture consisted of casein hydrolysate with added tryptophan supplemented with either ^{14}C-labeled algal amino acid mixture (New England Nuclear Corp., Boston, Mass.) to give 0.5 μCi/μg or ^{14}C-labeled leucine, isoleucine (35) to give 0.4 μCi/μg.

RESULTS

Electron Microscope Observations

The starting material for preparing the various fractions was a washed (broken) spheroplast preparation as indicated in Table I. The fractions P_1, P_2, and P_3 were obtained by differential centrifugation whereas Interface and Gradient Pellet were obtained from a combined P_1 and P_2 fraction (P_c) by sedimentation through sucrose. Besides the morphological characterization of the fractions some electron micrographs are shown (Figs. 9–11) which may help explain how the various structural components are formed.

Fractions Obtained by Differential Centrifugation

The initial spheroplast preparation is shown in Fig. 1. It contained, apart from an occasional intact rod, predominantly spheroplast-like elements. Several types of structures were observed. (a) Spheroplasts with densely packed cell content and a more or less loosened cell wall; (b) spheroplasts with far less protoplasmic material; and (c) cell envelope (cell wall and plasma membrane) fragments. This preparation became acellular upon further handling.

Fraction P_1 (Fig. 2) consisted mainly of coiled envelope fragments and cytoplasmic remants. In addition some sphroplast- and rodlike elements were occasionally present.

In fraction P_2 (Fig. 3), however, there appears to have been a strong selection towards envelope fragments. Some of these fragments which typify fraction P_2 are shown at higher resolution in Fig. 4
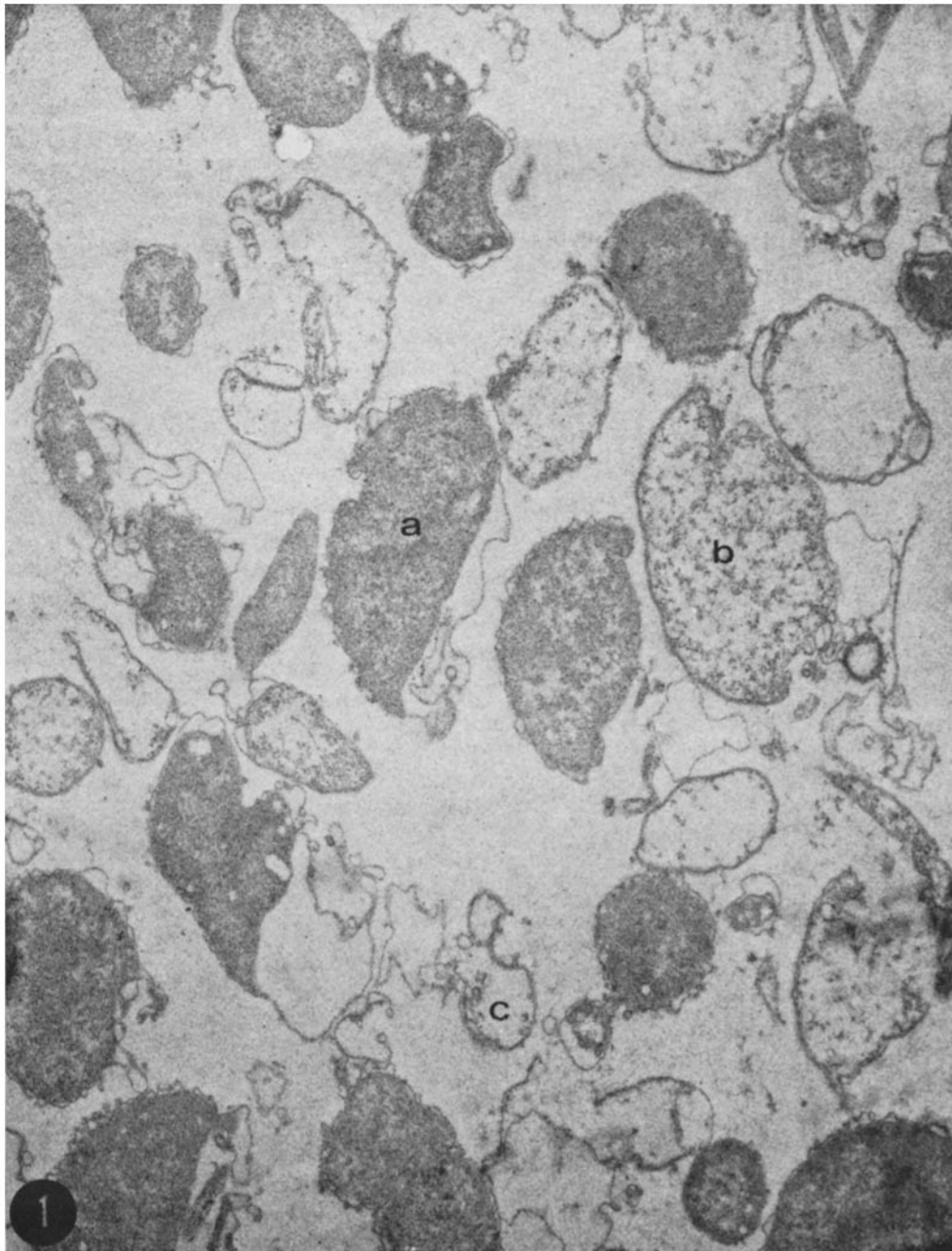

FIGURE 1   Initial spheroplast preparation. Note spheroplasts with densely packed (a) and loosely packed (b) cell content. At (c) an empty ghost is to be seen. The cell wall is partly separated from the plasma membrane. $\times$ 17,250.

(the fragments in P_1 are basically the same). Since the ruptured cell wall (predominantly lipopolysaccharide) tends to coil upon separation from the underlying plasma membrane (8, 9, 36–39, and Figs. 9 and 10) we consider the coiled fragments with the prominent triple-layered profiles to be the remainder of the cell wall after penicillin treatment. The plasma membrane (*pm*) generally ap-
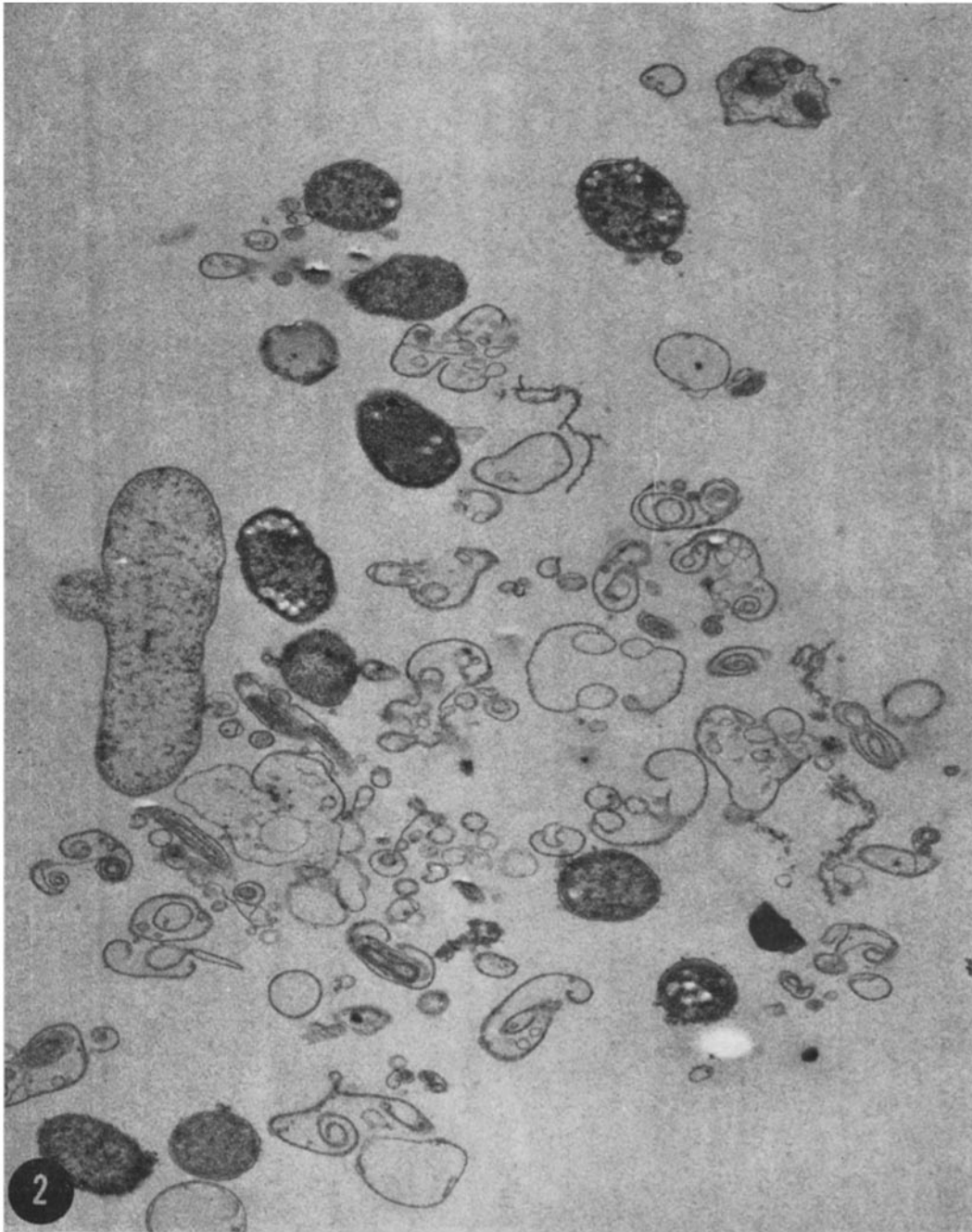

FIGURE 2   Fraction P_1. Obtained from broken spheroplasts by centrifugation for 10 min at 3500 g. This fraction contains broken spheroplast envelopes with cytoplasmic material occasionally attached. In addition, some rodlike elements are present. × 17,250.
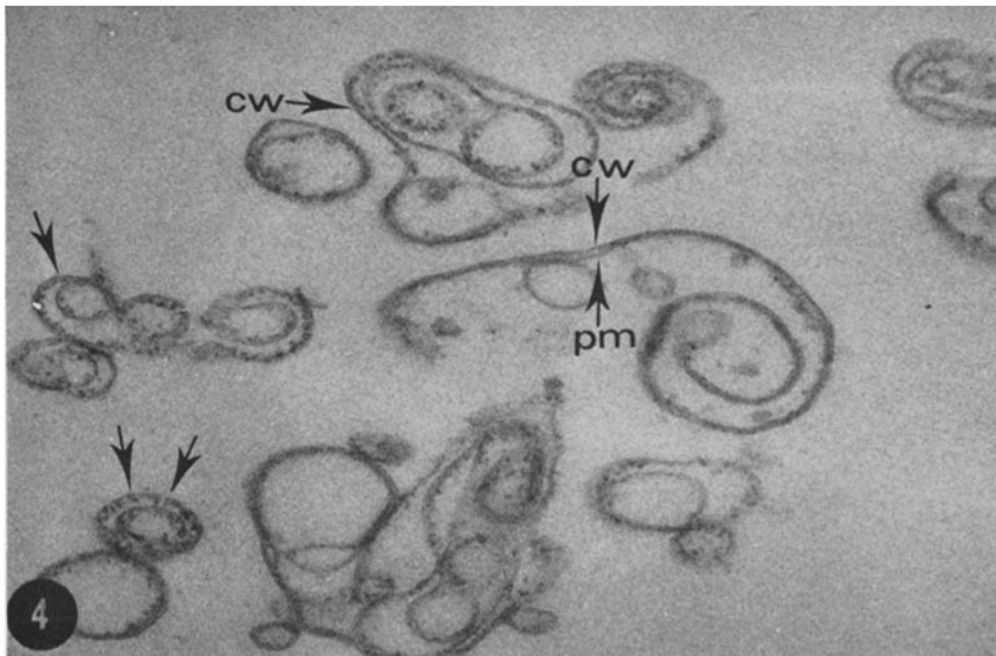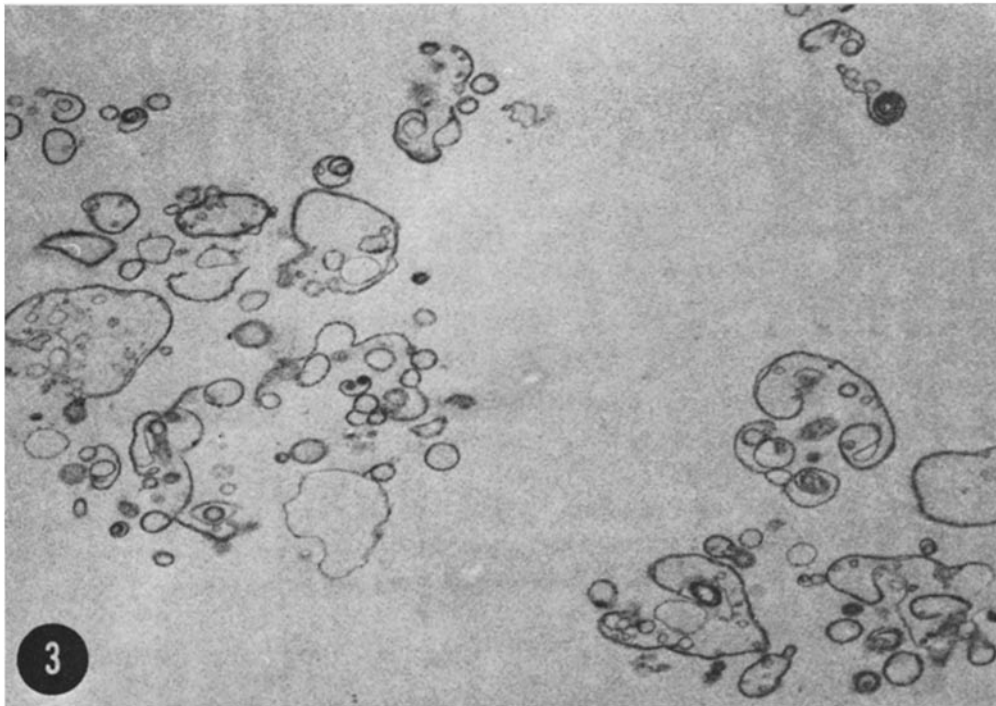
FIGURE 3 Fraction P_2. Obtained from the supernatant fraction of P_1 by centrifugation for 15 min at 20,000 g. When compared with P_1, the selection has been to envelope fragments. × 17,250.

FIGURE 4 Fraction P_2 at higher magnification. Note coiling of fragments. The diffuse plasma membrane (pm) may be seen closely apposed to the penicillin-affected cell wall (cw). Ribosome-like material (arrows) appears to be attached to the cell wall (cf. text and Figs. 9–12). × 58,000.

pears as a slightly diffuse layer (Figs. 4, 10, and 11) against the presumed cell wall. A striking difference with respect to the coiled cell wall structures isolated from lysozyme-EDTA spheroplasts (8, 9) is the presence of small black dots on the coiled profiles (arrows in Fig. 4). These dots are indistinguishable from ribosomes as far as size and staining properties are concerned. However, in many cases they seem to be attached to the cell wall (cf. Fig. 10), which would suggest that they became secondarily associated with this structure. On the other hand, the black dots might represent cell wall material not removed by the gentle penicillin method as contrasted to the lysozyme-EDTA method for preparing spheroplasts. They might for instance arise as a result of a local aggregation of the "closely packed elements" observed in the freeze-fractured *E. coli* cell envelope (40). At present, therefore, a definite conclusion with respect to the nature of the black dots on the cell wall is difficult to draw. Accumulations of dense particles are generally observed in the near vicinity of the plasma membrane of partially disrupted cells and envelope fragments (Figs. 1, 6, 9–11).

Fraction P₃ (Fig. 5) contains ribosome-like ma-

terial and very small envelope fragments. The latter appear mainly as vesicles.

Fractions Obtained by Centrifugation through Sucrose

The starting material for these preparations (P$_c$, Table I) is shown in Fig. 6. It contains predominantly envelope fragments with attached ribosome-like material and ruptured spheroplasts.

The Interface fraction (Fig. 7) reveals a strong selection towards empty envelope fragments, whereas in the Gradient Pellet fraction (Fig. 8) the selection has been towards spheroplasts in various stages of disruption and towards huge ribosomal complexes.

Spheroplast Fragmentation

The Figs. 9–11 represent an attempt to illustrate the way in which the various fragments described above may have originated. In Fig. 9, a spheroplast is shown with intact plasma membrane (*pm*) and disrupted cell wall (*cw*). This clearly shows how the broken cell wall remnant assumes a coiled configuration. Birdsell and Cota-Robles
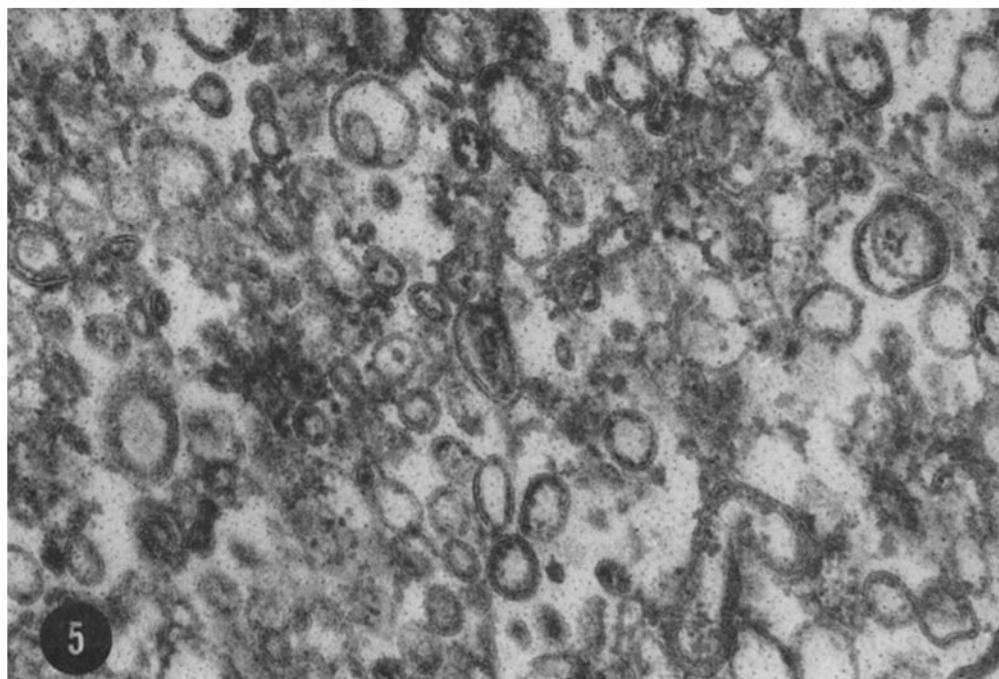
Figure 5 Fraction P₃. Obtained from the supernatant fraction of P₂ by centrifugation for 60 min at 105,000 g. This fraction contains very small envelope fragments and numerous ribosomes. × 82,500.
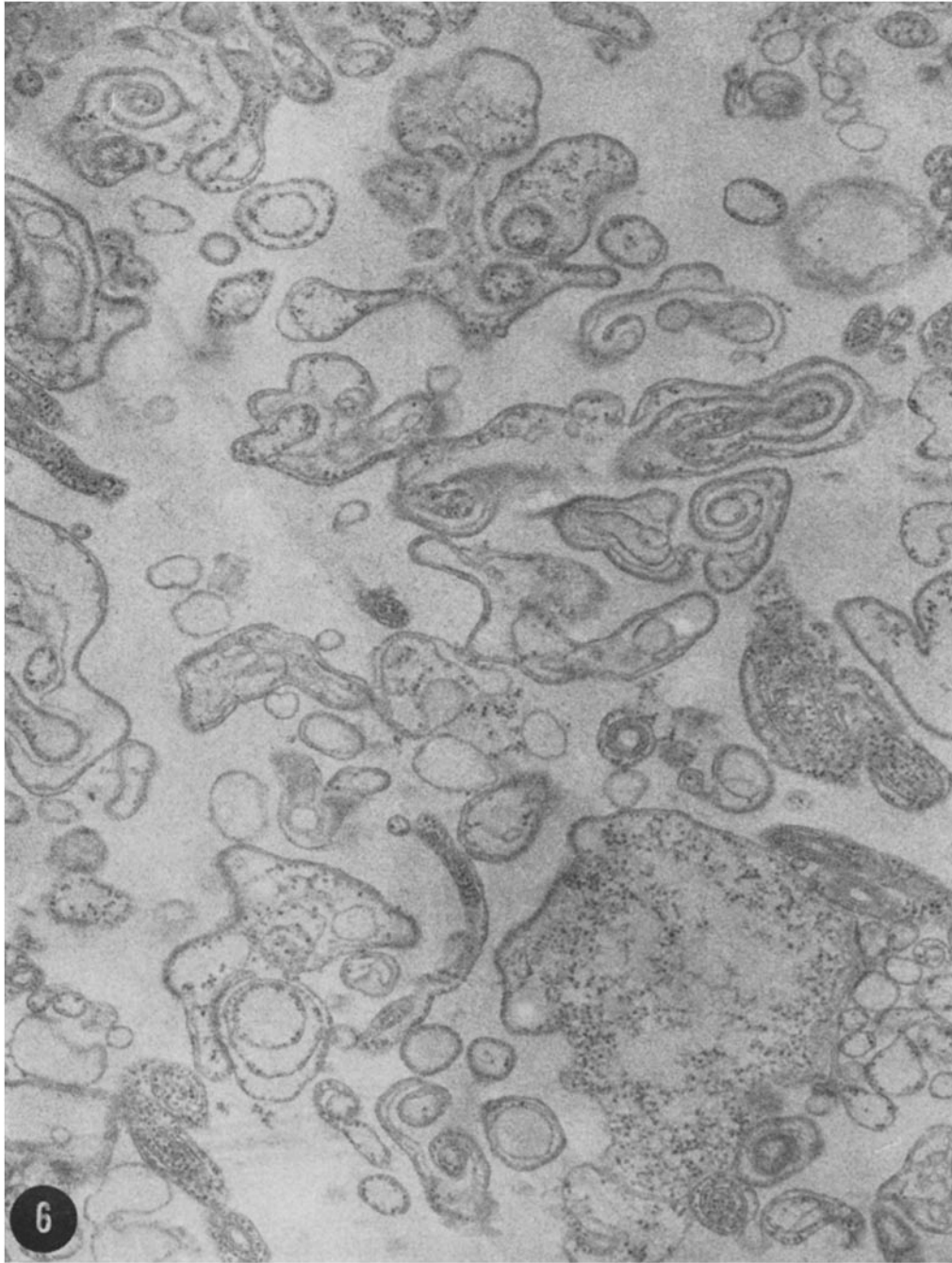
FIGURE 6 Fraction P_c (P_1 + P_2). Obtained from a broken spheroplast preparation by centrifugation for 15 min at 20,000 g. This fraction contains mainly cell envelope fragments with varying amounts of cytoplasmic material attached. Large, more or less intact structures are also occasionally present. × 47,500.
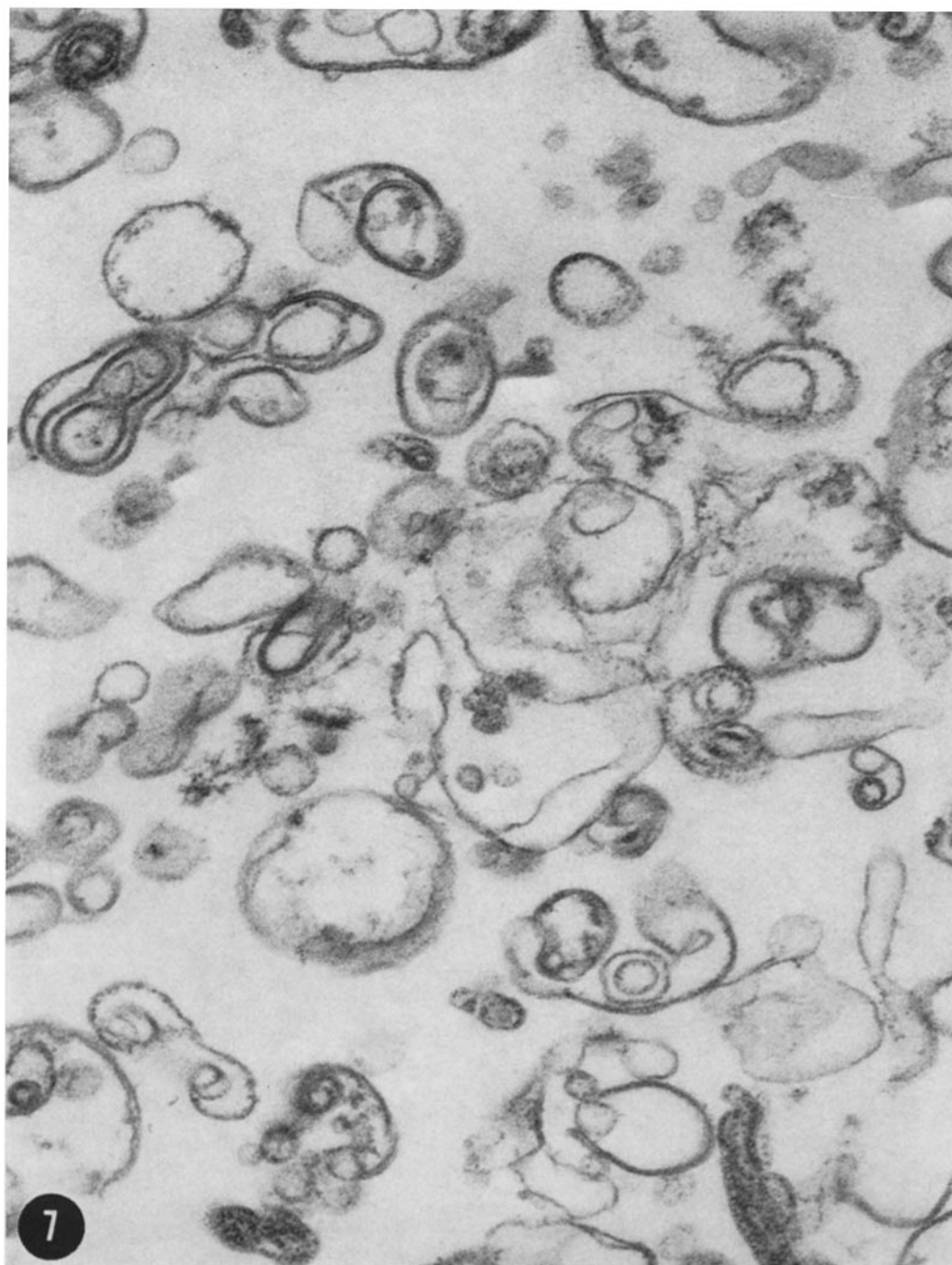
FIGURE 7   Interface. This fraction was obtained from P_c by centrifugation through a layer of 55%
(w/v) sucrose sitting on a layer of 70% (w/v) sucrose. It contains predominantly envelope fragments
with some cytoplasmic remnants. × 47,500.
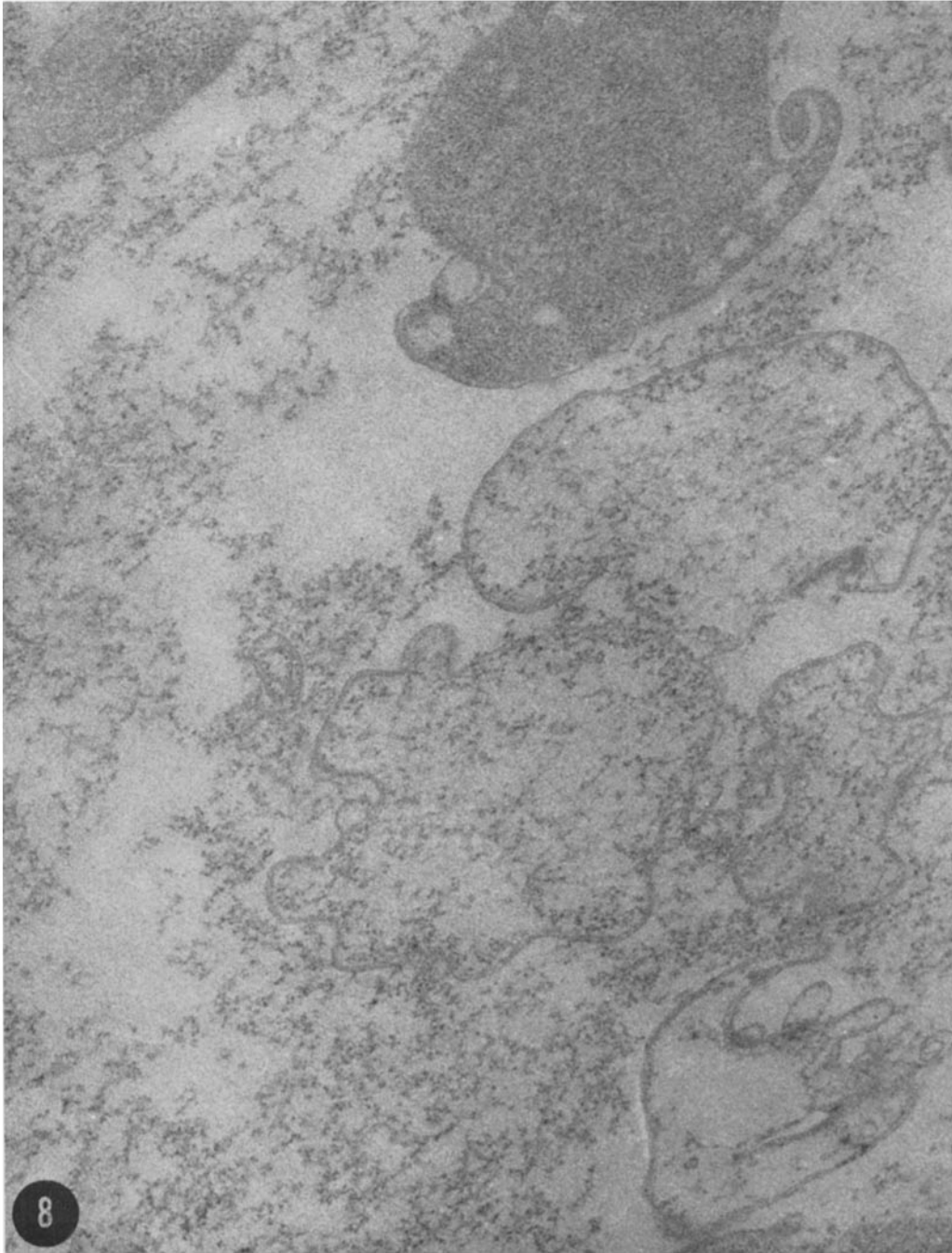
FIGURE 8 Gradient Pellet. This fraction has been pelleted through both the 55% and 70% sucrose layers. The selection has been towards spheroplast-like structures and huge ribosomal clusters. × 47,500.
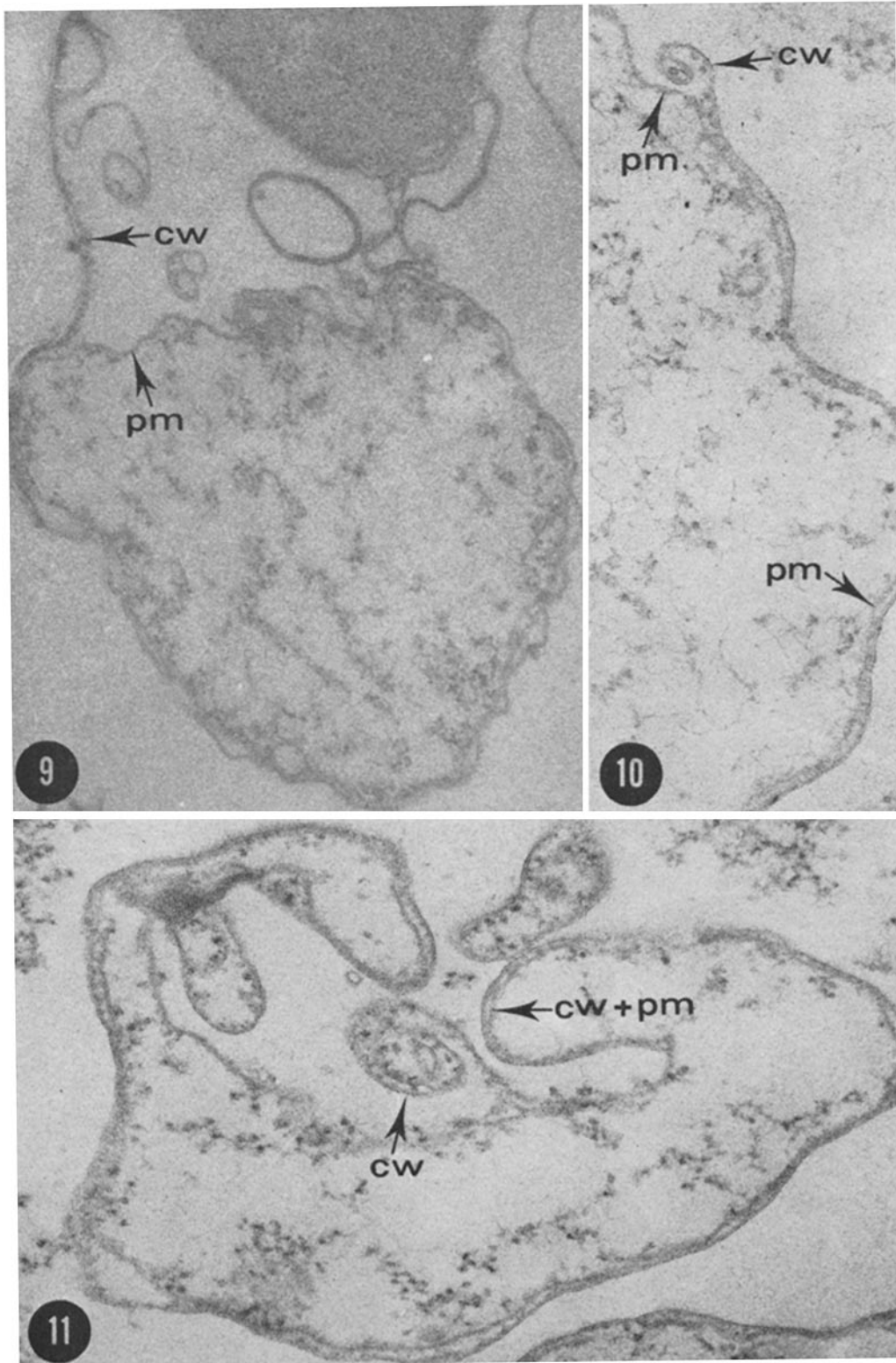
FIGURE 9   Spheroplast with broken cell wall (*cw*) and intact plasma membrane (*pm*). Note coiling of the cell wall. × 59,000.

FIGURE 10   Separation of the coiled cell wall (*cw*) from the plasma membrane (*pm*). Note ribosome-like particles on the cell wall profile. × 96,000.

FIGURE 11   In this spheroplast fragment it is shown how the plasma membrane can coil together with the cell wall (*cw* + *pm*). The coil (*cw*) is presumed to be a cell wall coil. Note large ribosomal clusters attached to the plasma membrane. × 96,000.

(37) also described coiling of the cell wall after rupturing the latter by adding EDTA to lysozyme spheroplasts. However, unlike our system, the coiling in their preparation resulted in closely packed profiles. In Fig. 10 the beginning coiling of the cell wall can be observed and in this case ribosome-like material is seen in association with the cell wall (cw). In another example (Fig. 11) one may observe how the plasma membrane can coil together with the cell wall (cw + pm). Large ribosomal clusters are seen here to be associated with the plasma membrane.

Distributions of Protein, Phospholipid, Nucleic Acids, Lipopolysaccharide, and Peptidoglycan

During the period of exposure of the log phase cells to penicillin, the number of cells remained almost constant but the content of protein (determined by the procedure of Lowry et al. [41]), RNA, DNA, and phospholipid increased. Table II shows that the relative content of protein and DNA increased during this period whereas the relative

content of RNA and phospholipid decreased. The finding of a lower number of visible cells than viable cells at the start of spheroplasting requires some explanation. Cell counts for seven different preparations were statistically analyzed for the data shown in Table II. With two of the seven, both visible and viable counts were performed. A Student's t test of all of the data indicates the significance of the difference of cell counts by the visible and viable procedures at P = 0.04. To the extent that this represents a real difference the following considerations would apply. In the visible assay, dividing and double cells were counted as single cells. Some of these cells could have been subsequently separated by the serial dilutions and vortex mixing and by the process of mechanically spreading the cell suspensions across the agar surface by means of glass rods.

The spheroplasts were collected by centrifugation and resuspended. Although about 30% of the preparation was not sedimented, the general composition of the preparation at this point was the same as that at the end of spheroplasting. Resuspension of the pellet caused the breakage of a high

TABLE II

Changes in Chemical Composition and Number of Whole Cells during Preparation and Washing of Spheroplasts

Stages of preparation	Distribution of components at different stages*				Per cent composition of fraction by weight‡				Relative number of whole cells§	
	Protein	RNA	DNA	Phospho-lipid	Protein	RNA	DNA	Phos-pho-lipid	Visible	Viable
Start of spher-oplasting	24.7	46.0	27.8	43.1	58.1	30.4	2.7	8.7	76 ± 5.4 (6)	100 ± 8.1 (3)
End of sphero-plasting	100	100	100	100	71.0	20.0	3.0	6.1	67 ± 6.4 (9)‖	32.8 ± 9.0 (3)
Spheroplasts Sharples pellet re-suspended	72.6	70.0	74.0	70.5	71.8	19.4	3.1	6.0	22 ± 1.2 (6)	2.3 ± 0.8 (3)
Final washed spheroplast preparation	54.9	44.5	63.5	76.0	71.6	16.4	3.5	8.5	11 ± 1.4 (5)¶	1.1 ± 0.6 (3)

* The amount present at the end of spheroplasting is set as 100%.

‡ The total weight of protein, RNA, DNA, and phospholipid is set as 100%.

§ The number of viable cells present at the start of spheroplasting (32.8×10^7 cells per ml) is taken as 100%. Reasons for the difference between the number of visible and viable cells at this stage are discussed in the text. The numbers shown represent relative number of cells ± SEM for the number of observations shown in parenthesis.

‖ At the end of spheroplasting the whole cells present were in the following forms: 5.5% ± 1.6 rods, 84% ± 3.6 spheroplasts, and 10% ± 3.4 intermediate forms (statistics for 10 preparations).

¶ In the final spheroplast preparation remaining whole cells were: 8.2% ± 4.3 rods, 88% ± 3.1 spheroplasts, and 4.2% ± 1.4 intermediate forms (statistics for four preparations).

TABLE III

Chemical Characteristics of E. coli Fractions —1

	Protein	RNA	DNA	Lipid (by phosphate)	Lipid (by ester)	Lipopolysaccharide (by KDO analysis)
	% ± SEM	% ± SEM	% ± SEM	% ± SEM	% ± SEM	% ± SEM
Broken spheroplasts	100 ± 3.3 (4)	100 ± 4.5 (2)	100 ± 5.6 (2)	100 ± 9.5 (3)	100 ± 4.2 (3)	100 ± 5.4 (4)
P_1	44 ± 1.5 (4)	41 ± 1.6 (2)	37 ± 4.4 (2)	60 ± 1.6 (3)	60 ± 2.1 (3)	55 ± 3.2 (4)
P_2	10 ± 0.3 (4)	7.6 ± 0.8 (2)	5.5 ± 0.8 (2)	31 ± 2.1 (3)	32 ± 2.7 (3)	30 ± 2.3 (5)
P_3	8.2 ± 0.2 (3)	23 ± 2.7 (2)	13.7 ± 1.2 (2)	7.3 ± 0.9 (3)	8.5 ± 0.4 (3)	6.1 ± 0.07 (4)
S_3	27 ± 0.4 (3)	13 ± 0.4 (2)	40 ± 2.7 (2)	0.2 ± 0.03 (2)	7.4 ± 2.8 (3)	1.4 ± 0.4 (6)
Total	89	85	96	98	108	93
P_e	56 ± 3.8 (4)	48 ± 3.2 (2)	43 ± 3.2 (2)	84 ± 3.9 (3)	95 ± 12 (3)	86 ± 1.0 (3)
S_e	36 ± 2.4 (4)	36 ± 1.2 (2)	47 ± 2.3 (2)	8.4 ± 0.7 (3)	13 ± 1.4 (3)	7.9 ± 0.7 (5)
Total	92	84	90	92	108	94
P_e-sucrose fractions 55/70 gradient						
Top	7.7 ± 0.3 (3)	9.3 (1)	11 ± 2.4 (2)	4.0 ± 0.6 (3)	12 ± 4.2 (3)	19 ± 0.9 (3)
Interface	14 ± 1.0 (3)	12 ± 1.6 (2)	15 ± 0.1 (2)	33 ± 1.4 (3)	45 ± 2.2 (3)	24 ± 1.6 (5)
Bottom	4.1 ± 0.3 (3)	3.4 (1)	4.4 ± 1.2 (2)	6.1 ± 1.8 (3)	14 ± 3.1 (3)	9.5 ± 0.1 (2)
Pellet	20 ± 2.6 (2)	25 ± 3.6 (2)	22 ± 1.7 (2)	31 ± 4.7 (3)	44 ± 8.1 (3)	22 ± 2.5 (4)
Total	46	50	52	74	115	75

Each value is the percentage of the broken spheroplast concentration of each substance present in the given fraction ± SEM for the number of observations in parenthesis. The concentrations in the broken spheroplasts were 15,995 µg of protein, 4125 µg of RNA, 895 µg of DNA, 1588 µg of lipid by the phosphate method, 1425 µg of lipid by the ester method and 560 nmoles KDO per ml as stored in liquid N_2. A description of the preparation of the fractions is given in Table I.

proportion of the cells so that when the preparation was again sedimented there was a disproportionate loss of soluble and light components. The final preparation of sedimented material showed an enrichment of phospholipid and DNA compared to the cells at the end of spheroplasting. The fact that 76% of the original phospholipid and 55% of the original protein were present in the final preparation whereas only about 10% of the original number of visible cells and about 1% of the original number of viable cells were present shows that the preparation at this point was essentially acellular. Electron microscope observations confirmed this conclusion.

Fractions were prepared, as described in Table I, from the final preparation of washed (broken) spheroplasts by either differential centrifugation alone or by differential centrifugation combined with centrifugation through sucrose layers. The process of storage in liquid nitrogen, thawing, dilution, and homogenization (before fractionation) further reduced the number of residual visible cells by one-half. Of the remaining cells, practically all ($>98\%$ by both visible and viable assays) sedimented at $3500\,g$ in the P_1 fraction. Table III shows the distribution of protein, RNA, DNA, phospholipid, and lipopolysaccharide among all of the derived fractions and Table IV shows the specific content of the first four of these components in membrane-envelope fractions. The specific phospholipid content shows that the P_2 and interface

fractions are the richest in membrane content. The high specific RNA content for these "free ribosome-poor" fractions indicates a high content of bound ribosomes. Visual confirmation of the abundant presence of envelopes with bound ribosome-like material is provided by electron microscopy. The P_1 fraction has a rather high RNA and DNA content (considering the small percentage of original whole cells present in the preparation). When the combined P_1 and P_2 fractions (P_c) are further fractionated by centrifugation through sucrose layers the subsequent chemical and electron microscope examinations show that a high proportion of the RNA is present in heavy aggregates of ribosomes that can sediment through the 70% sucrose layer and also in the form of envelope-bound ribosome-like material isolated at the sucrose layers interface. This distribution is similar to that found for DNA but a cytological confirmation of DNA presence is not as easily made as with the RNA. The P_3 fraction, which contains the free ribosomes and polysomes also contains an abundance of small membrane vesicles rich in cytochromes (39), oxidative enzymes (13), and DNA-synthesizing activity (Table VI).

If we are not dealing with a case of selective adsorption, the rather high DNA content found in particulate fractions attests to the gentleness of the of the preparative procedures. Especially interesting is the high DNA content in P_3 and in the sucrose interface fraction.

The distribution of lipopolysaccharide layer closely parallels that of the phospholipid. If the phospholipid distribution is indicative of the distribution of the plasma membrane then the data indicate that the plasma membrane and lipopolysaccharides are fractionating together. However, the possibility that additional phospholipid is nonconvalently associated with the lipopolysaccharide is indicated by newer investigations (9, 42, 43).

The production of spheroplasts is accomplished by interfering with the cross-linking of polysaccharide chains in the peptidoglycan layer (the layer located between the lipopolysaccharide layer and the plasma membrane). Since the penicillin method of spheroplast production does not hydrolyze the peptidoglycan and since the data of Table III indicate that the plasma membrane and lipopolysaccharide layers may remain associated, it would be expected that the remnants of the peptidoglycan layer would be retained in the envelope

TABLE IV

Specific Content of Nucleic Acid and Lipids in Various Fractions

	Protein	RNA	DNA	Lipid*	
				P	E
	mg/ml			*µg per mg protein*	
Broken sphero-plasts	16.0	258	56.0	99.2	91.0
P_1	7.00	244	47.6	136	125
P_2	1.58	199	30.9	314	298
P_3	1.30	724	94.9	90.1	96.4
P_c	8.87	226	43.1	150	155
55/70 interface	2.17	234	60.9	244	304

The preparation of the fractions is shown in Table I.

* Lipids were estimated both by phosphate, (P), and ester (E), methods.

fragments. However, the P_1, P_2, and P_c fractions had no detectable DAP content. The explanation of this finding was found when it was determined that intact whole cells contained 0.3 μmoles DAP per 12 mg protein but during the preparation of spheroplasts, 90% of the DAP was lost. The remaining 10% is probably mostly soluble since the membrane-envelope fractions (subsequently obtained) had no measurable DAP content. It seems most likely that when cross-linking of the peptide chains is prevented, hydrolysis of all or a part of the chains occurs.

Distribution of NADH Oxidase and Dehydrogenase

NADH oxidase activity is tightly bound to the membranes of *E. coli* (13). In addition to indicating

TABLE V

Per Cent Distribution of NADH Dehydrogenase and Oxidase Activities

	Dehydrogenase	Oxidase
Broken sphero- plasts	100 ± 3.3 (3)	100 ± 0.5 (3)
P_1	9.3 ± 0.4 (2)	42 ± 0.2 (2)
P_2	0.8 ± 0.00 (5)	24 ± 0.9 (2)
P_3	0.7 ± 0.00 (2)	3.4 ± 0.2 (2)
S_c	89 ± 2.8 (3)	3.8 ± 0.2 (2)
Total	100	73
P_c	10 ± 0.2 (3)	70 ± 3.0 (3)
S_c	64 ± 1.2 (3)	7.8 ± 0.6 (2)
Total	74	78
P_c-sucrose fractions-55/70 gradient		
Top	2.4 ± 0.2 (3)	6.4 ± 0.8 (3)
Interface	1.3 ± 0.1 (3)	32 ± 3.2 (3)
Bottom	0.6 ± 0.06 (4)	3.6 ± 0.1 (4)
Pellet	5.9 ± 0.1 (3)	3.3 ± 0.1 (4)
Total	10	46

Each value is the mean ± SEM for the percentage of the starting broken spheroplast activity in each fraction, with the number of observations shown in parentheses. The starting activity in the broken spheroplasts was 190 and 5.03 μmoles/min per ml for NADH dehydrogenase and oxidase, respectively. The NADH dehydrogenase was assayed by the ferricyanide method and the oxidase by the change in optical density at 340 nm as previously described (13).

the potential for respiration of a given acellular fraction, this activity can serve as a marker for the presence of membranes. On the other hand, NADH dehydrogenase activity is predominantly found in the soluble fraction of *E. coli* (13) and its activity can be used as an indicator of the extent of contamination of particulate fractions by soluble components. Although the recovery of oxidase activity was less than the recovery of dehydrogenase activity (Table V) or phospholipid (Table III), the distribution of NADH oxidase activity shown in Table V is generally consistent with the distribution of membranes as indicated by phospholipid content and electron microscopy. The exceptions were in the gradient bottom and pellet fractions which had been exposed to 70% sucrose. The bottom fraction contained only 3.6% of the total NADH oxidase activity whereas a similarly produced fraction contained 9.8% of the total phospholipid. In the case of the gradient pellet only 3.3% of the total oxidase activity was present whereas in a similarly produced fraction about one-third of the total phospholipid was present (Table III).

The NADH dehydrogenase activities show that the membrane-envelope fragments of the particulate fractions P_2, P_3, and interface fraction are essentially devoid of soluble components. The values shown in Table III actually indicate upper limits for the presence of soluble components because some dehydrogenase activity is not soluble but is associated with the NADH oxidase activity of the membranes.

DNA Synthesis

The incorporation of tritiated TTP into DNA by the broken spheroplast preparation, both in the presence and absence of added denatured calf thymus DNA was found to be dependent upon the presence of the four deoxynucleoside triphosphates. Omission of either the other three deoxynucleoside triphosphates or of deoxy-ATP, alone, abolished incorporation. The presence of 75 μg/ml of beef pancrease DNAase I (Worthington Biochemical Corp. [Freehold, N. J.], Code: DPFF ribonuclease-free DNAase) reduced incorporation to zero. The omission of mercaptoethanol had little or no effect. The final preparation of washed and broken spheroplasts had essentially the same specific DNA-synthesizing activity as the initial resuspended spheroplasts when assays of endogenous activity were performed. However, when the assays were carried out in the presence of added DNA, it was

TABLE VI

DNA-Synthesizing Activity

	No added DNA				Plus added DNA				Per cent stimulation by DNA
		Relative specific activity per unit				Relative specific activity per unit			
	Per cent distribution	Protein	DNA	Phospholipid	Per cent distribution	Protein	DNA	Phospholipid	
Broken sphero-plasts	100 ± 4.6 (5)	1.00	1.00	1.00	100 ± 2.4 (5)	1.00	1.00	1.00	251
P_1	20.3 ± 1.7 (2)	0.66	0.54	0.34	15.0 ± 0.8 (2)	0.48	0.41	0.25	156
P_2	4.3 ± 0.2 (3)	0.68	0.73	0.14	2.9 ± 0.1 (3)	0.47	0.53	0.09	133
P_3	19.9 ± 1.8 (3)	2.5	1.45	2.5	12.1 ± 0.9 (3)	1.52	0.88	1.5	114
S_3	41.2 ± 0.8 (4)	1.18	1.0	*	85.2 ± 6.0 (4)	2.38	2.1	*	602
Total	85.7	1.06	0.89		115	1.42			362
P_c	52.0 ± 6.9 (4)	1.28	1.2	0.58	26.4 ± 1.6 (4)	0.66	0.61	0.29	78
S_c	52.7 ± 4.9 (4)	0.90	1.1	*	135 ± 6.4 (4)	2.32	2.9	*	796
Total	105	0.99			161	1.53			440
55/70 gradient									
Top	13.6 ± 1.2 (5)	1.27	1.24	*	7.4 ± 0.4 (5)	0.71	0.68	*	92
Interface	14.2 ± 0.8 (3)	0.57	0.95	0.35	7.9 ± 0.6 (3)	0.32	0.53	0.20	94
Bottom	1.2 ± 0.4 (5)	0.34	0.27	0.12	1.4 ± 0.2 (5)	0.41	0.32	0.14	321
Pellet	0.45 ± 0.09 (4)	0.05			0.7 ± 0.31 (5)	0.07			462
Total	29.8	0.60			17.4	0.36			108
P_{3A}	88.9 ± 5.0 (4)	1.45			36.1 ± 3.3 (3)	0.60			43
S_{3A}	24.4 ± 0.8 (2)	0.53			64.4 ± 5.2 (2)	1.43			830
Total	113	1.06			100.5	0.95			212
P_{3B}	75.6 ± 2.8 (4)	1.45			35.8 ± 3.0 (4)	0.69			66
S_{3B}	20.1 ± 0.9 (4)	0.70			55.6 ± 0.6 (4)	1.98			876
Total	95.7	1.19			91.4	1.14			234

DNA-synthesizing activity was measured by the incorporation of TTP-^{3}H into DNA as described in the Methods section. Each value in the Distribution column is the percentage of the starting broken sphero-plast activity present in the given fraction ± SEM for the number of observations shown in parentheses. The activity of the starting broken spheroplast preparation was 205 and 720 pmoles per min per ml without and with added DNA, respectively. Its specific activity was 19.1 and 66 pmoles per min per mg of protein without and with added DNA, respectively, and these values were taken as 1.00 in determining the relative specific activity of other fractions. The specific activities based on DNA and phospholipid were calculated from the distribution of DNA-synthesizing activity found in these experiments and the distribution of DNA and phospholipid found with a similarly prepared set of fractions. P_{3A} and S_{3A} are the pellet and supernatant, respectively, from a 60 min, 105,000 g centrifugation of broken spheroplasts diluted with phosphate-free medium. P_{3B} and S_{3B} are the pellet and supernatant, respectively, from a similar centrifugation of undiluted spheroplasts.

* Because DNA polymerase is soluble and virtually no membranes (phospholipid) are found in the soluble fraction, the parameter of DNA-synthesizing activity per unit weight of phospholipid can be used only to compare different membrane-rich fractions.

found that the final preparation of washed, broken spheroplasts had only 60% of the specific activity of the original unwashed spheroplasts. This finding is consistent with the data of Table VI which show that added DNA stimulates the soluble activity much more than the particulate activity. This is strikingly shown by comparing P_{3A} with S_{3A}. When assayed in the absence of added DNA, 89% of the total activity can be sedimented whereas in the presence of added DNA, 46% is sedimented.

Table VI shows the distribution of DNA-synthesizing activity among the various acellular fractions. In the absence of added DNA about one-half of the total recovered activity is obtained in the conventionally prepared particulate fractions P_1, P_2, and P_3. Of particular interest is the P_3 fraction which shows a marked enrichment of specific activity figured on a protein, DNA, or phospholipid basis. If the DNA-synthesizing activity is membrane associated, the specific activity (protein basis) of the membranes in the P_3 fraction is probably higher than indicated here since an appreciable part of the protein of this fraction is present in the form of ribosomes. The addition of DNA, stimulated the DNA-synthesizing activity of the soluble fractions much more than that of the particulate fractions. Therefore, in

the presence of added DNA the relative quantitative picture among the various fractions was altered in favor of the soluble fractions.

Amino Acid Incorporation

The magnitude of incorporation of amino acids found by various laboratories using preparations of free ribosomes and polysomes isolated from *E. coli* is shown in Table VII. During a 60 min incubation at 37°C, single amino acids were incorporated to the extent of a few hundredths of a nanomole per milligram ribosomal protein. This incorporation could be stimulated up to 8-fold in most cases by the addition of suitable exogenous messenger and 350-fold in the case of phenylalanine. With a labeled algal hydrolysate, incorporation levels were from a few tenths to 1.7 nanomoles amino acid incorporated per milligram ribosomal protein.

Table VIII shows the level of incorporation reached during 20–30 min incubation at room temperature when envelope fractions P_1 and P_2 were used. The numbers were calculated with the assumption that all RNA present was in the form of ribosomes having the same relative amounts of RNA and protein as free ribosomes (i.e., 40% pro-

TABLE VII

Amino Acid Incorporation into E. coli Ribosome Fractions

Amino acid	Time	Temperature	Polynucleotide	Incorporation nmoles/mg ribosomal protein	Corrected to labeled algae mixture*	Reference
	min					
Leucine	60	37°	O‡	0.02	0.22	45
Leucine	60	37°	UC	1.5		45
Isoleucine	60	37°	O	0.01	0.09	45
Isoleucine	60	37°	UA	0.62		45
Serine	60	37°	O	0.02	0.30	45
Serine	60	37°	UC	1.6		45
Tyrosine	60	37°	O	0.02	0.32	45
Tyrosine	60	37°	UA	0.75		45
Proline	60	37°	O	0.02	0.26	45
Proline	60	37°	UC	0.6		45
Phenylalanine	60	37°	O	0.07	1.47	45
Phenylalanine	30	37°	U	24.6		45
Phenylalanine	45	37°	U	6.6		46
Phenylalanine	70	35°	U	29		47
Valine	60	35°	O	0.14–0.61	2.2–9.7	48
Algae mix	20	37°	O	0.62–1.71		49

* Factors obtained from Tissières et al. (44).

‡ O means no added polynucleotide.

tein and 60% RNA) and that all of the ribosomes were functionally active. If RNA is present outside of ribosomes, or if inactive ribosomes are present, the actual level of incorporation per ribosome would be higher than shown in Table VIII. The data show the high levels of incorporation attained by these preparations. The level of incorporation per residual viable cell was always higher in P_2 than in P_1 (about double) indicating that there are relatively more actively incorporating acellular fragments in P_2 than in P_1.

The possibility that the incorporation we observe in our particulate fractions is due to contamination by the soluble aminoacyl transferase system of Leibowitz and Soffer (50, 51) is eliminated by the observation that chloramphenicol (200 μg/ml) inhibited the incorporation of leucine-^{14}C and isoleucine-^{14}C by 94%. The aminoacyl transferase system is insensitive to levels of chloramphenicol as high as 1 mg/ml (51).

We have performed preliminary studies employing radioautography at the electron microscope level in order to obtain direct evidence for the incorporating ability of envelope fragments as opposed to contaminating whole cells. The data at present indicate that contaminating whole cells are relatively inactive and that an appreciable

TABLE VIII

Amino Acid Incorporation by Cell-Envelope Fractions

Experiment No.	Labeled amino acids*	Incubation time	Incorporation	
			P_2	P_1
		min	*nmoles/mg ribosomal protein*	
92	Algae mixture	20	21.6	273
97	Algae mixture	20	23.6	109
97	Algae mixture	20	46.0	268
98	Algae mixture	30	25.2	462
101	Algae mixture	30	6.68	
102	Algae mixture	29	16.4	
96	LEU-ILEU	20	13.9‡	87§
96	LEU-ILEU	20	10.2	97§
96	LEU-ILEU	20	11.8	181||
96	LEU-ILEU	20	15.9	176||

* Added to an unlabeled amino acid mixture described in Methods.
‡ Data for LEU-ILEU corrected to labeled algae mixture.
§ In suspending medium.
|| In cellular supernatant S_2.

fraction of incorporated radioactivity is associated with the membrane envelope fragments.[3]

DISCUSSION

We have tried to isolate envelope fractions by gentle techniques from *E. coli* in order to preserve their structural integrity and to study their biosynthetic capabilities. The first question that should be asked is whether we have succeeded in obtaining fractions that are measurably different from others that have been described. In Table IX we show comparative data for the recovery of nucleic acids in our and other preparations. We chose to compare DNA content because there is good reason to believe that DNA is normally attached to the cell membrane. Vigorous techniques of isolation should fragment the DNA and decrease the amount sedimenting with the envelope fractions. We chose RNA because the content of this nucleic acid would indicate the extent of ribosome association with the envelope fragments. We have previously shown that the extent of binding of active ribosomes to *E. coli* envelope fractions depends on the severity of the cell disruption technique (52, 53). Table IX shows that our envelope fractions contain a relatively high content of both RNA and DNA. These analytical data combined with the electron microscope analysis show that our preparations are unusually high in nucleic acid content and bound ribosomes.

The next important question is whether the high content of nucleic acids represents a meaningful structural relationship or merely insufficiently washed preparations. This question is not so easily or quickly answered. To prove that an association is biologically significant requires a much fuller characterization of (*a*) the nature of the association; (*b*) the enhancement of biological activity of the complex compared to the free component; (*c*) a unique character of the associated activity compared to the corresponding activity of the nonassociated or free activity. We have observed that the extent of contamination of the membrane-envelope fractions by the soluble enzyme, NADH dehydrogenase, is extremely small (Table V). Cell counts and electron microscope examination have also shown the absence of appreciable contamination by intact cells. The membrane-envelope fragments possess NADH oxidase activity, are active in DNA synthesis and in amino acid–in-

[3] Scharff, R., and R. W. Hendler. Unpublished observations.

TABLE IX

Nucleic Acid Content of Different Envelope Preparations

	This paper	Miura and Mizushima (9)	Suit (5)	Kaback and Stadtman (6)	Nagata, Shibuta, and Maruo (10)	Quigly and Cohen (11)
mg RNA/mg protein	0.234*	0.045	0.11,‡ 0.14,§ 0.24‖	0.02¶	0.12–0.19	0.13
mg DNA/mg protein	0.06,* 0.09**			0.002¶	0.02	0.006
% of total RNA	12*		2–5	2–4¶	20–40‡‡	2.5
% of total DNA	15,* 14**			2–4¶	20–30‡‡	0.6

* Interface fraction.
‡ From lysozyme-freeze-thaw spheroplasts.
§ From penicillin spheroplasts.
‖ From diaminopimelic acid starved cells.
¶ Personal communication from H. R. Kaback.
** P₃ fraction.
‡‡ Amount of bound nucleic acid increases with increase of Mg^{++} concentration. This preparation corresponds to our P_e fraction where we recover 48% of the total RNA and 43% of the total DNA.

corporating ability. The high content of DNA-synthesizing activity in P_3 relative to P_2 argues against a general adsorption phenomenon. P_2 contains more than four times the phospholipid present in P_3 and yet has only one-fifth the DNA-synthesizing activity. The distribution of synthesizing activity does not simply reflect the availability of DNA as shown by the amount of DNA-synthesizing activity per amount of DNA present in the P_3 fraction.

It has been postulated that the bacterial chromoid of *E. coli* is attached to the cell membrane at the point where it is replicated (2). Evidence has been provided that most of the rapidly formed RNA may also be membrane bound (1, 55, 56). Recent techniques of fractionating *E. coli* have produced a membrane-envelope fraction high in DNA and RNA content and in the ability to catalyze DNA-dependent RNA synthesis and DNA synthesis (5, 55, 56). These techniques have employed a combination of lysozyme, EDTA or EGTA, BRIJ 58 or sodium lauroyl sarcosinate and sometimes hydrolytic enzymes or mechanical shear forces. The sodium lauroyl sarcosinate treatment, originally described by Tremblay et al. (55) in an application with *Bacillus megaterium*, relies on the ability of Mg^{++} to coprecipitate the detergent along with the membrane complex containing the nucleic acids and nucleic acid–synthesizing enzymes. The use of enzymes, EDTA, and detergents, however, may dis-

tort the membrane and alter structural relationships.

Ganesan has described the isolation of a membrane-containing fraction from *B. subtilis* which is active in DNA synthesis (57, 58). Further purification yielded a complex containing polymerase, endonuclease, and ligase activities (59). It appears that the further purification, however, removed the complex from the membrane. Smith and Hanawalt (60) obtained a relatively heavy cell component from *E. coli* (pulse labeled with ^{32}P or bromouridine-^{3}H) which was enriched in its content of nascent DNA. The cells were first lysed with lysozyme and EDTA, dialyzed overnight against EDTA, treated with pronase, and subjected to mild shear on a Vortex mixer. The main criteria used for demonstrating attachment to cell membrane were the ease of sedimentation and the fact that deoxycholate treatment seemed to disrupt the complex. In later work (61), Fuchs and Hanawalt used a BRIJ 58, lysozyme lysis treatment and controlled shearing by sonication. The "growing point" for DNA was separated from the bulk of DNA by sucrose centrifugation. The complex sedimented faster than ribosomes in heavy sucrose gradients, but could not be banded at an isopycnic equilibrium position (indicating the absence of an appreciable content of lipid). Neither egg white lysozyme nor phospholipase A attacked the complex. Labeling with ^{32}P indicated a very low content of phospholipid in the complex. The presence

of membrane was not demonstrated. Sueoka and Quinn (62) used the same isolation procedure as Smith and Hanawalt (60) to isolate the nascent DNA-containing fraction. They provided evidence that the chromosome origin was actually present in the complex. Without providing anymore evidence that cell membrane was present or that the DNA-synthesizing activity was associated with membranous material, they referrred to the unit as a membrane complex. We have found that the particulate fraction, P_3, obtained at centrifugations between 20,000 and 105,000 g and which is usually thought of as a free ribosome preparation, is rich in membrane content and possesses a high capacity for DNA synthesis. Preliminary experiments indicate that the characteristics of the DNA synthesis by P_3 differ differ appreciably from those of the soluble fraction (Scharff and Hendler, unpublished observations).

We have previously shown that intact *E. coli*, pulse labeled with amino acids, can be fractionated to yield membrane-envelope–associated ribosomes with much higher specific radioactivity than that of free ribosomes (52, 53, 63). The current demonstration of high levels of amino acid incorporation by acellular fragments containing a high proportion of associated RNA represents a first step towards the refinement and study of an in vitro system for protein synthesis by a cell envelope–ribosome complex.

It must be emphasized that our finding of active protein synthesis in a membrane-envelope complex does not establish a unique structural-functional relation between membranes and ribosomes. It could be that mRNA is uniquely protected by the membrane from destruction by nucleases (64, 65).

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