JOURNAL OF BACTERIOLOGY, Jan. 1974, p. 290-301
Vol. 117, No. 1

Localization of Polyribosomes Containing Alkaline Phosphatase Nascent Polypeptides on Membranes of *Escherichia coli*

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Received for publication 20 August 1973

A procedure has been developed for extracting membranes from bacterial cells under conditions that keep a large fraction of bacterial polyribosomes intact. Freeze-thawing spheroplasts in the presence of deoxyribonuclease, followed by differential centrifugation, permits a separation of free and membrane-associated polyribosomes. The latter fraction contains as much as 40% of cell ribosomal ribonucleic acid (RNA) and 55% of cell messenger RNA (mRNA). Nascent polypeptides were divided almost equally between the two fractions, but 70 to 80% of alkaline phosphatase nascent chains, detected both chemically and immunologically, were derived from polyribosomes associated with the bacterial membrane. Analysis of the fractions for mRNA specific for the *lac* and *trp* operons by RNA-deoxyribonucleic acid hydridization showed somewhat larger amounts on membrane than on free polyribosomes, but enrichment for nascent alkaline phosphatase (a secreted protein) on membranes was consistently greater, suggesting that polyribosomes making secreted proteins are more tightly bound to membranes. Electron micrographs of the membrane preparations show relatively intact membranes with clusters of polyribosomes on their inner surfaces.

The selective secretion of proteins from the cell cytoplasm is a relatively common process in many types of cells. Although the molecular basis that can account for the secretion of some proteins but not others remains unknown, there is considerable data to suggest that, in eukaryotic cells, secreted polypeptides are synthesized on polyribosomes that are bound to the reticuloendothelium membrane (4, 25, 31, 41). Bacteria also secrete proteins from the cell cytoplasm; in some strains these proteins appear in the culture medium whereas in others the polypeptides are retained in a region of the cell envelope referred to as the periplasmic space (23). In exponentially growing cultures of *Escherichia coli* K-12, 5 to 8% of the cell protein is in the periplasm (15, 22). This value increases two- to threefold in *E. coli* mutants that have become constitutive for production of alkaline phosphatase, a normally repressed, periplasmic enzyme. About half of the increase can be accounted for by the alkaline phosphatase alone (22).

We searched for the basis for the selective secretion of bacterial proteins by examining the structure and formation of *E. coli* alkaline phosphatase. The active form of this enzyme contains two identical subunits of molecular weight 43,000 and tightly bound zinc atoms (5, 27, 29) and phosphate molecules (6a). From previous work that included studies with alkaline phosphatase-negative mutants (13, 32, 33), with *E. coli* spheroplasts capable of making polypeptides (34), and with inactive alkaline phosphatase protein containing amino acid analogues (6, 22, 35, 36), we concluded that it was the subunit form of the enzyme that was secreted and that secondary and tertiary structure of the polypeptide chain was not a crucial determinant in a mechanism for secretion.

In this work we devised a procedure for isolating relatively intact bacterial membranes and undegraded polyribosomes. The distribution of alkaline phosphatase nascent chains between the free and membrane-bound polyribosomes was measured both immunologically and chemically to determine if secreted bacterial protein were made preferentially on polyribosomes attached to membranes. Our results show that half of the bacterial polyribosomes can be recovered in a membrane fraction that contains almost all of the cell phospholipid but no deoxyribonucleic acid (DNA) or cytoplasmic enzymes. Seventy to eighty percent of alkaline

phosphatase nascent chains are on these polyribosomes, and this enrichment for a secreted protein is greater than that detected for *lac* and *trp* messenger ribonucleic acids (mRNA), which were quantitated by molecular hybridization techniques. Thus, secretion of polypeptides from the bacterial cytoplasm appears to resemble the mechanism described for eukaryotic cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains are derivatives of *E. coli* K-12. Strain CW3747 (ATCC 27256) and W3747 (ATCC 27257) have been described; the CW3747 produces alkaline phosphatase during exponential growth whereas the W3747 makes the enzyme only after growth stops in a limited P_i medium (34). Sud 24 is a fragile strain isolated by Mangiarotti et al. (19). Strain 129 is K-12, 1000 (F^-, thiamine$^-$). Strain AB-06 (segment 14) is derived from W3110 and carries a μ phage-induced mutation in the *lac* operon such that genes *y* and *a* are deleted (10). Cultures were grown at 37 C in either a minimal tris(hydroxymethyl)aminomethane (Tris)-salts-glucose medium (34), or a phosphate-buffered medium containing 0.2% glycerol (3). All experiments were carried out with exponentially growing cultures at a density of approximately 5×10^8 cell/ml.

Preparation of free and membrane-associated polysomes. To a 200-ml culture was added 16 ml of chloramphenicol (2.5 mg/ml), and cells were immediately chilled in a dry ice-acetone bath for about 30 s. Cells were harvested by centrifugation and pellets were suspended in 0.4 ml of a 25% sucrose solution containing 10 mM Tris-chloride, pH 8.1. Lysozyme (50 μliters of 5 mg/ml in 0.25 M Tris-chloride, pH 8.0) and ethylenediaminetetraacetic acid (EDTA) (50 μliters of a 20 mM solution, pH 8.0) were added at 4 C. Lysis was monitored at A_{540} before and 15 min after lysozyme treatment. If decrease in A_{540} indicated less than 75% lysis, an additional 50 μliters each of lysozyme and EDTA were added for a further 10 min of incubation. After the preparation of spheroplasts, 0.4 ml of deoxyribonuclease (DNase) (1 mg/ml in M $MgSO_4$), 0.35 ml of chloramphenicol (2.5 mg/ml), 0.04 ml of M mercaptoethanol, 0.2 ml of M Tris-chloride, pH 7.4, and 2.25 ml of water were added. This solution was frozen in dry ice-acetone and thawed at 23 C, but the temperature of the spheroplast solution was not allowed to increase above 4 C during thawing. After three cycles of freezing and thawing, the suspension was centrifuged 10 min at $27,000 \times g$.

The supernatant fraction, referred to as the cytoplasmic fraction, was layered on top of a discontinuous sucrose gradient made of 3 ml of 1.8 M sucrose containing 50 mM Tris-chloride, pH 7.4, 10 mM $MgCl_2$, 25 mM KCl (noted as TMK), and 2 ml of 0.5 M sucrose in TMK. This gradient was centrifuged for 12 to 18 h at 42,000 rpm in a 65 Spinco rotor. The pellet was suspended in 1 ml of TMK buffered and represents the "free" ribosomes and polyribosomes.

The original pellet fraction was suspended in 4 ml of TMK containing, in addition, 10 μliters of M mercaptoethanol per ml of buffer. This fraction was applied to a discontinuous gradient consisting of 4 ml of 1.8 M sucrose in TMK and 3 ml of 0.5 M sucrose in TMK and was centrifuged for 12 to 16 h in a SB 206 rotor in the International Equipment Co. B-60 ultracentrifuge at 30,000 rpm. Two milliliters from the interface of the sucrose layers were carefully collected; this is referred to as the cell membrane fraction. To this fraction were added 0.5 ml of 10% Triton X-100 in TMK, 0.32 ml of 2 M KCl, 2.15 ml of TMK, and 30 μliters of M mercaptoethanol. After mixing, this solution was layered on 4 ml of 1.8 M sucrose in TMK and centrifuged for 16 to 22 h in the 65 Spinco rotor at 42,000 rpm. Under these conditions, bacterial polysomes form a pellet at the bottom of the centrifuge tube. To display the pattern of polysomes, the pellets were dissolved in 1 ml of TMK-mercaptoethanol buffer and a sample was layered onto a 10 to 30% linear sucrose gradient in TMK. Fractions were collected with an ISCO model 640 collector equipped with model UA-2 ultraviolet analyzer set at 254 nm.

Isolation of nascent polypeptide chains. The pelleted polysome fractions were suspended in 1 ml of buffer containing 50 mM Tris-chloride, pH 7.4, 150 mM NaCl, and 15 mM EDTA. This solution was centrifuged for 5 h at 42,000 rpm in a 65 Spinco rotor, and the supernatant fraction was used for analyses.

Detection of the amino-terminal segment of alkaline phosphatase. The solution of ^{14}C-labeled nascent chains derived from polysomes were lyophilized and mixed with 20 mg of purified unlabeled alkaline phosphatase dissolved in 1.56 ml of 99% formic acid and 0.49 ml of water. This solution was added to 20 mg of cyanogen bromide and stirred in a stoppered tube for 24 h at 23 C. The reaction was stopped with 20 ml of water, and the mixture was lyophilized. The degraded protein was dissolved in 2 ml of 0.1 M NH_4HCO_3 and applied to a G-25 Sephadex column (diameter = 2 cm, H = 45 cm). Elution was carried out with 0.1 M NH_4HCO_3, and those fractions which eluted just after the void volume and just prior to the total volume of the column were pooled and lyophilized. This fraction was resuspended in 2 ml of 0.1 M NH_4HCO_3 and digested with 50 μg of TPCK-treated trypsin (Worthington Biochemical Co.) for 2 h, 37 C. The tryptic digest was reapplied to the G-25 column and the fractions which eluted between a V_e/V_0 of 1.40 and 1.55 were pooled and lyophilized. This fraction contains the amino-terminal cyanogen bromide fragment (noted CB VII) of the pure enzyme (15a). The lyophilized fraction was dissolved in pyridine-acetic acid-water (16.6 ml: 166.6 ml:5 liters; final pH 3.5) and applied to Whatmann 3 MM paper. Electrophoresis was carried out at 3 kV, 140 mA for 80 min in water-cooled Varsol tanks (Savant, Hickville, N.Y.). A marker CB carried out at 3 kV, 140 mA for 80 min in water-cooled Varsol tanks (Savant, Hickville, N.Y.). A marker CB VII was included to detect the position of authentic amino-terminal peptide. That portion of the electropherogram corresponding to the position of the marker was cut out and sewn into another piece of Whatmann 3 MM for descending chromatography in

butanol-acetic acid-water (2:0.5:2.5). After 20 h of chromatography, the paper was dried and a 1-cm strip was placed in scintillation vials containing 5 ml of toluene-2,5-diphenyloxazole-1,4-bis-(5-phenyloxazolyl) benzene scintillant. Those strips corresponding to the mobility of CB VII marker were removed from the vial, dried, and eluted with 0.1 N acetic acid. These eluates were hydrolyzed with 6 N HCl and subjected to automatic amino acid analysis by the methods of Moore and Stein (21).

Quantitative measure of alkaline phosphatase nascent polypeptides with specific antibodies. Samples from the EDTA-treated polysomes that were obtained from cultures of cells labeled with ^{14}C-amino acids were mixed with preparations of rabbit antisera and precipitins collected after 16 h at 4 C. Details of the precipitation procedure have been published (22, 36); however, an important modification has been included here. Each reaction mixture contained 0.05% sodium dodecyl sulfate (SDS) in addition to antiserum, antigen, and buffer. The antisera used in these experiments were obtained from rabbits injected with a denatured inactive form of the alkaline phosphatase; these antibodies do not form a precipitin with active dimeric enzyme. (H. Morris and M. J. Schlesinger, manuscript in preparation).

Analysis of alkaline phosphatase nascent chains by gel filtration of CNBr fragments. Studies on the amino acid sequence of alkaline phosphatase have shown that the large polypeptide, containing about 420 amino acids, can be cleaved into eight fragments by cyanogen bromide (CNBr), and these can be separated by gel filtration on Sephadex G-75 (15a). Preparations of membrane-associated nascent chain labeled with ^{14}C-amino acids were precipitated with antisubunit antibodies, and these precipitins were dissolved with 70% formic acid. Similar precipitins were prepared with ^{3}H-labeled active enzyme. This latter sample was obtained from bacteria (CW3747) labeled for 60 min with ^{3}H-amino acid and subsequently converted to spheroplasts. The periplasmic fractions were brought to 2% SDS, 0.01 M mercaptoethanol, and heated at 70 C for 30 min in order to denature active enzyme to subunits. Unlabeled, purified enzyme (10 mg) was added to each preparation separately, and the eight fragments were prepared. Prior to chromatography, the ^{3}H- and ^{14}C-samples were mixed in a ratio of 5:1 counts/min.

Isolation of RNA from polysomes. RNA was extracted from polyribosomes with phenol in 0.01 M sodium acetate, pH 4.5, at 23 C, followed by DNase and Pronase treatment (17).

Hybridization of RNA with DNA on filters. Preparation of denatured DNA and hybridization to RNA on filters in 50% formamide was performed as described (17).

Induction for lac and trp operons. *lac* mRNA was obtained from 200 ml of cultures grown in phosphate-buffered medium. The inducer, 0.5 mM isopropyl-thiogalactoside, was added, followed 5 min later by either 250 μCi of ^{3}H-uracil (20 Ci/mmol) or 100 μCi of ^{3}H-uridine (280 Ci/mmol). After 2 min, chloramphenicol was added and the culture was chilled in acetone-dry ice. Derepression of the *trp* operon was carried out simultaneously with induction of *lac* in the following procedure. A 200-ml culture at a density of 5 $\times$ 10^{8} cells/ml was given 0.2 ml of indole propionic acid (20 mg/ml), followed 3 min later with 2 ml of isopropylthiogalactoside (12 mg/ml). Five minutes later, 200 μCi of ^{3}H-uridine was added, followed 2 min later by chloramphenicol.

Preparation of electron micrographs. For direct observation of the membrane fraction, a drop of sample banding in the discontinuous sucrose gradient was diluted fivefold with the TMK buffer and applied to a glow-discharged 20A film supported on a 200 mesh copper grid (11). After 10 min, the excess solution was blotted away with filter paper and the grid was rinsed once with 30% ethanol. The specimen was stained with uranyl acetate (4% in 30% ethanol) for 3 min, the excess was then blotted away, and the grid was washed sequentially four to five times with 30% ethanol. To prepare thin sections, the membrane fraction was incubated with 4% glutaraldehyde overnight, centrifuged, and washed with 0.8% sodium acetate. It was fixed with 1% osmium tetroxide for 2 h, rinsed with sodium acetate, and stained with 0.5% uranyl acetate. After dehydration, the material was embedded in Epon and sectioned. A Philips 200 electron microscope was used for viewing the sections.

Assays for organic solvent-extracted phosphate. The method of Ames (2) was used for extracting phospholipids. The phosphate solution was ashed with magnesium nitrate and P_i was determined in the residue (1).

Assays for enzymatic activities. The procedure for measuring the amounts of alkaline phosphatase (13) and β-galactosidase (17) using formation of *p*-nitrophenol from *p*-nitrophenylphosphate and *o*-nitrophenylgalactoside, respectively, have been published. Activities (units) are expressed as micromoles of nitrophenol formed per minute at 37 C. The assay for 3-phospho-glyceraldehyde dehydrogenase is that of D'Alessio and Josse (9). Protein was measured by the Folin procedure (18).

Materials. *lac* DNA was isolated from ϕ80dlac and kindly supplied by D. Kennell. *trp* DNA was from ϕ80dtrp and kindly supplied by C. Yanofsky. All chemicals were reagent grade. The *p*-nitrophenyl phosphate, *o*-nitrophenyl galactoside, and chloramphenicol were from Sigma Chemical Co. The radioactive isotopes used were as follows: ^{14}C-amino acids and ^{14}C proline, 260 mCi/mmol, and ^{3}H-thymidine, 62 mCi/mmol (New England Nuclear); ^{3}H-uridine 28Ci/mmol (Schwarz-Mann).

RESULTS

Composition of membrane preparations. Our initial goal was to extract and subfractionate bacterial cells in a manner that would preserve both membranes and polyribosomes. Disruption of cells was to be sufficiently mild to recover all membrane phospholipid but complete to the extent that cytoplasmic enzymes would be released as soluble protein. In addition, a series of centrifugation steps were ar-

ranged to separate non-membrane-associated polyribosomes and ribosomes from the membrane fraction. We first treated spheroplasts, prepared with lysozyme and EDTA in sucrose solution, to mild pressure shock with a French pressure cell, but under the minimal conditions for solubilizing total cytoplasmic enzymes we discovered that half the membranes were fragmented into small non-sedimentable particles. The method finally devised is described in detail in Materials and Methods. Figure 1 provides an abbreviated diagram of the fractionation procedure and Table 1 shows the distribution of cell protein, phospholipid, and three cell enzymes among the major subfractions. For a standard preparation in which about 80% of the cells were converted to spheroplasts, 80% of the phospholipid was recovered in a membrane fraction which had 34 to 40% of cell protein. Virtually all the three cellular enzymes analyzed were released into the soluble fraction.

Table 2 shows the distribution of RNA as measured by incorporation of labeled uridine for either long (three generations) or short (2 min) time periods, protein and nascent polypeptide chains as measured by a 30-s pulse of labeled amino acids, and DNA as measured by uptake of labeled thymidine over three generations. The values recorded are representative samples for 10 experiments in which conversion of cells to spheroplasts ranged from 70 to 90%. In the cytoplasmic fraction, about half of the RNA, both long and short labeled, was recovered as ribosomes and polyribosomes. The radioactivity from the pellet of the first centrifugation was recovered primarily in the membrane (see Fig. 1) and a pellet; the latter corresponded closely with an amount expected in the fraction of cells not converted to spheroplasts. In the membrane fraction, 70% of the long-labeled and 60% of the

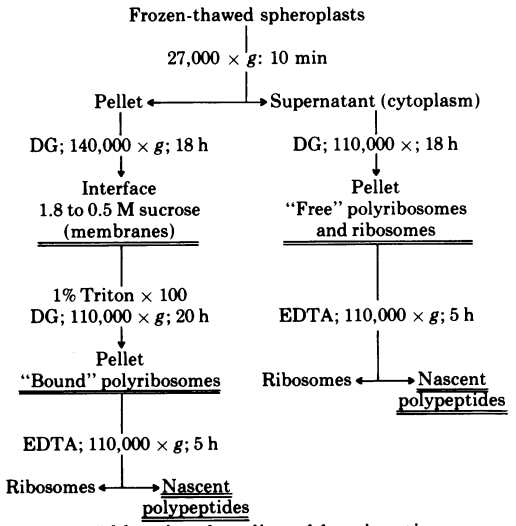

FIG. 1. *Abbreviated outline of fractionation procedure for isolating free and membrane polyribosomes. DG refers to discontinuous sucrose gradient.*

TABLE 1. *Distribution of cellular enzymes and phospholipid in membrane preparations*

Cell fraction	Protein (mg)	Phospholipid[a] (nmol)	Enzymatic activities (total units[b])		
			Glyceraldehyde 3-phosphate dehydrogenase	β-Galactosidase	Alkaline phosphatase
Spheroplasts	2.03	676	6.2	0.045	0.44
Cytoplasm	1.65	0	5.1	0.036	0.45
Membranes	0.83	544	<0.3	<0.003	<0.01

[a] Determined as phosphate extracted with methanol-chloroform (see Materials and Methods).
[b] Expressed as micromoles of product per minute at 37 C.

TABLE 2. *Distribution of nucleic acids and protein in membrane preparations*[a]

Cell Fraction	RNA[b] (×10³ counts/min)		Protein[c] (×10³ counts/min)	DNA[d] (×10³ counts/min)
	Long label	Pulse label		
Whole cells	3,122	1,832	9,174	2,603
Cytoplasm (1st supernatant)	1,746	688	5,702	244
"Free" polyribosomes	836	309	198[e]	ND[f]
Membrane plus unbroken cells (1st pellet)[a]	1,706	1,002	4,386	36
Membranes	716	370	1,868	7.4
"Bound" polyribosomes	501	210	188[e]	ND

[a] Refer to Fig. 1 and Materials and Methods for the details of fractionating the cells. The data recorded were obtained from separate labeling experiments with exponential cultures of *E. coli* K-12.
[b] For long label, ^{3}H-uridine (100 μCi) was added for three cell generations; for pulse label, ^{3}H-uridine (100 μCi) was added for 2 min; 75% of cells in the experiment recorded were estimated to be converted to spheroplasts.
[c] ^{14}C-amino acids (12.5 μCi) were added for 30 s; 80% of cells estimated to be converted to spheroplasts.
[d] ^{14}C-thymidine (10 μCi) was added for three cell generations.
[e] The figures are those for protein released from polyribosomes after treatment with EDTA and recentrifuging the ribosomes, thus they represent nascent polypeptide chains.
[f] ND, Not determined.

pulse-labeled RNA was recovered as polyribosomes after Triton X-100 treatment. Of the total RNA recovered as ribosomes and polyribosomes in this fractionation, about 40% (from long-labeled RNA) were derived from the membranes, and a similar value was noted for pulse-labeled RNA. In some experiments, we observed as much as 60 to 70% of the pulse-labeled RNA in polyribosomes in the membrane fraction, but we do not know whether the variations represent differences in the strain examined, the growth medium employed, or other factors. Most of the protein labeled after a 30-s pulse was recovered in the soluble fraction of the cytoplasmic fraction. Only 5% of the incorporated isotope was recovered as nascent polypeptide chains and these were distributed evenly between cytoplasmic and membrane-associated polyribosomes (Table 2). Because of the presence of DNase in the initial extraction procedure, only 0.2% of the incorporated thymidine was found in the membrane fraction.

An analysis of the polyribosome fractions after centrifugation in a linear sucrose gradient shows a typical pattern of polyribosomes loaded with nascent chains (Fig. 2). The polyribosomes derived from the cytoplasmic fraction show a larger amount of 70*S* ribosomes and di- and tri-somes than do those obtained from the membrane, suggesting that they are partially degraded (Fig. 2, A and C). After incubation with EDTA, both polyribosome fractions degrade to 30*S* and 50*S* ribosomal subunits with release of nascent polypeptide chains (Fig. 2, B and D). The quality of the membrane-associated polyribosomes and membranes in these preparations is strikingly revealed in an electron micrograph of membranes that were placed directly on carbon films, washed, and negatively stained (Fig. 3). In this figure, those polyribosomes not associated with the membrane presumably were released as a result of washing the sucrose from the preparation.

Alkaline phosphatase nascent chains on membrane-associated polysomes. The polyribosomes isolated from membranes and from the cytoplasmic fraction were examined for the presence of alkaline phosphatase nascent polypeptides. In one series of experiments antibodies directed against a denatured form of alkaline phosphatase were added to ^{14}C-labeled nascent chains and the antigen-antibody precipitins were analyzed. Two controls were included in the experiment: (i) ^{14}C-polypeptides from *E. coli* K-10 grown under conditions in which virtually no alkaline phosphatase was formed were tested with the antisubunit antibodies, and (ii) the nascent chains were reacted with antibodies directed against trinitrophenyl gamma globulin. Of the total nascent alkaline phosphatase chains detectable in both cytoplasmic and membrane polyribosomes, 61 to 89% were obtained from the membrane (the first two columns of Table 3). When compared to the

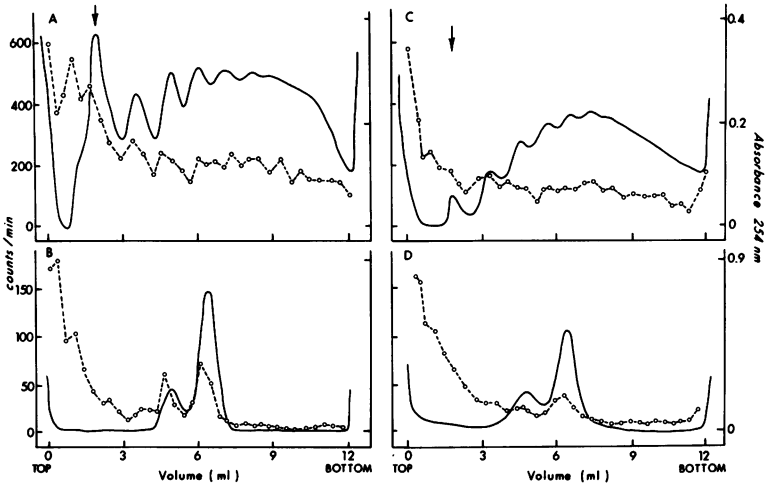

FIG. 2. *Membrane and free polyribosome patterns after centrifugation in sucrose gradients. Polyribosomes (panels A and C) were centrifuged in a 10 to 30% linear sucrose gradient at 145,000 × g for 2 h. The samples (B and D) treated with EDTA prior to loading onto the gradient were centrifuged 10 h at 145,000 × g. The absorbancy (——) was recorded during the collection of the gradient (see Materials and Methods) and samples of 50 μliters were added to Brays scintillation fluid for determination of radioactivity* (O). *In this particular experiment, ^{14}C-amino acids were added to the culture for 30 s. Panels A and B were derived from free polyribosomes and C and D were from membrane polyribosomes. The arrow shows the position of 70S ribosomes.*

distribution of total nascent chains, the enrichment of alkaline phosphatase on membrane was 1.3- to 1.8-fold.

Alkaline phosphatase nascent polypeptides were also detected by a chemical analysis of the amino-terminal region of the protein. Studies on the primary structure of this enzyme have shown that the amino terminal region can be isolated as a tetrapeptide containing threonine, proline, glutamic acid, and methionine (15a).

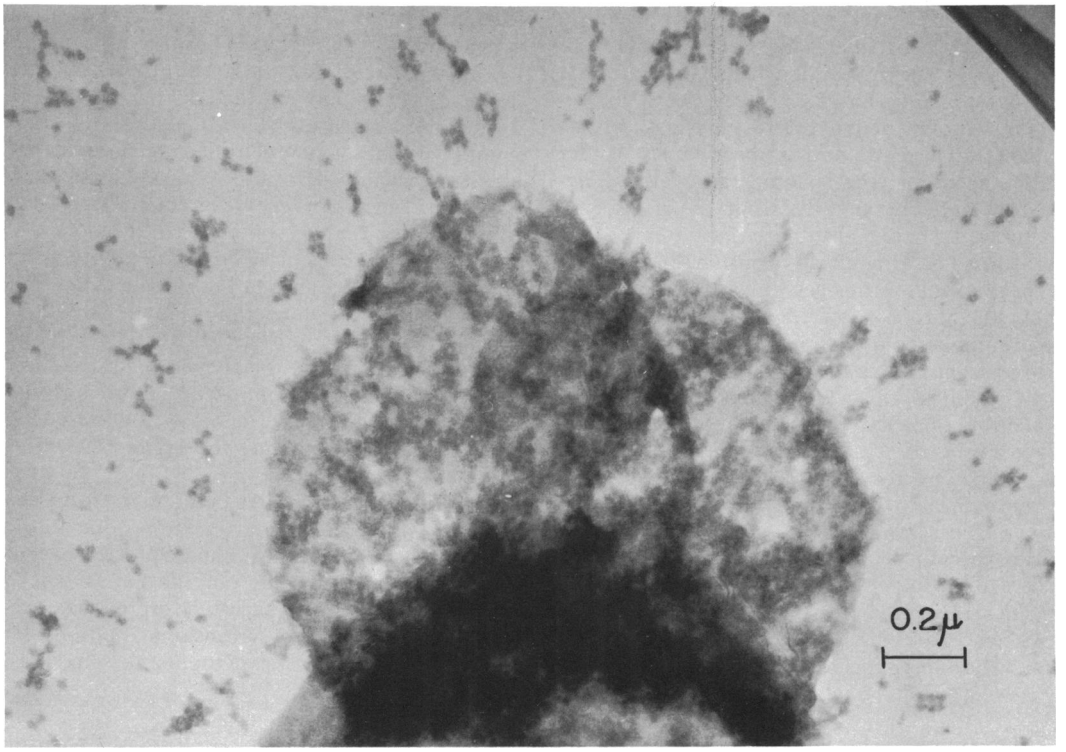

FIG. 3. *Electron micrograph of intact membranes. Samples from the interface of 1.8 to 0.5 M sucrose of the discontinuous gradient were applied directly to electron micrograph grids (see Materials and Methods). Magnification ×66,800.*

TABLE 3. *Distribution of alkaline phosphatase nascent chains*

Strain	Net amount[a] of ^{14}C-polypeptide recovered in antibody-antigen precipitins (counts/min)		Amino-terminal tetrapeptide (counts/min/nmol of proline)		Nascent chains on membrane polyribosomes (%)		
	Membrane	Cytoplasm	Membrane	Cytoplasm	Total	Specific	Enrichment[b]
CW3747	1,232 (5.8)[c]	167 (1.8)			70	89	1.27
CW3747	12,160 (6.8)	4,200 (1.6)			40	74	1.85
CW3747	25,120 (5.1)	9,240 (1.8)			48	73	1.52
CW3747	4,215 (6.6)	1,870 (2.6)			47	69	1.47
CW3747	5,420 (5.7)	3,420 (1.9)			35	61	1.74
K-10	1,640 (0.5)	<100					
CW3747			54.5	12.8	61	83	1.36
W3747			5.9	3.7			

[a] The ^{14}C-protein precipitated with the nonspecific antibody "control" has been subtracted; in all cases this background level was on the order of 0.5 to 1.0% the total counts added to the antigen-antibody reaction mixture.

[b] Enrichment = percentage of alkaline phosphatase ÷ percentage of total nascent chains.

[c] The values in parentheses represent the percentage of the total labeled protein that was precipitated by the specific antibody.

The nascent chains, labeled with ^{14}C-proline, were mixed with carrier protein and subjected to a procedure that ultimately led to purification of the amino-terminal tetrapeptide. The final purification step is depicted in Fig. 4, and the amount of ^{14}C-proline, recorded as specific activity, in the pure peptide is in Table 3. In this analysis the amounts of carrier pure enzyme added to both membrane and cytoplasmic nascent chains were identical, and orders of magnitude in excess of the amount of nascent alkaline phosphatase. Under these conditions, the specific radioactivity accurately reflects the distribution of labeled chains and further eliminates differences that might arise as a result of variations in recovery of the peptide. From several experiments, we detected 75 to 85% of the nascent chains on membrane-associated polyribosomes. Of the total nascent chains recovered from both sets of polyribosomes, those recognized antigenically as alkaline phosphatase accounted for 4% of the labeled protein. This value agrees closely with the value of 5% recorded for the fraction of label in the complete alkaline phosphatase enzyme synthesized by the CW3747 strain during exponential growth.

The following argue strongly that the polypeptides recovered from polyribosomes are indeed nascent chains of alkaline phosphatase. First, the polypeptides were recovered from polyribosomes that were centrifuged through 1.8 M sucrose and subsequently released by EDTA. Second, the chemical analysis was based on the amino-terminal peptide. For a 30-s pulse, most of the added label in the amino-terminal peptide would be expected to remain on the polyribosome because the rate of protein synthesis under our experimental conditions (12 to 15 amino acids per s [8]) would allow for not quite the entire polypeptide (420 residues) to be formed and released. Third, analysis of fragments of these polypeptides, generated by cleavage with CNBr and separated on G-75 Sephadex, shows a very different pattern than that recorded for the complete, intact polypeptide (Fig. 5). The ^{3}H-CNBr fragments from complete subunits show a profile matching that of the unlabeled, carrier protein indicated as OD_{230}. The distribution of ^{14}C-CNBr fragments derived from the polyribosomes does not match the completed chains, and more material is in smaller fragments. This is illustrated by the variations in values of ^{3}H-to-^{14}C ratios ranging from 50 to 9.

lac and trp mRNAs on membrane-associated polyribosomes. To determine if the enrichment of alkaline phosphatase nascent peptides on membrane polyribosomes was a feature unique for a secreted protein, we assayed the different polyribosome fractions for mRNAs that code for cytoplasmic enzymes.

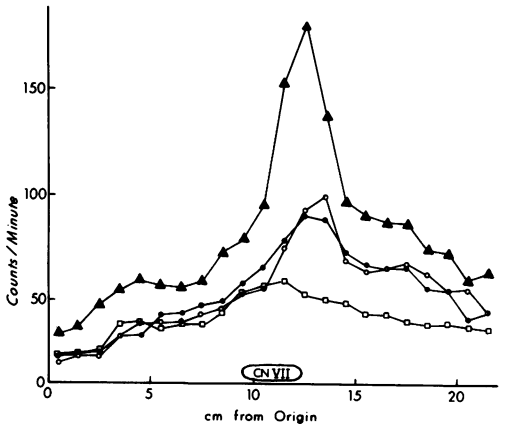

FIG. 4. *Purification of the amino-terminal cyanogen bromide fragment from alkaline phosphatase by paper chromatography. See Materials and Methods for details. Nascent polypeptides were analyzed from strains W3747 cytoplasmic (□) and membrane (○) polyribosomes and strain CW3747 cytoplasmic (●) and membrane (▲) polyribosomes. The circled CNVII refers to the position of a pure sample of the cyanogen bromide amino-terminal fragment stained for the presence of peptide.*

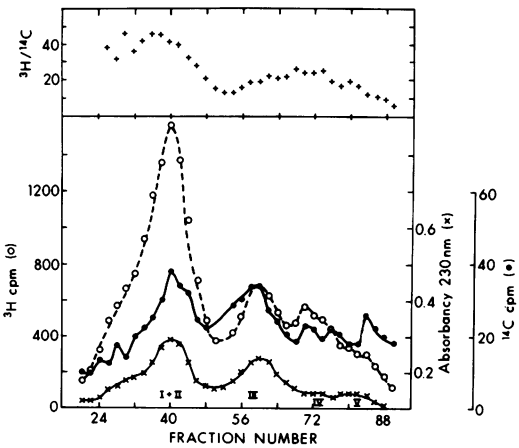

FIG. 5. *Analysis of cyanogen bromide-treated "complete" and "nascent" polypeptides of alkaline phosphatase. Complete chains were from periplasmic fractions of cells labeled 60 min with ^{3}H-amino acid (○); nascent chains were from polyribosomes of cells labeled for 30 s with ^{14}C-amino acids (●). Absorbancy at 230 nm (×). Roman numerals refer to the different, distinct cyanogen bromide fragments. See Materials and Methods for details.*

The technique of nucleic acid hybridization was used to measure the amounts of *lac* and *trp* mRNA extracted with phenol from the isolated polyribosomes. Two bacterial strains were tested for *lac* mRNA; one of them contains a μ phage-induced deletion of the *y* and *a* genes; the former codes for the membrane-bound M protein of the *lac* operon (10). The total amount of ^{3}H-uridine-labeled mRNA from the whole cell, membrane, and cytoplasmic fractions that formed RNase-resistant hybrids with the respective DNA fractions is presented in Table 4. When compared to the distribution of total mRNA between bound and free polyribosomes, the enrichment factor for *lac* and *trp* mRNA on the bound polyribosomes was 1.1 to 1.4. These values are maximal and probably too high because the free polyribosomes appear to have degraded to a greater extent than the membrane polyribosomes (see Fig. 2).

Attempts to detect a selective interaction between alkaline phosphatase polysomes and membranes. The biochemical analyses suggested that there was enrichment on membranes for those polyribosomes making a secreted protein. Even so, 50% or more of an mRNA coding for a nonsecreted protein was isolated from membrane-associated polyribosomes. There was the possibility, however, that polysomes carrying secreted peptides were more tightly bound than the others in a manner analogous to that reported for some mammalian cells (28, 30, 40; M. Zauderer, personal communication). We were encouraged to look for qualitative differences in the bacterial membrane-associated polysomes on the basis of electron micrographs of thin sections of the glutaraldehyde-fixed membrane fractions. A representative sample of membranes analyzed in this way is presented in Fig. 6. Three types of vesicles are observed: one shows few if any polyribosomes and is essentially a nude-bilayer membrane, a second shows polyribosomes in a matrix almost filling the vesicle, and the third shows a small number of polysomes distributed along the inside periphery of the vesicles (arrows, Fig. 6).

We attempted to separate those vesicles with peripheral membranes from the others by equilibrium density centrifugation in a much narrower gradient using, in one case, a linear gradient of 50 to 60% sucrose and, in the second, a discontinuous gradient with equal steps of 5% intervals from 30 to 50% and a cushion of 60% sucrose. However, in both cases, a single symmetrical peak of label (RNA or protein) was detected banding at 54.5% sucrose.

Another series of experiments designed to detect tightly bound polyribosomes containing alkaline phosphatase nascent chains included the following: (i) additional freeze-thaw treatment of isolated membranes, but under conditions in which when half the mRNA and nascent protein were released, a concomitant amount of alkaline phosphatase material was lost from membranes; (ii) mild incubation of the membrane fraction with RNase (2 μg/ml at 4 C for 5 min), based on the assumption that monosomes carrying alkaline phosphatase would be anchored to the membrane through an interaction of the peptide chain with mem-

TABLE 4. *Distribution of lac and trp mRNA between cytoplasm and membrane fractions*[a]

Strain	RNA hybridized to *lac* DNA (counts/min)[b]			RNA hybridized to *trp* DNA (counts/min)			Percent on membranes		
	Whole cell	Membrane	Cytoplasm	Whole cell	Membrane	Cytoplasm	Total[c]	Specific	Enrichment
129, induced with IPTG	ND[d]	3,853	1,497				49	79	1.42
06, induced with IPTG	8,551	4,880	1,452				72	77	1.07
129, induced with indole-propione acid and IPTG	456	267	84	9,218	7,117	1,130	69	76-85	1.1-1.24
129, not induced	79	48	29	198	197	83			

[a] See Materials and Methods for details of the extraction of RNA and the methods for measuring hybridization.

[b] The counts per minute from the actual sample measured have been corrected to account for the total amount of hybridized RNA in that fraction.

[c] A 2′ pulse-labeled polysomal RNA consists almost exclusively as mRNA.

[d] ND, Not done.

branes; but, again, even though half of the RNA and half the amount of nascent chains were released from the membranes, no enrichment of the remaining peptides for antigenically competent alkaline phosphatase was detected; (iii) mild incubation of membrane with trypsin (2.5 μg/ml at 4 C for 5 min), but after a 50% loss of half the peptides and virtually no loss of RNA, the relative amount of antigenic alkaline phosphatase peptides also decreased by 50%.

Finally, we attempted a reconstruction experiment by mixing all cellular polyribosomes (obtained by lysing spheroplasts with 1% Triton X-100) with membranes that were depleted of all ribosomes (by including EDTA in our normal procedure). The polyribosomes, labeled with both ^{3}H-uridine (long label) and ^{14}C-amino acids (pulse label), were centrifuged through the discontinuous sucrose gradient normally used for preparing the membranes and suspended in TMK buffer prior to mixing with membranes. Mixing was done at either 0 or 30 C, and the membranes were reisolated in the discontinuous gradient. We detected between 15 and 30% of the polyribosome counts per minute on the membrane fraction; however, we could not detect significant amounts of antigenic alkaline phosphatase in the in vitro-prepared membrane polyribosomes. We also were unable to detect differences in binding whether we used polyribosomes or membranes from strain W3747 or CW3747 even though the latter strain secretes three times more protein into the periplasm than the former (22).

Polysomes on membranes isolated in the absence of lysozyme and chloramphenicol. There are reports claiming that the association of bacterial ribosomes is an artifact arising from the presence of lysozyme in the extraction procedures (24). To rule out possible artifacts in this work, we prepared membranes from a fragile mutant of *E. coli*. With this strain, cell lysis can be achieved in the absence of lysozyme (19). We also tested the effect of chloramphenicol by analyzing polysomes in a culture not treated with this drug. Results are presented in Table 5 and show that, in the particular experiments, the distribution of RNA in polysomes and ribosomes derived from either a cytoplasmic or membrane fraction was not affected by

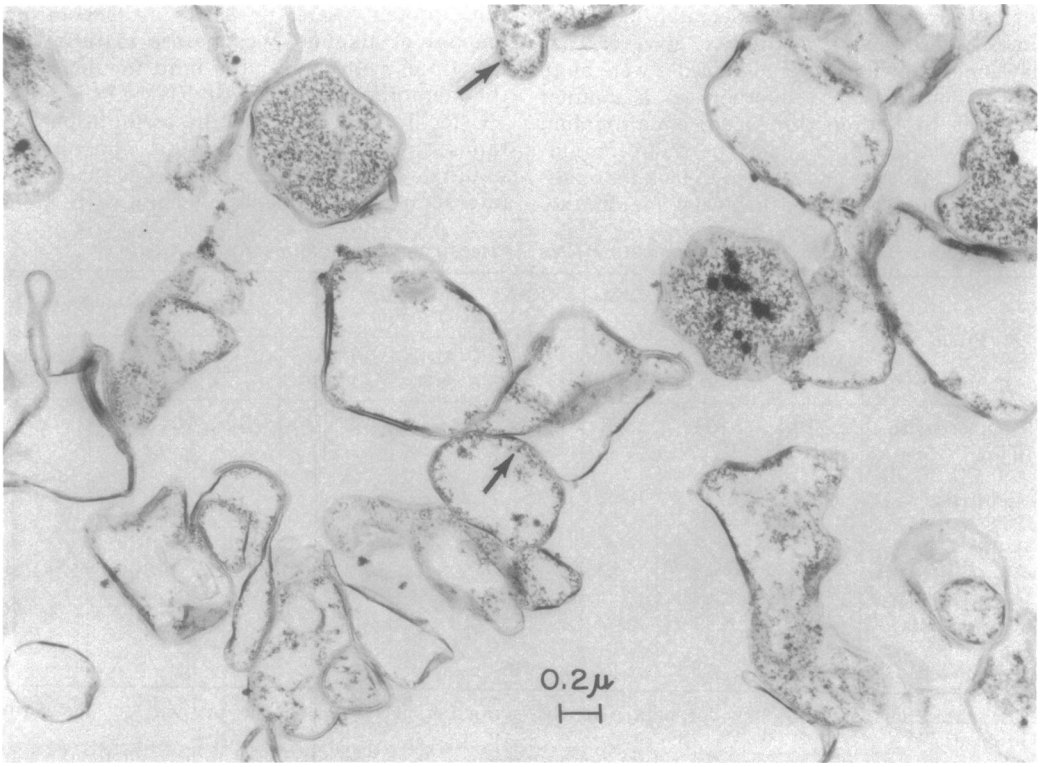

FIG. 6. *Electron micrograph of thin section of glutaraldehyde-fixed membranes. Magnification* ×*39,000. Arrows show membranes with peripheral polyribosomes.*

TABLE 5. *Effect of lysozyme and chloramphenicol on distribution of polyribosomes between cytoplasm and membrane*

Strain	Conditions	Cytoplasmic polyribosomes ($\times 10^3$ counts/min)	Membrane polyribosomes ($\times 10^3$ counts/min)
Sud 24	+ lysozyme	926	491
	− lysozyme	930	452
K-10	+ chloramphenicol	1094	433
	− chloramphenicol	1244	393

the presence of lysozyme or chloramphenicol.

DISCUSSION

These experiments show that preparations of isolated *E. coli* membranes contain a large amount of the cell's polyribosomes. Consistently, we observed from 40 to 60% of the cellular ribosomal and mRNA on membranes and a similar distribution of nascent polypeptides. Values as high as 70% of pulse-labeled RNA were recovered in a membrane fraction, and it may well be that most of the bacterial polyribosomes interact in some manner with the cell membrane. There have been numerous reports that bacterial polyribosomes are associated with membranes. Our colleague, D. Schlessinger, reported a binding of polyribosomes from *Bacillus megaterium* to membranes and measured their capacity to form protein (37, 38). Although he did not characterize his "bound" polyribosomes with respect to the membrane fraction nor analyze the distribution of specific mRNAs, much of his data are similar to those presented here. Hendler has presented data showing that preparations of *E. coli* membranes can effectively synthesize protein (14). The polyribosome fractions isolated in the experiments reported here were also capable of incorporating ^{14}C-amino acids into trichloroacetic acid-precipitable material (data not presented). Both cytoplasmic and membrane polyribosomes responded in a similar manner when supplemented with an S-100 fraction and an adenosine triphosphate-generating system. Incorporation of amino acids reached a maximum level after 20 min at 37 C. We were unsuccessful in detecting the formation of significant amounts of antigenic alkaline phosphatase chains in these in vitro protein synthesis experiments.

Data has been presented which rule out the possibility that the binding is artifactual as a result of using lysozyme or chloramphenicol in the extraction procedure. We cannot, however, eliminate the possibility that the membrane vesicles have entrapped the large aggregates of polyribosomes observed in the electron micrographs. During preparation of these membranes, soluble enzymes leave the vesicles, and DNase thoroughly degrades cellular DNA. The latter result precludes any possible binding of polyribosomes to membranes mediated by DNA. The membrane polyribosomes were also susceptible to added RNase and trypsin, proteins that are unlikely to penetrate intact vesicles.

Our goal in this project was to determine if polyribosomes making secreted proteins are localized on the cell membrane. The data reported clearly show a significant enrichment of nascent alkaline phosphatase chains on membrane polyribosomes. In addition, we found mRNAs on membrane-associated polyribosomes that code for cytoplasmic enzymes as well, but the enrichment of the latter kind of polyribosomes on membranes was not as great as that observed for the secreted enzyme.

To account for our results we considered two hypotheses. (i) All polyribosomes are associated in some manner with membranes, but those making secreted protein are more tightly bound than the others. Cytoplasmic polyribosomes are artifacts of the preparation and fractionation of cell extracts. (ii) Polyribosomes making secreted proteins are bound to membranes whereas those making cytoplasmic proteins form within membranes during the fractionation. Attempts to resolve these possibilities by altering the fractionation procedures and by reconstructing in vitro membrane-associated polyribosomes have so far been unsuccessful. The electron micrographs of thin sections of the membranes are consistent with the notion that a unique set of polyribosomes may be associated with membranes, but our biochemical data do not permit us to conclude that these polyribosomes include those making alkaline phosphatase polypeptide chains.

Data have been published which suggest that biosynthesis of secreted bacterial proteins occurs on sites distinct from those making intracellular proteins (7, 12). We once attempted to visualize these postulated membrane sites in a series of experiments carried out with M. Bayer, Fox-Chase Cancer Institute. Cultures of *E. coli* K-10 and two alkaline phosphatase-negative mutants were grown under conditions of both repression and derepression for alkaline phosphatase. Pellets of bacterial cells (10^{10} cells) were freeze-etched, and electron micrographs were prepared. Extensive areas of bacterial

plasma membranes from the different cultures were examined, but no consistent, clear structures emerged that could be considered sites unique for secretion of proteins.

Studies on secretion of proteins in the eukaryotic cell have led to the generalization that polyribosomes bound to membrane of the rough endothelium reticulum are those involved in forming the secreted polypeptide (26, 28, 39). Some recent data suggest, however, that secreted immunoglobulins are first made on nonmembrane polyribosomes and further show that the immunoglobulin polypeptide initially formed in the cell is distinct from the secreted protein with respect to the amino-terminal portion of the protein (20). On this basis, a model has been proposed in which information in the amino-terminal section of the polypeptide is the determinant for the secretion process (20). This portion of the molecule interacts with the membrane, either as a result of its hydrophobic structure or by binding to a specific receptor protein already in the membrane. With the nascent polypeptide chains anchored in the membrane, the polyribosome becomes membrane bound, and subsequent synthesis of the protein provides the energy for transport. Parts of this model have been presented by others who referred to the secretory process as a vectorial discharge of the growing nascent chains (4, 26). We have preliminary evidence that the amino-terminal portion of the alkaline phosphatase nascent chain is distinct from that of the isolated, pure enzyme, and we are currently analyzing this part of the nascent polypeptide. The data presented here showing 70 to 90% of nascent alkaline phosphatase protein on membrane-bound polyribosomes lend support to this kind of model for secretion of an *E. coli* protein.

ACKNOWLEDGMENTS

We thank David Kennell for his advice and assistance with the nucleic acid hybridization experiments and for providing *lac*-DNA preparations. We thank Charles Yanofsky for kindly supplying ϕ80dtrp DNA. We are indebted to Sigrid Thach and J. R. Williamson for preparing electron micrographs.

This study was supported by a Public Health Service grant (2 RO1-AI-08593) from the National Institute of Arthritis and Metabolic Disease. One of us (R.C.) was partially supported by a Fellowship from the World Health Organization.

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