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Biochemistry

Extracellular labeling of nascent polypeptides traversing the membrane of *Escherichia coli*

(alkaline phosphatase/protein synthesis/protein secretion/membrane-bound polysomes)

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ABSTRACT **To provide direct evidence for the hypothesis that secreted proteins may traverse membranes as growing chains, we labeled spheroplasts of *Escherichia coli* with a reagent (acetyl[^{35}S]methionyl methylphosphate sulfone) that reacts with amino groups but does not cross the membrane. After fractionation, about 6% of the label in the membrane–polysome fraction was found to be attached to the polysomes. This attachment was via peptidyl-tRNA, as shown by several tests: release of most of the label from purified polysomes at low Mg^{2+}; subsequent loss of about 25,000 daltons on cleavage by dilute alkali; release by puromycin; and release, accompanied by a marked increase in average molecular weight, on peptide chain completion. Moreover, a significant fraction of the completed chains was identified serologically and by molecular weight as a major periplasmic protein, alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1]. This work provides direct evidence that: (*i*) secreted proteins thread through the membrane as growing peptide chains; and (*ii*) membrane-associated polysomes in bacteria are functionally attached to membrane and not merely trapped on disruption of the cell.**

Over the past decade considerable attention has been focused on the mechanism of secretion of extracellular proteins (1, 2). In animal cells a special role for the ribosomes bound to the endoplasmic reticulum was recognized early by Palade and coworkers (see ref. 1), who observed a parallel between the abundance of such ribosomes and the secretion of proteins. Working with extracts in the same laboratory, Redman and Sabatini (3) found that puromycin released incomplete chains to the interior of the microsomes, and they accordingly suggested that the secreted protein passes through the membrane as a growing chain. Blobel and coworkers (4–6) have provided further evidence for this model: in extracts freed of membrane the mRNA from pancreatic cells yields precursors of several secretory proteins, while homologous membrane present during chain growth cleaves an NH_2-terminal segment of about 20 amino acid residues from these precursors, converting them into the final secreted products. Moreover, the eliminated residues are predominantly hydrophobic and are rather uniform in various proteins, which suggested that this "signal" region initiates passage across the membrane. A similar mechanism of protein secretion has been proposed for bacteria (7) on the basis of the evidence from animal cells. This proposal is supported by recent evidence that some secreted bacterial proteins are synthesized as precursors from which a hydrophobic segment (8), or a segment carrying a terminal phosphatide (9, 10), is later cleaved.

In contrast to chains that might protrude into the endoplasmic reticulum in animal cells, chains protruding across the membrane in bacterial spheroplasts or protoplasts should be readily accessible to the external medium. We have therefore undertaken to test the model of chain extrusion by radioactively labeling exposed amino groups on *Escherichia coli* spheroplasts. The reagent used, ^{35}S-labeled acetylmethionyl methylphosphate sulfone (AMMP), is an analogue of one designed by Bretscher (11), formylmethionyl methylphosphate sulfone, which cannot penetrate animal cells. When the labeled spheroplasts were disrupted and fractionated, the polysomes recovered from the membrane–polysome fraction contained radioactivity, and it was shown by several criteria to be attached as peptidyl-tRNA. Moreover, when the labeled polysomes were allowed to complete and release their chains, a portion of the labeled product was identified as a periplasmic protein, alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1]. These results show that at least some secreted proteins cross the bacterial cell membrane (and presumably other membranes) as growing chains.

These findings have additional significance for bacterial physiology. Since electron microscopic sections cannot distinguish membrane-bound and free polysomes in bacteria, it has not been certain whether the membrane-associated ribosomes observed in bacterial lysates are functionally attached or have been artificially trapped in the vesicles formed during cell disruption. A functional binding was suggested earlier by the finding (12) that the membrane-associated polysome fraction of *E. coli* contained two to four times as much alkaline phosphatase as did the free polysome fraction. The present results decisively establish the existence in bacteria of membrane-bound polysomes with a special functional role.

MATERIALS AND METHODS

Bacterial Strains and Growth. *E. coli* K12 CW3747, Met$^-$ and constitutive in the synthesis of alkaline phosphatase, was obtained from the American Type Culture Collection (no. 27259). Cells were grown at 37°, with vigorous aeration (unless otherwise indicated), in minimal medium A (13) supplemented with 0.4% glucose and 0.2% Casaminoacids.

Synthesis of Acetyl[^{35}S]methionyl Methylphosphate Sulfone (AMMP). [^{35}S]Methionine of very high activity (400–600 Ci/mmol), obtained in aqueous solution from New England Nuclear Corp., was converted to the *N*-acetyl derivative by dissolving 1 mCi in 100 μl of 1 M aqueous pyridine and adding 3 equivalents of acetic anhydride over a 20-min period at 0°. The extent of acylation was monitored by applying a 1-μl sample to a Quanta-Gram thin-layer chromatography plate and determining the radioactivity comigrating with a methionine or with an *N*-acetylmethionine standard in a butanol/acetic acid/water solvent (4:1:1). Generally more than 95% of the methionine was acetylated.

After acylation, pyridine was removed under reduced

Abbreviations: AMMP, acetylmethionyl methylphosphate sulfone; buffer A, 10 mM Tris·HCl (pH 7.6)/50 mM KCl/10 mM $Mg(OAc)_2$; M_r, molecular weight; $NaDodSO_4$, sodium dodecyl sulfate.

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pressure, the solution was acidified (pH 1.5) with HCl, the *N*-acetylmethionine was extracted with 10 volumes of ethyl acetate, and the ethyl acetate was dried with anhydrous Na_2SO_4 and removed under reduced pressure. *N*-Acetylmethionine was converted to the methylphosphate sulfone according to the procedure of Bretscher (11).

Preparation and Extracellular Labeling of Spheroplasts. Chloramphenicol (200 μg/ml) was added to an exponentially growing culture (20 ml), the culture was poured onto excess ice, and the cells were pelleted by brief centrifugation. Spheroplasts were formed by the freeze-thaw-lysozyme procedure of Ron *et al.* (14), in the presence of chloramphenicol and 25% sucrose, and was usually greater than 95% as monitored by microscopy.

The spheroplasts were resuspended in 0.5 ml of a 25% sucrose solution containing 10 mM $Mg(OAc)_2$/10 mM NaH_2PO_4 (pH 8.0). Approximately 40 μCi of [^{35}S]AMMP (600 Ci/mmol) was added and the mixture was shaken gently for 20 min at 0°. The spheroplasts were pelleted by low-speed centrifugation, washed three times with 25% sucrose solution in buffer A [Tris·HCl (10 mM, pH 7.6)/KCl (50 mM)/$Mg(OAc)_2$ (10 mM)], and lysed by suspension in 1–2 ml of buffer A. Electrophoretically pure DNase (Worthington, 5 μg/ml) was added, and unlysed spheroplasts and debris were removed by centrifugation at 1200 × *g* for 10 min.

Preparation of Membrane-Associated and Free Polysomes. The lysate was layered on a discontinuous sucrose gradient made of 3 ml of 1.8 M sucrose and 2 ml of 1.0 M sucrose, each in buffer A. After centrifugation for 18.5 hr at 42,500 rpm in an SW 50.1 rotor, three fractions were collected: the pellet (free ribosomes), a band about 1 cm from the top of the gradient (free membranes), and 2 ml from the interface of the sucrose layers (membrane-associated polysomes). The pellet was dissolved in buffer A, and the free membrane fraction was diluted with buffer A and concentrated by centrifugation; both fractions were dialyzed against 1000 volumes of buffer A and stored at −76°. The interface fraction was similarly concentrated and was then purified by centrifugation through a second discontinuous sucrose gradient under the same conditions.

The polysomes in this fraction were freed of membrane by washing three times with 1% Na deoxycholate. In the first washing the interface fraction was mixed with 0.1 volume of chilled 10% deoxycholate, layered on a discontinuous gradient, and centrifuged for 18 hr at 42,500 rpm. Under these conditions the polysomes, relatively freed of membrane, were pelleted. In the two subsequent washings the polysomes were suspended in buffer A plus deoxycholate and repelleted. The final pellet was dissolved in buffer A, dialyzed against 1000 volumes of the same buffer, and stored at −76°.

Chain Completion and Immunological Detection of Alkaline Phosphatase. One A_{260} unit of purified polysomes from the labeled membrane–polysome fraction was allowed to complete its nascent chains by incubation for 25 min at 37° in 0.1 ml of a protein-synthesizing system, as described (15). (One A_{260} unit is that amount of material that has an absorbance at 260 nm of 1 when dissolved in 1 ml of solvent at a pathlength of 1 cm.) The molecular weight (M_r) distribution of the label was determined by sodium dodecyl sulfate ($NaDodSO_4$)/disc gel electrophoresis (16); radioactivity was measured on 1-mm slices incubated in toluene-based scintillation fluid containing 3% Protosol. In an identical sample alkaline phosphatase was identified by adding an optimal proportion (5 μl) of rabbit antiserum. The volume was brought to 1.0 ml with 0.14 M NaCl/0.01 M NaH_2PO_4, pH 7.4. After incubation at 32° for 3 hr, 175 μl of goat antiserum to rabbit globulin was added and the mixture was incubated at 4° for 16 hr. Precipitates were collected by centrifugation at 30,000 × *g* for 10 min, washed three times with cold 0.14 NaCl/0.01 M NaH_2PO_4, and analyzed by $NaDodSO_4$/disc gel electrophoresis and measurement of the radioactivity, as above.

Table 1. Distribution of RNA and lipid among various fractions

Fraction	Radioactivity, cpm [^{3}H]Lipid	[^{14}C]RNA
Pelleted material (free polysomes)	200	76,500
Interfacial material (membrane-associated polysomes)	86,500	87,600
Top of gradient (free membranes)	221,200	1,200
Deoxycholate-treated interfacial material	250	84,700

Cells of *E. coli* CW 3747 were grown in the presence of [^{3}H]oleic acid and [^{14}C]uracil for two generations. Cells were harvested, converted to spheroplasts, and fractionated as described in *Materials and Methods*. Deoxycholate treatment (bottom line) consisted of three washings with 1% deoxycholate (*Materials and Methods*).

Labeling of RNA, Lipids, and Nascent Peptide. To label ribosomes we grew cells for two generations with either [^{14}C]uracil (1.8 μM, 0.06 μCi/ml) or [^{3}H]uracil (0.75 μM, 1 μCi/ml). To label membranes we grew cells for two generations with 1.2 μM radioactive oleic acid, containing either ^{14}C (0.06 μCi/ml) or ^{3}H (1.0 μCi/ml). To label nascent peptides we pulsed cells with ^{3}H-labeled amino acids (35 Ci/mmol) for 15 sec, added chloramphenicol (200 μg/ml), and poured the culture over ice.

Reagents. Rabbit antiserum to alkaline phosphatase was kindly provided by Michael H. Malamy of Tufts Medical School, and goat antiserum to rabbit globulin by Laurence Levine, Brandeis University. S-100 fractions were prepared from *E. coli* MRE600 as described (15). [^{14}C]Oleic acid, [^{3}H]oleic acid, [^{3}H]uracil, [^{14}C]uracil, and Protosol were obtained from New England Nuclear Corp. All other chemicals were of reagent grade.

RESULTS

Separation of Membrane-Associated and Free Polysomes. To fractionate *E. coli* spheroplasts in a manner that preserved polysome integrity yet efficiently separated free from membrane-associated polysomes, we stabilized the polysomes in the growing cells with chloramphenicol, converting the cells to spheroplasts, disrupted the latter gently, and fractionated the lysate in a discontinuous sucrose gradient, described in detail above. As Table 1 shows, when cells labeled for two generations with [^{14}C]uracil and [^{3}H]oleic acid were fractionated by this procedure, the pellet (free polysomes) contained 50% of the RNA and negligible amounts of lipid; a fraction near the top of the gradient (free membranes) contained approximately 70% of total cell lipid and less than 1% of the RNA; and the membrane–polysome fraction contained 50% of the RNA and 30% of the lipid. These results verify the clean separation of free and of membrane-associated polysomes in the fractionation procedure used.

When the membrane–polysome fraction was treated with a 1% solution of deoxycholate and run on a second discontinuous gradient, the polysomes formed a pellet. As measured by [^{3}H]oleic acid, this pellet retained 5–15% of the lipid initially in that fraction. (This retention was evidently not sufficient to prevent pelleting.) After two further washings with 1% deoxycholate, the polysomes retained less than 0.4% of the initially associated membrane, and very little of the RNA was lost. Three

Table 2. Extracellular labeling of cellular components with [^{35}S]AMMP

Fraction	Radioactivity, cpm [^{35}S]-AMMP	Radioactivity, cpm [^{3}H]Oleic acid
Intact spheroplasts	2.0×10^6	3.0×10^5
Free membrane	1.4×10^6	2.2×10^5
Free polysomes	<200	<200
Membrane-associated polysomes	0.6×10^6	8.0×10^4
Membrane-associated polysomes after treatment with deoxycholate	4.0×10^4	<200

Cells were labeled with [^{3}H]oleic acid for at least two generations prior to harvesting. Spheroplasts were formed and were labeled with [^{35}S]AMMP (as described in *Materials and Methods*). They were fractionated as in Table 1.

washings with deoxycholate were therefore used in the subsequent experiments, in which it was essential to distinguish any external label attached to polysomes from that attached to membrane constituents.

Distribution of Extracellular Label in Spheroplasts. To label the amino groups accessible at the cell surface of the chloramphenicol-inhibited spheroplasts, incubation with [^{35}S]AMMP for 20 min at 0°, as described in *Materials and Methods*, was found to be optimal. Under these conditions about 20% of the label reacted with the cells, and there was little polysome breakdown.

As Table 2 shows, the free polysome fraction contained less than 0.001% of the ^{35}S reacted with the spheroplasts. This finding verifies that the labeling observed in the other fractions was entirely extracellular, as expected. It is further seen that 65% of that label was attached to free membrane and 35% to the membrane–polysome fraction. The bulk of the ^{35}S in the latter fraction was attached to membrane proteins and lipids, which were removed by three washings with deoxycholate; about 6% remained with the polysomes. As was observed above for [^{3}H]oleic acid, additional deoxycholate treatments removed little further ^{35}S.

Evidence for Attachment of Label to Polysomes as Peptidyl-tRNA. The presumed attachment of the extracellular label to the polysomes via growing chains was first tested by treating the purified (i.e., deoxycholate-treated) ^{35}S-labeled polysomes with 100 μg/ml of puromycin, in the presence of GTP and bacterial elongation factor G. In various experiments this treatment released 70–95% of the label, which no longer sedimented with the ribosomes. In a further test the purified polysomes were dialyzed against 10^{-5} M Mg^{2+}, which also released more than 75% of the ^{35}S-labeled material. For further characterization, part of the latter released material was treated with dilute alkali in order to cleave the tRNA from the peptide, and the M_r distribution of label in the treated and the untreated samples was determined by Sephadex gel filtration. As Fig. 1 shows, the labeled material released by low Mg^{2+} had a heterogeneous M_r distribution, with an average of 53,000, while after the alkali treatment the average M_r decreased by approximately 25,000, as expected for loss of tRNA.

As a further test, the purified ^{35}S-labeled polysomes were incubated under conditions that allowed resumption of protein synthesis, in the presence of ^{3}H-labeled amino acids, and about 75% of the ^{35}S was found to be released, presumably by chain completion. Moreover, the M_r distribution, now determined by $NaDodSO_4$/disc gel electrophoresis, was very similar for the

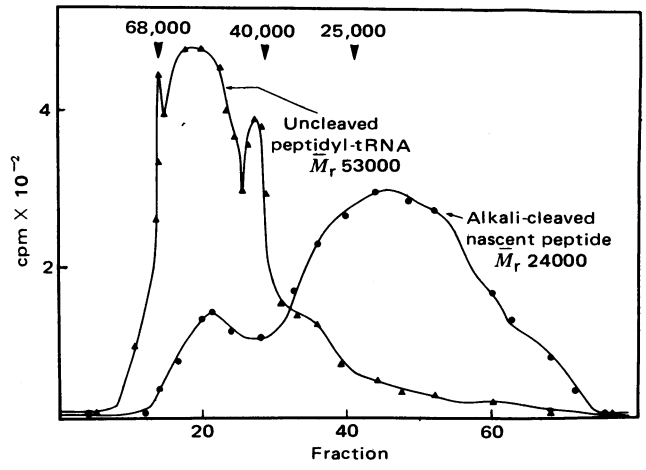

FIG. 1. Attachment of [^{35}S]AMMP to polysomes via nascent peptidyl-tRNA. To release peptidyl-tRNA from purified, deoxycholate-washed polysomes, we dialyzed 2 A_{260} units of polysomes containing 20,000 cpm of ^{35}S, in 0.2 ml, against 200 ml of 10 mM KOAc (pH 5.0)/10^{-5} M $Mg(OAc)_2$ for 16 hr at 0°. Half of the mixture (▲) was adjusted to 500 mM KCl and applied to a 1 × 60-cm Sephadex G-75 column which had been equilibrated with 10 mM KOAc (pH 5.0)/10 mM $Mg(OAc)_2$/500 mM KCl. The column was eluted with the same buffer, and 0.5-ml fractions were collected. Radioactivity was measured in an Isocap 300 counter after 10 ml of a Triton/xylene scintillation fluid was added. The other half of the dialyzed mixture (●) was treated with 0.1 M NaOH at 37° for 10 min to cleave tRNA from peptidyl-tRNA. The mixture was neutralized and analyzed on a Sephadex G-75 column as above.

^{35}S and the ^{3}H (data not shown). Finally, as Fig. 2 shows, the average M_r was markedly increased in the completed ^{35}S-labeled chains, compared with the chains released by puromycin.

Fig. 2 also reveals several discrete M_r peaks in the completed chains, including one at 43,000. This value suggested the presence of the monomer of alkaline phosphatase, a major secreted protein for which the bacterial strain being used is constitutive. In addition, when the completed chains were analyzed by Sephadex gel filtration at high-salt concentration, which should convert alkaline phosphatase to monomers, a

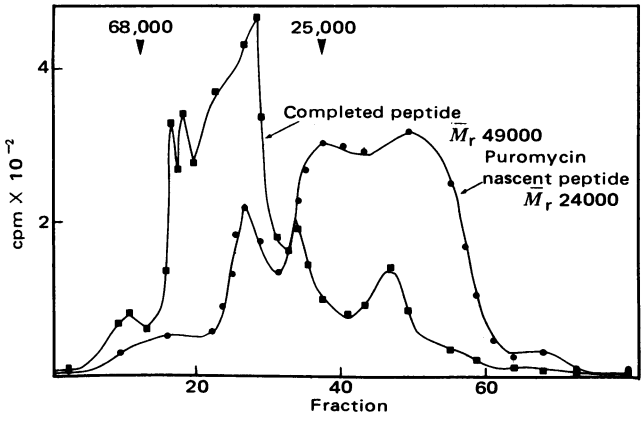

FIG. 2. Increase of mean M_r of ^{35}S-labeled peptides on chain completion. To release nascent peptides we incubated 1 A_{260} unit of purified membrane polysomes, containing 10,000 cpm, in 0.1 ml of buffer A with 20 μg of puromycin, 10 μg of elongation factor G, and 100 μg of GTP for 30 min at 37°. Another sample of the purified polysomes was incubated in a protein-synthesizing system to allow chain completion (see *Materials and Methods*). Both the puromycin-released nascent peptides (●) and the completed peptides (■) were analyzed by disc gel electrophoresis (see *Materials and Methods*).

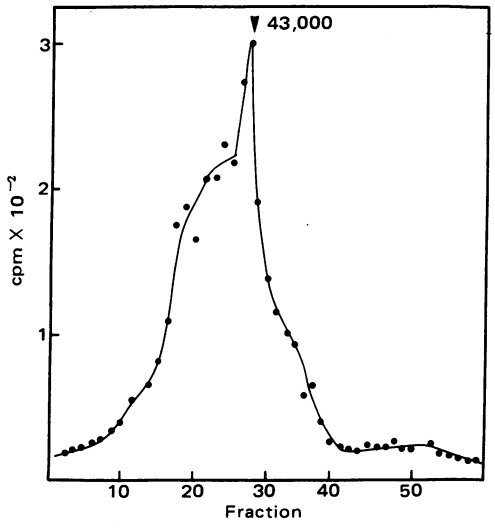

FIG. 3. Identification of ^{35}S-labeled alkaline phosphatase. One A_{260} unit of purified membrane polysomes, containing 10,000 cpm of ^{35}S, was incubated in a protein-synthesizing system. The mixture was reacted with antiserum to alkaline phosphatase and the precipitate was analyzed by disc gel electrophoresis (see *Materials and Methods*). The marker at 43,000 daltons was commercial alkaline phosphatase from *E. coli*.

similar peak was seen; while at low salt, which should preserve the dimers, this peak was replaced by a broad peak of M_r higher than 70,000 (data not shown).

Immunological Detection of Extracellularly Labeled Nascent Chains of Alkaline Phosphatase. For further identification of alkaline phosphatase, purified ^{35}S-labeled polysomes were allowed to complete their chains and the mixture was incubated with antiserum to the enzyme. In most experiments about 20% of the labeled completed peptides reacted with this antiserum, while less than 2% reacted with normal serum. In other controls the labeled polysomes were incubated with the antiserum prior to runoff, and only negligible (<1%) labeled material reacted (presumably because of specificity of the antiserum for the dimer of alkaline phosphatase).

To further characterize the ^{35}S-labeled material precipitated by the antiserum, we redissolved it by $NaDodSO_4$ and analyzed it by disc electrophoresis. Two peaks are seen (Fig. 3): a sharp one at 43,000 (the M_r of the alkaline phosphatase monomer), and a shoulder at 46,000–48,000. This pattern fits recent evidence that in a membrane-free DNA-directed system alkaline phosphatase is synthesized as a precursor, with an NH_2-terminal hydrophobic extension that can be cleaved by the outer membrane of the cell (8).

DISCUSSION

In this paper *E. coli* cells were fixed in protein synthesis by the addition of chloramphenicol, converted to spheroplasts, treated with a ^{35}S-labeled reagent (AMMP) that reacts with amino groups and cannot penetrate the membrane, and fractionated. Failure of AMMP to penetrate the cells was demonstrated by the extremely low level of labeling of the free polysome fraction compared with the membrane-associated polysomes (Table 2). An alternative possibility would be that the reagent did penetrate the cells and preferentially labeled the polysomes near the membrane. However, this mechanism can be excluded. It would attach label to ribosomes as well as to growing chains, whereas, in fact, release of the bulk of the chains from the membrane-bound polysomes by puromycin resulted in release of 70–95% of the label. In contrast, with free polysomes extracted from the cells and then labeled, puromycin released only 5% of the label (unpublished observations).

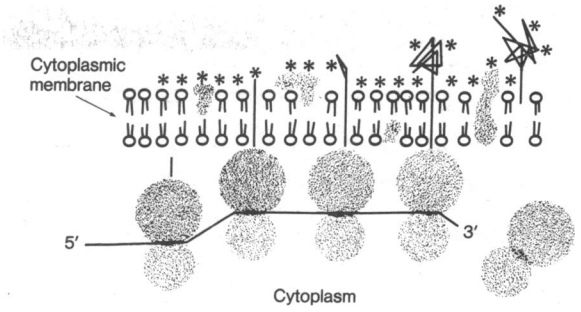

FIG. 4. Proposed model for secretion of proteins across membrane as growing chains and for their extracellular labeling. Asterisks, [^{35}S]AMMP.

When the membrane components were eliminated from the membrane–polysome fraction, by exhaustive washing with deoxycholate, about 6% of the label in this fraction remained with the polysomes. (A higher proportion has been obtained in preliminary experiments with the diazonium salt of [^{125}I]iodosulfanilic acid, which labels tyrosine and histidine residues.) The ^{35}S was bound to the ribosomes as peptidyl-tRNA, as shown by several tests: release by low Mg^{2+}; loss of M_r equivalent to tRNA on treatment with dilute alkali; release by puromycin; and release accompanied by increase in M_r on allowing chain completion. This last finding excludes the possibility that the label in the purified polysomes is due to contamination by completed membrane proteins or periplasmic proteins.

In the light of the evidence that membrane-bound ribosomes synthesize the membrane proteins in algal cells (17), it is probable that some of our labeled polysomal chains are also membrane proteins that are being incorporated into the cell membrane while still growing. However, we have also identified a significant fraction of the labeled nascent chains, serologically and by M_r, as the monomer and the larger precursor of a major periplasmic protein, alkaline phosphatase (Fig. 3). Hence, for at least one secreted protein in bacteria this work provides convincing, direct evidence for a 10-year-old hypothesis (3): that proteins are secreted across a membrane as growing chains (Fig. 4).

The extracellular label found on nascent peptides amounted to about 1–4 molecules of AMMP per 1000 membrane-associated ribosomes, or 10–40 per cell. It seems likely that this figure seriously underestimates the number of growing chains traversing the membrane. The cause does not appear to be early exhaustion of the reagent, since increase in its concentration or a further addition after the standard incubation led to only slightly more labeling (unpublished). Possible explanations include interference with excess of the reagent by the fragments of outer membrane and wall retained by the spheroplasts; blockage of the terminal NH_2 group by a formyl or other group (though ϵ-NH_2 of lysine should still be labeled); and loss of labeled chains during the extensive manipulation. In addition, if there should be a long segment of chain between the ribosome and the outer surface of the plasma membrane, many of the chains being secreted either would not yet be accessible to the labeling reagent or would no longer be attached to a ribosome.

The initial threading of secreted polypeptides into the membrane, or their induction of the formation of an appropriate pore in the membrane (4), may be reasonably explained by the presence of a hydrophobic initial "signal" segment on precursors of these proteins (6). However, the source of energy

for vectorial transport of the rest of the chain is obscure. With a suitable firm attachment of the ribosome to the inner surface of the membrane the energy of elongation might itself force the chain through the membrane, or if the ribosome is embedded across the membrane its nascent chain could be released on the outside. However, in *E. coli* either of these firm attachments to the membrane would evidently require a conformational effect of the growing chain, for we have observed that the attachment is lost when the nascent chains are released from the membrane-bound ribosomes by puromycin (unpublished). Since this finding also suggests quite a different alternative—that the ribosome may be attached to the membrane in bacteria solely by the nascent peptide—alternative sources of energy for the vectorial transport must also be considered. Thus, the spontaneous folding of the chain outside the membrane might exert tension on it, or the membrane might play a more elaborate role and itself apply energy to ensure the unidirectional transport of the chain that crosses it.

In addition to establishing a mechanism of protein secretion, the data presented here provide an answer to another old question: whether the membrane-associated ribosomes observed in the vesicles of disrupted bacteria are functionally attached or have been artificially trapped. Functional attachment is suggested by the reported (12) greater abundance of alkaline phosphatase chains on membrane-bound than on free polysomes, which we have confirmed (unpublished observations); and we have further found that a cytoplasmic protein, elongation factor G, is predominantly synthesized on free polysomes. The functional binding of ribosomes to the membrane in bacteria is now unequivocally demonstrated by the extracellular labeling of nascent chains attached to such ribosomes.

Note Added in Proof. For a detailed model of the passage of the hydrophobic and hydrophilic portions of a peptide chain across the bacterial membrane, see Lampen (18). Synthesis of a precursor of the *E. coli* outer membrane lipoprotein (19) and differential synthesis of excreted and intracellular proteins by membrane-bound and free polysomes of *E. coli* (20) have recently been reported.

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