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Interaction of secreted nascent chains with surrounding membrane in *Bacillus subtilis*

(membrane-bound polysomes/peptide length spanning membrane/dansylation/Pronase digestion/protein secretion)

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ABSTRACT To determine the length of secreted nascent polypeptide chain that is surrounded by membrane, we digested labeled nascent chains protruding from protoplasts of *Bacillus subtilis* with Pronase and isolated the residual ribosome-attached chains from the membrane–polysome fraction. Gel chromatography revealed a sharp major peak that had been protected by membrane plus bound ribosomes. The ribosomes themselves protected half as great a length. Because no free chain between the ribosome and the membrane was detected by Pronase treatment, the difference between the two protected lengths should measure the length protected by the membrane. More accurate measurements of these lengths, obtained by dansylation of the exposed NH_2 terminus of the isolated fragments, yielded a difference of 21 amino acids. This value corresponds to an extended chain of 75 Å, which is approximately the thickness of the bacterial cell membrane. We earlier presented evidence that bacterial ribosomes are attached to membrane solely by their secreted chain. The present results further show that after loss of the extracellular segment of the chain its attachment persists, at 37° as well as 0°C. These findings suggest that the chain does not slip through a passive membrane but is actively held within a channel.

Both in animal cells (1) and in bacteria (e.g., refs. 2 and 3) secreted proteins are synthesized as precursors with an extra NH_2-terminal "signal" segment (1, 4), which is cleaved during secretion (2, 5, 6). This segment is predominantly hydrophobic (2, 3, 7, 8). Because cytoplasmic proteins lack such a segment and are synthesized on unattached ribosomes (9, 10), the signal segment evidently directs the ribosome to the membrane. The hydrophobic nature of this segment further suggested that it enters the membrane and thereby initiates a process of threading the growing chain through it (5, 7). We have been able to demonstrate such cotranslational secretion by reacting a nonpenetrating radioactive reagent with amino acid residues on the outer surface of spheroplasts of *Escherichia coli* (11) or protoplasts of *Bacillus subtilis* (unpublished data): label was found on chains of secreted proteins that were still attached to ribosomes inside the cell. Moreover, the nascent chain appears to provide the only attachment of the ribosome to the membrane in bacteria (unpublished data; ref. 12) in contrast to the direct attachment reported for animal cells (13, 14) and chloroplasts (15).

The present work undertook to throw further light on the interaction of nascent chain and membrane by determining the length of chain protected by the membrane from Pronase digestion. The results revealed protection of a quite uniform length, of about 21 residues. Moreover, since these chains remain attached to the membrane after loss of their extracellular portion, it appears that they are held by forces in a surrounding channel and not simply by folding of their extracellular portion.

MATERIALS AND METHODS

Bacteria and Protoplasts. *B. subtilis* ATCC strain 6051a, constitutive for α-amylase, was grown at 37°C with vigorous aeration in 25 ml of minimal medium A (16) with 0.4% glucose, supplemented with 0.2% Casamino acids unless otherwise stated, and with labeled precursors as indicated. The cells (1.2×10^{10}) were pelleted in the cold by brief centrifugation and resuspended in 2 ml of 100 mM Tris·HCl, pH 8.0/20% (wt/vol) sucrose/200 μg of chloramphenicol per ml. Protoplasts were formed by adding 400 μg of lysozyme and incubating at 37°C for 5 min. Conversion was usually greater than 95%, as monitored by microscopy. The protoplasts were centrifuged at 4°C and lysed by suspension in 2 ml of buffer A [10 mM Tris·HCl, pH 7.6/50 mM KCl/10 mM $Mg(OAc)_2$]. Electrophoretically pure DNase (5 μg/ml) was added, and unlysed protoplasts and debris were removed by centrifugation at 3000 × *g* for 10 min.

Labeling of Nascent Peptides and Ribosomes. To label nascent peptides we pulsed a culture (25 ml) growing in minimal medium (5×10^8 cells per ml) with 20 μCi of a mixture of 15 ^{3}H-labeled amino acids (35 Ci/mmol) or with 20 μCi of [^{35}S]methionine (500 Ci/mmol) for 15 sec, and added chloramphenicol (200 μg/ml). The culture was quickly poured over ice and treated as described below. To label ribosomal RNA we incubated the cells for two generations with 25 μCi of [^{3}H]uracil (50 Ci/mmol).

Reagents. Pronase was obtained from Calbiochem; [^{35}S]-methionine, ^{3}H-labeled amino acids, and [^{3}H]uracil were obtained from New England Nuclear. Polyamide sheets were from Cheng Chin Trading Co. (Taipei, Taiwan). All other chemicals were of reagent grade.

RESULTS

With bacteria the secreted growing chains protruding from a protoplast membrane should be accessible to Pronase, and so it should be possible to determine the length of nascent chain that the membrane protects from cleavage. However, to obtain this value the length protected by protoplasts must be corrected for the length protected by the ribosome. Moreover, because our earlier observations (unpublished data; ref 12) indicated that, in bacteria, ribosomes are attached to membrane solely by the growing chain, it is also necessary to determine whether or not a significant length of chain is present between ribosome and membrane.

Effect of Pronase on Attachment of Ribosomes to Membrane. To test whether the ribosomes are uniformly held close against the membrane or whether there may be a detectable intervening length of chain, we isolated membrane–polysome complexes with [^{3}H]uracil-labeled ribosomes and then treated them with Pronase. The conditions used were similar to those shown by Malkin and Rich (17) to digest nascent peptide up to the ribosome without significantly digesting the latter; we

Abbreviation: buffer A, 10 mM Tris·HCl, pH 7.6/50 mM KCl/10 mM $Mg(OAc)_2$.

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confirmed the absence of significant digestion of ribosomes under our conditions. As Table 1 shows, ribosomes were not released from membrane by Pronase, though they could be released by puromycin. It thus appears that the ribosome is held very close to the membrane.

Estimation of Length of Chain Protected by Membrane and by Ribosomes. To determine the total length of nascent chain protected by membrane plus ribosome we pulsed cells with a mixture of ^{3}H-labeled amino acids, treated them with chloramphenicol to fix the nascent chains, and converted them to protoplasts. These were treated with Pronase, lysed, and fractionated, and the membrane–polysome complexes were isolated: the yield was essentially the same as that from protoplasts with intact extracellular chains. The complexes were incubated at 0°C for 10 min with 30 μg of RNase per ml to remove any "dangling" ribosomes; i.e., those indirectly attached to membrane via a shared mRNA (about 15%, unpublished data). The remaining membrane-bound ribosomes were largely freed of membrane components by washing with deoxycholate, and the residual nascent chains were recovered (see legend of Fig. 1). The M_r distribution of the chains was determined by Sephadex G-25 gel filtration. For comparison, the intact nascent chains were recovered from the membrane–polysome complexes from untreated protoplasts.

In addition, to determine how much of the protection of these residual chains was provided by the ribosome, we derived polysomes with labeled nascent chains from membrane–polysome complexes (by washing three times with deoxycholate) and similarly treated them with Pronase; the M_r distribution of their residual nascent chains was similarly determined. Free polysomes, treated and examined in the same way, give the same results as membrane-derived polysomes.

As Fig. 1 shows, the ribosomes protected about 30 residues per chain (M_r 3300), as previously reported for mammalian ribosomes (17); there was very little smaller or larger material. In the parallel experiments with protoplasts, about 50% of the residual chains, protected from cleavage by membrane plus ribosomes, appeared as quite a sharp peak, at M_r 6000 (about 55 amino acid residues). There was also a heterogeneous distribution of smaller sizes (presumably early chains) and larger sizes (presumably incomplete membrane proteins inaccessible to Pronase). Hence the sharpness of the major peak indicates that the membrane protects quite a uniform length of nascent secreted chains.

Since this peak contained about 55 residues and the peak from ribosomes contained about 30, it appears that a length of about 25 residues spans the membrane during secretion. Analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis yielded similar sharp peaks, with M_r values very similar to those obtained with Sephadex (results not shown).

Dansylation. Determination of the length of such small polypeptides by gel filtration and gel electrophoresis may be subject to error due to conformational effects. To obtain a more accurate measurement we pulse-labeled cells with [^{35}S]methionine and treated the protoplasts and membrane-derived polysomes with Pronase as above. The chains from the treated protoplasts were fractionated by gel filtration and the major peak was isolated, while the chains from the ribosomes, shown in Fig. 1 to have a virtually uniform length, were used without fractionation. These two materials were dansylated and then analyzed for dansylated and undansylated methionine residues, as described in Table 2. [Under the conditions used, dansylation should be complete (18).] Since the methionine residues should be randomly distributed along the protected peptide fragments and since only those at the NH_2 terminus would be dansylated, the ratio of total to derivatized methionine residues should provide a direct measure of the length of the protected peptide.

As Table 2 shows, the ratio of total to dansylated [^{35}S]methionine was 49 for the membrane-protected peptides isolated from Pronase-treated protoplasts and 28 for the peptides isolated from Pronase-treated polysomes. These results define the

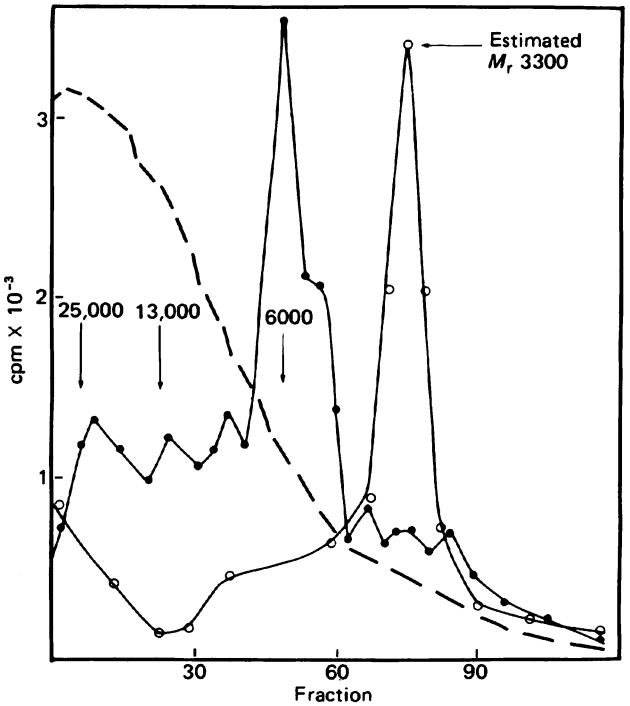

FIG. 1. Length of nascent chain protected against proteolytic cleavage. Protoplasts from 10^{10} cells with ^{3}H-labeled nascent chains were resuspended in 0.5 ml of a 25% sucrose solution containing 10 mM $Mg(OAc)_2$, 10 mM Tris·HCl (pH 8.0), and 100 μg of Pronase per ml. After incubation at 0°C for 30 min, phenylmethylsulfonylfluoride (1.74 mM) and *o*-phenanthroline (0.11 mM) were added to prevent further proteolysis. The protoplasts were washed (by centrifugation and resuspension in the same medium without Pronase), pelleted, lysed, and fractionated. To recover the residual nascent chains we isolated the membrane–polysome complexes, freed them of membrane by washing once with 1% deoxycholate in buffer A, and dialyzed them against 10 μM $Mg(OAc)_2$. The ribosomes were removed by centrifugation and the supernatant was treated with 0.1 M NaOH at 37°C for 10 min to cleave the chains from tRNA. The product was concentrated by lyophilization. Membrane-derived polysomes from labeled cells were similarly treated with Pronase and the residual chains were recovered. As a control (dashed line), nascent chains were also isolated from membrane-bound polysomes from untreated protoplasts. All products were examined by Sephadex G-25 filtration (1 × 25 cm). Approximately 120 fractions were collected (10 drops each) and radioactivity was determined. Markers were insulin (M_r 6000), cytochrome *c* (M_r 13,000), and *E. coli* crude tRNA (M_r 25,000). ●, Protoplasts treated with Pronase; ○, derived polysomes treated with Pronase.

Table 1. Release of [^{3}H]ribosomes from the membrane–polysome fraction of *B. subtilis*

Treatment	% released
No Pronase, 0°C	10 ± 8
Pronase, 0°C	14 ± 12
Puromycin, 37°C	73 ± 21

The assay for release of labeled ribosomes from the membrane–polysome fraction has been described (13). Approximately 5000 cpm of membrane-associated polysomes, labeled with [^{3}H]uracil, was incubated in buffer A with or without Pronase (100 μg/ml), or with puromycin, elongation factor G, and GTP (13), for 30 min. Data are the mean (and the range) of five separate experiments.

Table 2. Dansylation of nascent peptide fragments from protoplasts and polysomes treated with Pronase

Fragments protected by	Radioactivity, dpm: Nondansylated [^{35}S]methionine	Radioactivity, dpm: Dansylated [^{35}S]methionine	Ratio (peptide length)
Membrane plus ribosomes			
Exp. I	25,251	524	49.2
Exp. II	24,532	505	49.6
Ribosomes alone			
Exp. I	23,128	841	28.5
Exp. II	18,972	713	27.6

Protoplasts and derived polysomes, from cells pulse-labeled with [^{35}S]methionine, were both treated with Pronase as in the legend to Fig. 1. The residual nascent peptide fragments were obtained from the membrane–polysome fraction from the treated protoplasts, and the gel filtration peak at M_r 6000 (Fig. 1) was isolated. This material and the total residual chains from the treated polysomes were oxidized (to convert the methionine quantitatively to the sulfone) and were dansylated, hydrolyzed, mixed with unlabeled standards (methionine sulfone and its dansylated derivative), and chromatographed on polyamide sheets, as described (18). The two standards were identified on the chromatograms by reaction with ninhydrin and by UV fluorescence, respectively. These areas were scraped off the polyamide sheet and dissolved in H_2O, and radioactivity was measured. Random areas of equal size from the polyamide sheet were also assayed for radioactivity to establish a background level (48–56 cpm), which was subtracted from the above values. The data are from two chromatographic separations and are corrected for counting efficiency (all about 80%). The length of the polypeptide chain is calculated as the ratio of total to dansylated [^{35}S]methionine.

length of nascent chain spanning the membrane (i.e., the difference) as 21 amino acids.

Effect of Temperature on Attachment of Nascent Chain to Membrane. The above results not only provide an estimate of the length of nascent chain surrounded by membrane, but also shed light on the mechanism of secretion, for they show that the chains remain attached to the membrane even after the extracellular regions have been removed. However, since the exposure to Pronase and the subsequent manipulations were carried out at 0–4°C, this persistent attachment might be an artifact, due to the rigidity of the membrane in the cold. Accordingly, Pronase-treated protoplasts were exposed to 37°C (in the presence of chloramphenicol) and then analyzed. As Table 3 shows, there was no significant release of labeled ribosomes from the membrane. Similar results were obtained with Pronase treatment of membrane–polysome complexes, from which the Pronase removed about 30% of the nascent peptide (data not shown) but no ribosomes (Table 3). Since we have earlier presented evidence that ribosomes are not directly attached to membrane in bacteria (unpublished data; ref. 12), their persistent attachment after loss of the extracellular segment of the nascent chain indicates that the region of the chain passing through the membrane is firmly held there and cannot readily slip back.

DISCUSSION

To determine the length of the secreted chains that span the cell membrane, we labeled the nascent chains in *B. subtilis* with a mixture of radioactive amino acids and determined the length of the segments that protoplasts protected from digestion by Pronase. Part of this length would have been protected by the membrane, part by the ribosome, and possibly part by its location between the two. The last component is evidently very

Table 3. Continued attachment of labeled ribosomes to membrane after treatment with Pronase

Treatment	Retention of polysomes, %: 0°C, 30 min	37°C, 10 min	37°C, 30 min
Protoplasts incubated with:			
No addition	90 ± 8	86 ± 6	86 ± 10
Pronase	86 ± 6	84 ± 6	81 ± 9
Membrane–polysome complexes incubated with:			
No addition	90 ± 8	86 ± 8	84 ± 6
Pronase	86 ± 8	83 ± 8	80 ± 9

Protoplasts with ^{3}H-labeled ribosomes were incubated with and without Pronase for 30 min at 0°C as in the legend to Fig. 1. Pronase was removed by centrifugation and the protoplasts were incubated in buffer A at 0° or 37°C, as indicated, in the presence of 200 μg of chloramphenicol per ml. In a parallel experiment the membrane–polysome fraction, obtained from similarly labeled, untreated cells, was also incubated with and without Pronase, at 0° or 37°C. The retention of polysomes on the membrane was measured by fractionation as described (13). The 100% value for the protoplasts (30,000 cpm) was that of a sample lysed and fractionated immediately after protoplast formation. For the isolated membrane–polysome complexes the sample size was such that before incubation and fractionation on Sepharose the value (100%) was also 30,000 cpm. Data are the mean and the range of three separate experiments.

short or nonexistent, since ribosomes were not released from the isolated membrane–polysome complexes by Pronase (Table 1), though they were released by puromycin (Table 1) and hence were not trapped inside vesicles.

To measure the length of chain protected by the ribosome, we treated polysomes with labeled nascent chains with Pronase. The residual, protease-resistant chains exhibited a quite uniform M_r (about 3300, equivalent to 30 residues), both in gel filtration (Fig. 1) and in sodium dodecyl sulfate gel electrophoresis. This value is very similar to that obtained by Malkin and Rich (17) for the M_r of the Pronase-resistant segment of nascent chains on eukaryotic ribosomes. More precise measurements of length, based on the ratio of dansylatable (i.e., NH_2-terminal) to total methionine residues in these fragments, gave a value of 28 residues (Table 2).

To measure protection by membrane plus ribosomes, we treated protoplasts with labeled nascent chains with Pronase to remove the protruding segments and isolated the protected residual chains from the membrane–polysome complexes recovered after lysis. On chromatography, these chains showed a sharp major peak, in addition to some smaller and larger chains (see below). The M_r of the peak corresponded to about 55 residues (M_r 6000; Fig. 1). When the chain length of this peak was measured more accurately by dansylation, as described above, the value was found to be 49 residues (Table 2). As we have seen, the ribosome alone protected 28 residues. The difference between the two values indicates that during protein secretion a quite uniform length of 21 residues spans the membrane.

If Pronase did not cleave all amino acids equally, it could lead to a nonrandom distribution of terminal amino acids and, hence, to inaccuracy in the estimation of length by dansylation. However, any such error should cancel out in the calculation of the difference between the lengths protected by protoplasts and by ribosomes.

A sequence of 21 amino acid residues would have a length of 75 Å in a completely extended polypeptide chain or of 35 Å in an α helix. The thickness of a phospholipid bilayer in synthetic liposomes is 35–40 Å (19), which is compatible with an

α helix, and a chain surrounded by lipid would be expected to form an α helix (20). Nevertheless, an extended chain seems much more likely for the segment in the membrane, on several grounds. First, Lα lamellar phase phospholipid bilayers, including those consisting of *E. coli* phospholipids, have a thickness of about 60 Å, determined by x-ray diffraction and by freeze-fracture electron microscopy (21). Moreover, the incorporation of membrane protein increases the thickness to 75 Å (21); this value is also observed for bacterial membranes, measured either in thin sections of fixed material (e.g., ref. 22) or in freeze-fractured unfixed cytoplasmic membranes (B. Witholt, personal communication). Finally, since a uniform mechanism of secretion of all regions of the chain (after the initiating segment) is logically expected, it is relevant that some regions of secreted proteins, containing proline, could not form an α helix, while all regions could form an extended chain. It therefore seems very likely that the chain traverses the membrane in the extended form rather than as an α helix. Since the protected length would then be greater than the thickness of the phospholipid bilayer, it would require a surrounding organized channel, presumably of protein (see also ref. 5). The several reported leader sequences of secreted proteins all have at least this length.

If the existence of chains threading through the membrane had not already been definitively established by their extracellular labeling, their extracellular digestion by Pronase would have led to the same conclusion (except that specific chains could not be identified). Cotranslational entry has also recently been demonstrated for membrane proteins by cleavage during synthesis (6), the finding of incomplete pulse-labeled chains (which can be chased) in the outer membrane of *E. coli* (23), and the glycosylation of a viral membrane protein by a membrane enzyme during chain elongation (24–26).

After Pronase treatment of pulse-labeled protoplasts, the membrane–polysome complexes would be expected to contain labeled peptide not only in the protected segment of secreted proteins, but also in the growing proteins being incorporated into the membrane; since these could be folded in the membrane, they could be larger. Hence the presence of about half the residual label in a sharp peak and half in a range of larger molecules (Fig. 1) is compatible with the assumption that all the secreted chains are in the sharp peak. Since the larger chains would carry more label per chain, it appears that in this strain (constitutive for secretion of α-amylase) the membrane-bound ribosomes may be secreting more chains than they are incorporating in membrane. Whether the free cytoplasmic polysomes are forming additional membrane proteins is not known.

The protection of a segment of secreted proteins by membrane eliminates one conceivable mechanism of protein secretion (11), which is already unlikely from morphological evidence: embedding of the ribosome in the membrane so that the chain is already secreted when it leaves the ribosome. In another mechanism, favored in a recent review (27) and also suggested in our earlier publication (11), folding of the extracellular chain would provide the energy for ensuring unidirectional transfer. However, it seems unlikely that such folding plays an essential role in secretion, for ribosomes are apparently attached to membrane solely via their nascent chain (unpublished data; ref 12) and they remain attached after loss of the extracellular segment of that chain, even on prolonged incubation at 37°C (Table 3). The present results therefore support a model in which the channel in the membrane is not passive, as implied in earlier models (5, 13, 27), but plays an energetic role in holding and hence, presumably in moving the chain.

Note Added in Proof. The unpublished observations on *B. subtilis* noted above are in Smith, W. P., Tai, P.-C. & Davis, B. D. (1979) *Biochemistry*, in press.

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1. Milstein, C., Brownlee, G., Harrison, T. & Mathews, M. (1972) *Nature (London) New Biol.* **239**, 117–120.
2. Inouye, H. & Beckwith, J. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1440–1444.
3. Inouye, S., Wang, S., Sekizawa, J., Halegoua, S. & Inouye, M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1004–1008.
4. Blobel, G. & Sabatini, D. D. (1971) in *Biomembranes*, ed. Manson, L. A. (Plenum, New York), Vol. 2, pp. 193–196.
5. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 835–851.
6. Chang, C. N., Blobel, G. & Model, P. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 361–365.
7. Schechter, I. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2256–2260.
8. Devillers-Thiery, A., Kindt, T., Scheele, G. & Blobel, G. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 5016–5020.
9. Randall, L. L. & Hardy, S. J. S. (1977) *Eur. J. Biochem.* **75**, 43–53.
10. Tai, P.-C., Smith, W. P. & Davis, B. D. (1978) *Limited Proteolysis in Microorganisms*, Fogarty Conference, National Institutes of Health, 1978 (U.S. Government Printing Office, Washington, DC), in press.
11. Smith, W. P., Tai, P.-C., Thompson, R. C. & Davis, B. D. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2830–2834.
12. Smith, W. P., Tai, P.-C. & Davis, B. D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 814–817.
13. Adelman, M. R., Sabatini, D. D. & Blobel, G. (1973) *J. Cell Biol.* **56**, 206–229.
14. Sabatini, D. D. & Kreibich, G. (1976) in *The Enzymes of Biological Membranes*, ed. Martonosi, A. (Plenum, New York), Vol. 2, pp. 531–579.
15. Chua, N.-H., Blobel, G., Siekevitz, P. & Palade, G. E. (1976) *J. Cell Biol.* **71**, 497–514.
16. Davis, B. D. & Mingioli, E. S. (1950) *J. Bacteriol.* **60**, 17–28.
17. Malkin, L. I. & Rich, A. (1967) *J. Mol. Biol.* **26**, 329–346.
18. Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138.
19. Huang, C. & Mason, J. U. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 308–310.
20. Tanford, C. (1973) *The Hydrophobic Effect* (Wiley, New York).
21. Costello, J. J. & Gulik-Krzywicki, T. (1976) *Biochim. Biophys. Acta* **455**, 412–432.
22. Osborn, M. J., Gander, J. E. & Parisi, E. (1972) *J. Biol. Chem.* **247**, 297–303.
23. de Leij, L., Kingma, J. & Witholt, B. (1978) *Biochim. Biophys. Acta*, in press.
24. Katz, F. N., Rothman, J. E., Lingappa, V. R., Blobel, G. & Lodish, H. F. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3278–3282.
25. Rothman, J. E. & Lodish, H. F. (1977) *Nature (London)* **269**, 775–780.
26. Toneguzzo, F. & Ghosh, H. P. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 715–719.
27. DiRienzo, J. M., Nakamura, K. & Inouye, M. (1978) *Annu. Rev. Biochem.* **47**, 481–532.