*Chapter 4*

# Lipoprotein of the Outer Membrane of *Escherichia coli*

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## I. INTRODUCTION

### A. Is the Outer Membrane Foreign to You?

To those interested in the cytoplasmic membrane of eukaryotic cells, or even in the cytoplasmic membrane of prokaryotic cells, the outer membrane of *Escherichia coli* may sound like a foreign and an uninviting system to study. The title of this article itself may be just enough to repel these people. In fact, the *E. coli* outer membrane is a peculiar system in many respects. However, as will be seen in this chapter, the outer membrane offers an excellent system for the study of many of the fundamental questions concerning biomembranes, which are common not only to prokaryotic but also to eukaryotic membranes, including: the functions and structures of membrane proteins; molecular mechanism of biosynthesis and assembly of membrane components; interactions between membrane proteins; regulation of membrane biogenesis; and mechanism of translocation of proteins across the membrane. Progress toward the elucidation of these problems has been considerably more substantial in the study of the *E. coli* outer membrane system as opposed to studies of eukaryotic membranes. The advantages in utilizing the *E. coli* outer membrane to examine the problems mentioned above are: (1) The outer membrane consists of few major proteins; (2) it is very easy to isolate these major proteins in large quantities; (3) the functions and structures of the major proteins have therefore been well characterized; and (4) A



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finely detailed genetic map has consequently been established for *E. coli*, and many different genetic approaches are thus available for studying the functions, structures, biosynthesis, and assembly of these major outer-membrane proteins.

Among the major outer-membrane proteins of *E. coli*, the lipoprotein has been most extensively investigated. In fact, it is probably the most extensively studied membrane protein of all the different systems, especially those concerned with the mechanism of biosynthesis and assembly. To many readers, it may be surprising to hear that the lipoprotein is the most abundant protein in *E. coli*. Its complete chemical structure has been determined. Furthermore, the lipoprotein precursor, the prolipoprotein, has been isolated, its complete amino acid sequence has been established, and the partial base sequence of its mRNA has been determined. The lipoprotein gene has also been identified, and many mutants of the lipoprotein have been isolated.

In this article, I would like to review much of the work completed so far on the lipoprotein and to describe the latest progress in many different fields of research on the lipoprotein.

## B. What Is the Outer Membrane?

Before describing the outer-membrane lipoprotein of *E. coli*, I would like to describe the structure of the *E. coli* envelope. *E. coli*, as well as other gram-negative bacteria, is surrounded by an envelope consisting of two distinct membranes as observed by electron microscopy: the outer membrane and the inner, or cytoplasmic, membrane (Murray *et al.*, 1964; DePetris, 1967). The cell wall, or peptidoglycan layer, is located between the two membranes (Murray *et al.*, 1965) (see Fig. 1). Both the outer and the cytoplasmic membranes show the typical features of a unit membrane having a thickness of about 75Å. These unit membranes consist mainly of phospholipids and proteins, thus forming the lipid bilayer organization. As does the cytoplasmic membrane of eukaryotic cells, the *E. coli* cytoplasmic membrane displays many important functions, such as energy metabolism, active transport, and synthesis of lipids, peptidoglycan, and lipopolysaccharide. On the other hand, the functions of the outer *E. coli* membrane are still somewhat obscure. However, one can picture the function of the outer membrane when the stability of *E. coli* cells is compared to that of animal cells. As shown in Fig. 1, animal cells need only the cytoplasmic membrane because they live in osmotically isotonic environments. On the other hand, bacteria usually inhabit hypotonic environments, which result in lysis of animal cells. In order to prevent

lysis in a hypotonic environment, bacteria have evolved the cell wall, which consists of a rigid network of peptidoglycan. The peptidoglycan is highly cross-linked, and the bacterial cell is probably surrounded by a single supermacromolecule of the peptidoglycan, which gives structural rigidity to the cell. However, the cross-linked network is loose enough to permit all materials required for growth to pass through the layer.

Among the prokaryotes, a significant difference exists between the cell walls of gram-negative and gram-positive bacteria. As can be seen in Fig. 1, gram-positive bacteria have very thick, multilayered cell walls. On the contrary, gram-negative bacteria such as *E. coli* and *Salmonella typhimurium* have a single layer of peptidoglycan. The membrane systems of gram-negative bacteria, however, are more differentiated than those of gram-positive organisms, having an extra membrane system outside the peptidoglycan layer. This differentiation of membrane systems also occurs in eukaryotic cells, which contain several different membrane systems, such as the lysosomal membrane, the mitochondrial membrane, the microsomal membrane, the chloroplast membrane, and the nuclear membrane, besides the cytoplasmic membrane. The functions

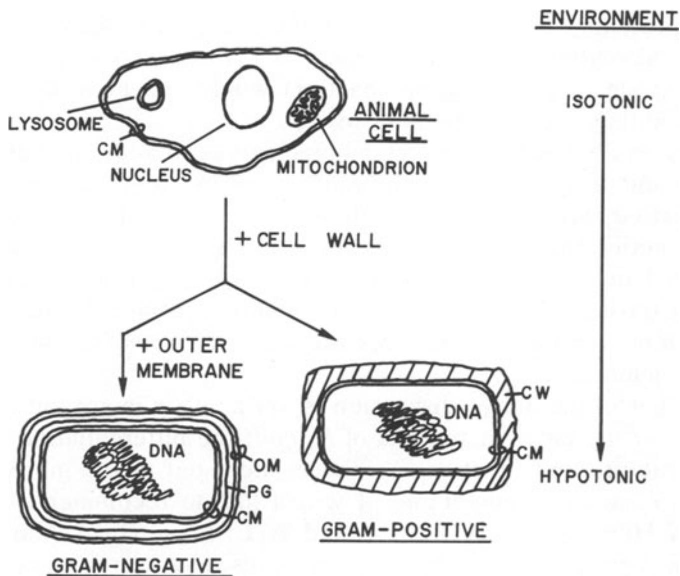



Fig. 1. Schematic illustration of the membrane structures of eukaryotic and prokaryotic cells. CM, cytoplasmic membrane; CW, cell wall; OM, outer membrane, PG, peptidoglycan.

of the outer membrane of gram-negative bacteria are, in part, very similar as to those of the eukaryotic lysosomal membrane. The lysosomes, found in animal cells, are organelles surrounded by a single membrane. Because they contain many hydrolytic enzymes, such as phosphatases, glucosidases, nucleases, proteases, and lipases—which are able to hydrolyze all classes of essential components if released inside the cells—lysosomes are sometimes called "suicide bags." The lysosomal enzymes are used to digest foreign materials, such as bacteria, when taken in by the cell.

Bacteria also need the hydrolytic enzymes found in lysosomes for their survival, because they have to digest macromolecules or small organic compounds in order to use these as nutrients. However, because bacteria do not contain lysosomes, these enzymes must be kept separate, by other means, from other cellular components, in order to prevent self-digestion. Gram-negative bacteria have evolved an elaborate system whereby they keep these enzymes in the space between the outer membrane and the cytoplasmic membrane. This space is called the periplasmic space and is analogous to the lysosome. Materials are transported through the outer bacterial membrane, digested by the lytic enzymes, and then incorporated into the cell through the cytoplasmic membrane. Actually, there are other proteins besides the lytic enzymes within the periplasmic space, such as specific amino-acid-binding proteins and specific sugar-binding proteins which are involved in the transport of these compounds through the cytoplasmic membrane. On a functional basis, the periplasmic space of gram-negative bacteria would appear to be more sophisticated than the eukaryotic lysosome.

As seen above, the outer membrane serves to keep the periplasmic enzymes and proteins in the periplasmic space. At the same time it serves as a selective barrier to the cell exterior. Thus, certain substances, such as some antibiotics, cannot penetrate through the outer membrane. On the other hand, the outer membrane appears to have passive diffusion pores for those materials required for cell growth, since the active transport systems for these substances are located exclusively in the cytoplasmic membrane.

Implicit in the above discussion is yet another important aspect in research on the outer membrane of *E. coli*: the differentiation of membrane structure and function. To those interested in the more specific aspects of bacterial membranes, I would like to recommend the book *Bacterial Membranes and Walls* edited by L. Leive (1973, Marcel Dekker, New York). There are also two previous review articles concerning the lipoprotein of the *E. coli* outer membrane (Inouye, 1975; Braun, 1975).

## II. STRUCTURE

### A. Bound Form of the Lipoprotein

In 1969, Braun and Rehn reported the existence of a peculiar lipoprotein covalently linked to the peptidoglycan of *E. coli* (Braun and Rehn, 1969). They showed that the lipoprotein binds to one of 10 to 12 repeating units of the peptidoglycan, that its molecular weight is about 7000 daltons, and that it lacks histidine, tryptophan, glycine, proline, and phenylalanine. Since then, Braun and co-workers have investigated the chemical nature of the linkage between the lipoprotein and the peptidoglycan (Braun and Sieglin, 1970; Braun and Wolff, 1970); its amino acid sequence (Braun and Bosch, 1972*a,b*); and the lipid structure covalently linked to the amino terminus (Hantke and Braun, 1973). Fig. 2 shows the complete chemical structure of the lipoprotein bound to the peptidoglycan, as elegantly determined by Braun and co-workers.

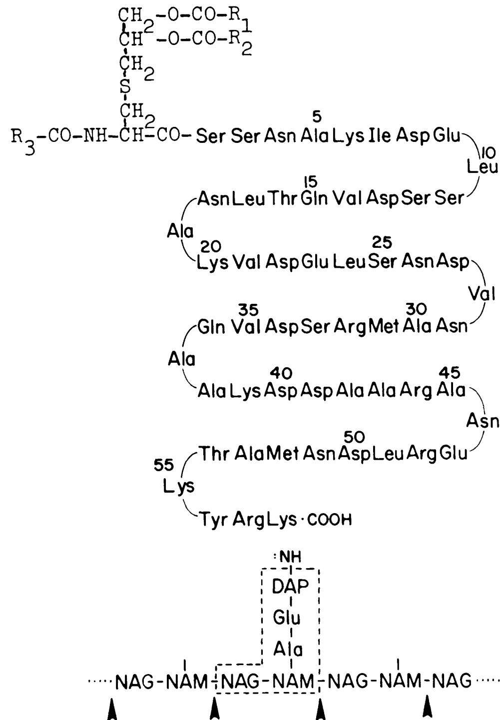



Fig. 2. The complete chemical structure of the bound form of the *E. coli* lipoprotein (Braun, 1975). The amino-terminal amino acid is glycerylcysteine [S-(propane-2′,3′-diol)-3-thioaminopropionic acid], to which two fatty acids are attached by ester linkages and one fatty acid by an amide linkage. Arrows indicate the bonds which are cleaved by lysozome. DAP, diaminospimelic acid; NAM, *N*-acetylmuramic acid; NAG, *N*-acetylglucosamine.

As can be seen in Fig. 2, the lipoprotein consists of 58 amino acid residues. It is linked, by the $\epsilon$-amino group of the carboxyl-terminal lysine residue, to the carboxyl group of meso-diaminopimelic acid (DAP) of the peptidoglycan; the D-alanine residue(s) usually attached at this position is replaced by the lipoprotein. The most interesting feature of the lipoprotein is its amino-terminal portion, which consists of glycerylcysteine [*S*-(propane-2′,3′-diol)-3-thioaminopropionic acid]. To this glycerylcysteine two fatty acids are attached by two ester linkages, and one fatty acid by an amide linkage, as shown in Fig. 2.

### B. Free Form of the Lipoprotein

It was independently found that the lipoprotein also exists in the *E. coli* membrane without being covalently bound to the peptidoglycan. When the *E. coli* membrane function is analyzed by SDS-gel electrophoresis, one can find a few major protein peaks, as shown in Fig. 3A. Among these peaks, the peak-11 protein appears to have the smallest molecular weight, estimated to be about 7500 from its mobility relative to the internal molecular weight standards (Inouye and Guthrie, 1969).

This peak was later concluded to be the free form of the lipoprotein, which is not linked to the peptidoglycan, from the following criteria: (1) When the *E. coli* membrane fraction is treated with T4 phage lysozyme, a new peak appears to the left side of peak 11, as shown by an arrow in Fig. 3B (Inouye *et al.*, 1972). This is the only change resulting from the treatment and the same peak also appears as a result of the treatment with other muramidases, such as hen and goose egg-white lysozymes. (2) The membrane proteins shown in Fig. 3B are double labeled with [$^{3}$H]arginine and [$^{14}$C]histidine. Surprisingly, both the new peak and the peak-11 protein, in contrast to all other peaks, have an extremely low content of histidine (Inouye *et al.*, 1972), suggesting that the peak-11 protein and the new-peak protein are composed of the same polypeptide.

These results are explained as shown by a schematic diagram in Fig. 4. The lipoprotein exists in two different forms, a free form and a bound form. One-third of the lipoprotein molecules are covalently linked to the peptidoglycan (bound form). When the untreated *E. coli* membrane fraction is subjected to SDS-gel electrophoresis, only the free form migrates through the gel to give peak F (Fig. 4A), which corresponds to peak 11 in Fig. 3. Since the peptidoglycan is a supermacromolecule and totally insoluble even in hot SDS solution, the bound form remains on top of the gel. When the membrane fraction is treated with lysozyme, the peptidoglycan is digested to small pieces and the bound form of the lipopro-

tein becomes soluble in the SDS solution. However, the bound form still contains a fragment of the peptidoglycan cleaved by the lysozyme treatment. As a result, the bound form gives rise to a separate peak (peak B) distinct from the peak of the free form (peak F) in SDS-gel electrophoresis, as shown in Fig. 4B. Thus peak B corresponds to the new peak shown by an arrow in Fig. 3B, and from the ratio of the new peak to

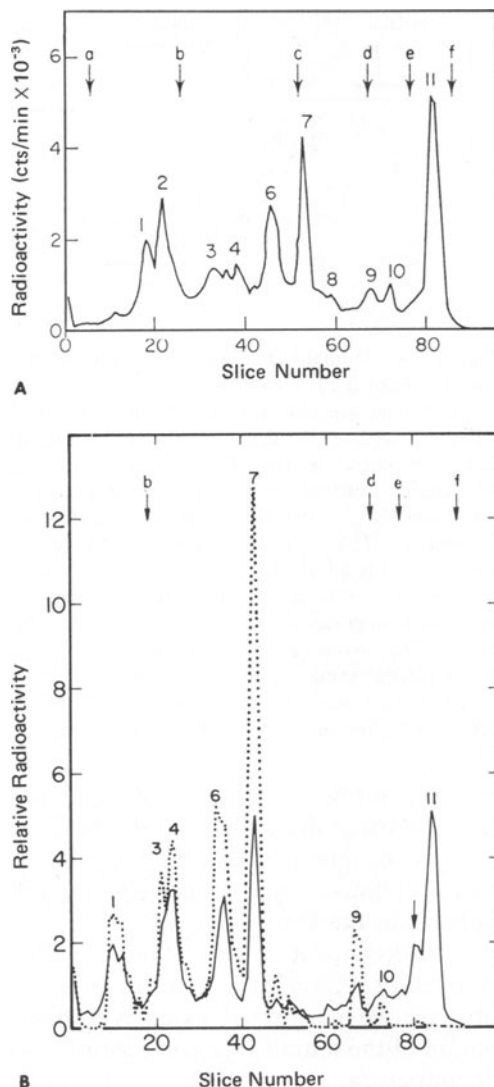



Fig. 3. SDS-gel electrophoresis of membrane fractions with and without T4 phage lysozyme treatment. (A) A culture of *E. coli* MX74T2 was labeled with [$^3$H]arginine. The membrane fraction was prepared and analyzed by SDS-gel polyacrylamide gel electrophoresis according to the method of Inouye and Pardee (1970). (B) A culture of *E. coli* MX74T2 was labeled with both [$^3$H]arginine and [$^{14}$C]histidine. The membrane fraction was prepared and treated with T4 phage lysozyme as previously described (Inouye *et al.*, 1972). ——, [$^3$H]arginine; -------, [$^{14}$C]histidine. Arrows with letters indicate the positions of internal molecular weight standards (Inouye, 1971); a, dimer; b, monomer of 1-dimethylaminonaphthalene-5-sulfonyl (DANS) bovine serum albumin; c, dimer; d, monomer of DANS-hen egg-white lysozyme; e, cytochrome c; f, DANS-insulin. The number on each peak is the same as in the previous paper (Inouye, 1971). A large arrow in (B) indicates the appearance of a new peak.

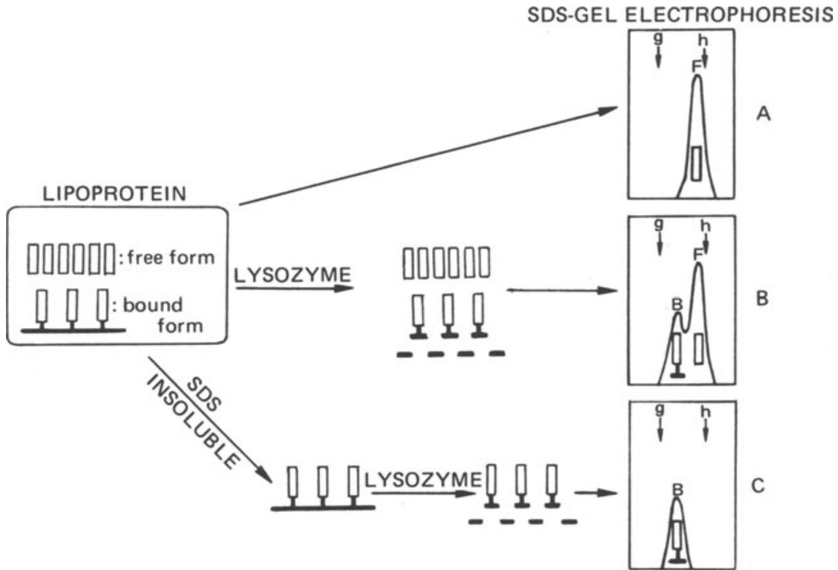



Fig. 4. A schematic diagram of the separation of the free and the bound forms of the lipoprotein of the *E. coli* envelope by SDS-polyacrylamide gel electrophoresis. (A) The *E. coli* envelope was subjected to SDS-gel electrophoresis without lysozyme treatment. The free form migrates in the gel to position F between the internal molecular weight standards: f, cytochrome c; g, DANS-insulin. (Only this region is illustrated in the diagram.) The lipoprotein attached to the peptidoglycan cannot migrate in the gel because of its high molecular weight. (B) The envelope is treated with lysozyme, followed by SDS-gel electrophoresis. The peptidoglycan is hydrolyzed into small pieces. As a result, the bound form, which is attached to a small fragment of the peptidoglycan, becomes soluble in SDS and migrates in the gel. Because of the presence of the small peptidoglycan fragment, the lipoprotein migrates a little slower than the free form, giving rise to a new peak at position B. (C) The envelope fraction is first treated in 4% SDS at 100°C for 5 min. After the treatment, the sample is centrifuged to collect the peptidoglycan. The peptidoglycan is then digested with lysozyme, followed by SDS-gel electrophoresis. Only one peak appears, representing the bound form, at position B.

peak 11 in Fig. 3B, it was concluded that the amount of the bound form is one-third of the total lipoprotein. Conversely, when the peptidoglycan, purified by precipitation with 4% SDS at 100°C, is digested with lysozyme, followed by SDS-gel electrophoresis, only the bound-form peak appears at the B position (Fig. 4C).

Peak-11 protein was purified and paracrystallized in our laboratory (Inouye *et al.*, 1976; DeMartini *et al.*, 1976). Using the purified peak-11 protein, we performed extensive chemical analysis, including determination of the amino acid composition, fatty acid analysis, contents of the peptidoglycan components, and peptide analysis after cyanogen bromide

cleavage (Hirashima *et al.*, 1973*b*; Inouye *et al.*, 1976). From these studies, we clearly demonstrated that the protein of peak 11 has exactly the same chemical structure as the bound form of the lipoprotein shown in Fig. 2, except that it does not contain any components of the peptidoglycan. The carboxyl-terminal structure was also shown to be Tyr-Arg-Lys (Inouye *et al.*, 1976). The same conclusion was also obtained by Braun *et al.* (1975). This should be emphasized because this carboxyl-terminal sequence would not be present if the peak-11 protein were a product of the bound form after cleavage from the peptidoglycan with such hydrolytic enzymes as lysozymes, amidases, or proteases (Braun and Sieglin, 1970; Braun and Wolff, 1970; Inouye *et al.*, 1973).

### C. Location and Amount of the Lipoprotein

It is of great interest to determine whether the lipoprotein exists in the outer membrane or in the cytoplasmic membrane. In order to examine this, the outer membrane and the cytoplasmic membrane are first separated by sucrose-density gradient centrifugation. Since the outer membrane contains lipopolysaccharides, its density is greater than that of the cytoplasmic membrane. The two can thus be easily separated (Miura and Mizushima, 1969; Osborn *et al.*, 1972). The existence of the lipoprotein can be examined either immunologically, with the use of anti-lipoprotein serum (Bosch and Braun, 1973), or by SDS-gel electrophoresis of the membrane proteins from both membrane fractions (Lee and Inouye, 1974). It was found by both methods that the free-form as well as the bound-form lipoprotein exists almost exclusively in the outer membrane. This result indicates that the amino-terminal end of the lipoprotein is inserted into the outer membrane facing the outside of the cell, since the carboxyl-terminal end is linked to the peptidoglycan layer.

The number of the bound-form lipoprotein molecules per cell was estimated by Braun and co-workers (Braun and Rehn, 1969; Braun *et al.*, 1970). Using *Salmonella typhimurium,* they found that from 75,000 to 240,000 lipoprotein molecules are covalently bound to the peptidoglycan, depending upon the growth conditions. The lipoprotein is bound to every tenth peptidoglycan repeating unit (for both *S. typhimurium* and *E. coli*), and the size of the cell varies depending upon the growth conditions. Therefore, cells harvested in the logarithmic growth phase in a rich medium have 240,000 molecules of the bound-form lipoprotein, whereas 125,000 and 75,000 bound-form lipoprotein molecules exist in cells grown in glucose minimal salt medium at the logarithmic growth phase and the stationary phase, respectively.

Since there is twice as much free-form lipoprotein as bound-form lipoprotein in the cell, the number of free-form lipoprotein molecules is estimated to be 480,000 per cell growing in rich medium. Therefore, the total number of lipoprotein molecules is 720,000, suggesting that the lipoprotein is the most abundant protein in the cell on the basis of molecular numbers.

### D. Conformation of the Lipoprotein

One can easily purify both the bound form (Braun and Rehn, 1969) and the free form of the lipoprotein (Inouye *et al.*, 1976). Braun and coworkers released the bound-form lipoprotein from the peptidoglycan by either lysozyme or trypsin treatment in order to solubilize it in SDS solution. The lysozyme-released lipoprotein (lipoprotein I) contains 2 or 3 peptidoglycan repeating units (see Fig. 2) covalently linked at the carboxyl-terminal end of the lipoprotein, whereas the trypsin-released lipoprotein (lipoprotein II) is free of the peptidoglycan units and lacks the carboxyl-terminal peptide $\overset{56}{\text{Tyr}}\text{-Arg-}\overset{58}{\text{Lys}}$. Circular-dichorism spectra of both proteins are essentially identical, and their $\alpha$-helical contents are calculated to be about 80%. Furthermore, from infrared spectra Braun concluded that lipoprotein II has $\beta$-structure to the extent of approximately 15% of the total conformation.

On the other hand, the infrared spectrum of the purified free-form lipoprotein indicates that the free-form lipoprotein has an $\alpha$-helical conformation, but no indication of the existence of $\beta$-structure (Lee *et al.*, 1977*a*). Circular-dichroism spectra were measured in various SDS concentrations, and the $\alpha$-helical content was found to be as high as 88% in 0.01%–0.03% SDS in the presence of $10^{-5}$ M $Mg^{2+}$. From these observations, Inouye (1974) and Braun (1975) proposed different molecular models. These models will be discussed in Section IVB.

## III. BIOSYNTHESIS

### A. Specific Biosynthesis *in Vivo*

As mentioned in the previous section, the lipoprotein lacks histidine as well as proline, glycine, phenylalanine, and tryptophan. Thus, one can design an interesting experiment to examine whether the lipoprotein can be produced in cells starved for histidine. When the cells of an *E. coli*

strain requiring histidine for growth (a histidine auxotroph) are starved for histidine, about 95% of total protein synthesis is suppressed and the cells almost completely stop growing (Hirashima and Inouye, 1973). This is because almost all *E. coli* proteins contain histidine. However, to our surprise, about 90% of the lipoprotein continued to be produced after 1 hr starvation for histidine, with no significant production of any of the other membrane proteins (Fig. 5B). More strikingly, even after 4 hr starvation, the lipoprotein was still being synthesized at 40% of the rate of normally growing cells (Fig. 5C).

Since there is no interference from production of other membrane proteins, the exclusive biosynthesis of the lipoprotein in the absence of histidine provides an excellent system for investigating the *in vivo* biosynthesis and assembly of the outer-membrane protein. Furthermore, this system has been used for the isolation of lipoprotein mutants (see

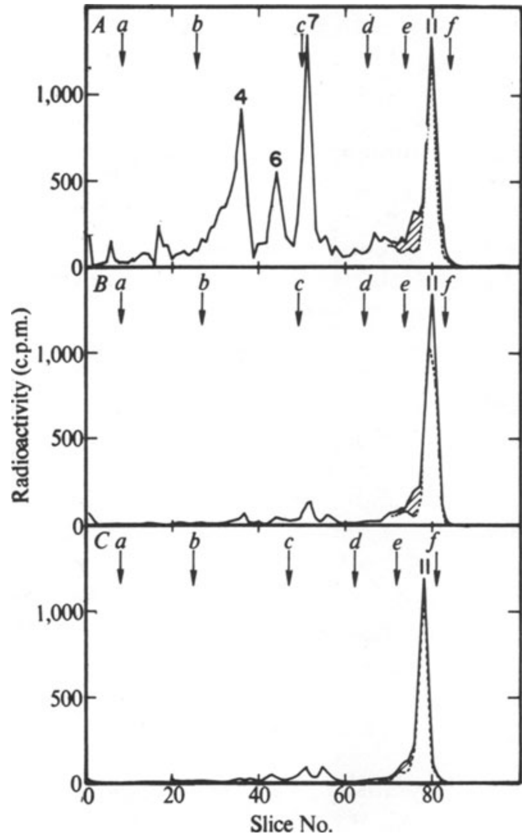



Fig. 5. SDS-gel electrophoresis of the membrane fraction of an *E. coli* histidine auxotroph labeled with [$^{14}$C]arginine in the presence and absence of histidine (Hirashima and Inouye, 1973). (A) Labeled in the presence of histidine for 1 hr. (B) Labeled during a 1-hr histidine starvation. (C) Labeled for 2 hr after 2 hr of histidine starvation. The positions of the internal molecular weight standards are indicated by small arrows as shown in Fig. 3.

Section V), and for specific *in vivo* labeling of the lipoprotein with nuclear magnetic resonance (NMR) probes (see Section VI).

### B. Effects of Antibiotics

#### *1. General Considerations*

An interesting approach to studying the mechanism of biosynthesis of membrane proteins is to examine the effects of various antibiotic inhibitors of protein and RNA synthesis. We have extensively investigated the effects of five ribosome-directed antibiotics (kasugamycin, tetracycline, chloramphemicol, sparsomycin, and puromycin), and an RNA synthesis inhibitor (rifampicin), on the biosynthesis of *E. coli* membrane proteins (Hirashima *et al.,* 1973*a*). We found that membrane protein synthesis is strikingly more resistant to kasugamycin and puromycin than cytoplasmic protein synthesis. In contrast, membrane protein synthesis is more sensitive to tetracycline and sparsomycin than cytoplasmic protein synthesis. Tetracycline is an especially potent inhibitor specific for membrane protein synthesis; with 0.3 $\mu$g tetracycline/ml, almost 90% of membrane protein synthesis is inhibited, whereas the inhibition of cytoplasmic protein synthesis is only 45% at the same concentration. Chloramphenicol shows no differential inhibitory effect. These results suggest that membrane and cytoplasmic proteins are being synthesized by somewhat different mechanisms.

The effects of the antibiotics can be further tested on the production of individual membrane proteins (Hirashima *et al.,* 1973*a*) as follows: Cells are labeled with a radioactive amino acid in the presence or absence of an antibiotic, and the membrane fraction is then prepared from the cells. The membrane proteins are separated by SDS-polyacrylamide gel electrophoresis; the incorporation of the radioactive amino acid into the individual membrane proteins is estimated; and the incorporations in the presence and absence of the antibiotic are compared. In this way, the biosynthesis of one of the major outer membrane proteins, the tolG protein (peak 7 in the paper by Hirashima *et al.,* 1973*a*; see also review by Inouye, 1975), was found to be more resistant to kasugamycin, chloramphenicol, and sparsomycin than the other major outer-membrane proteins.

#### *2. Unusual Resistance to Puromycin*

The biosynthesis of the lipoprotein shows an unusual resistance to puromycin. As shown in Fig. 6b, with 300 $\mu$g puromycin/ml there is no

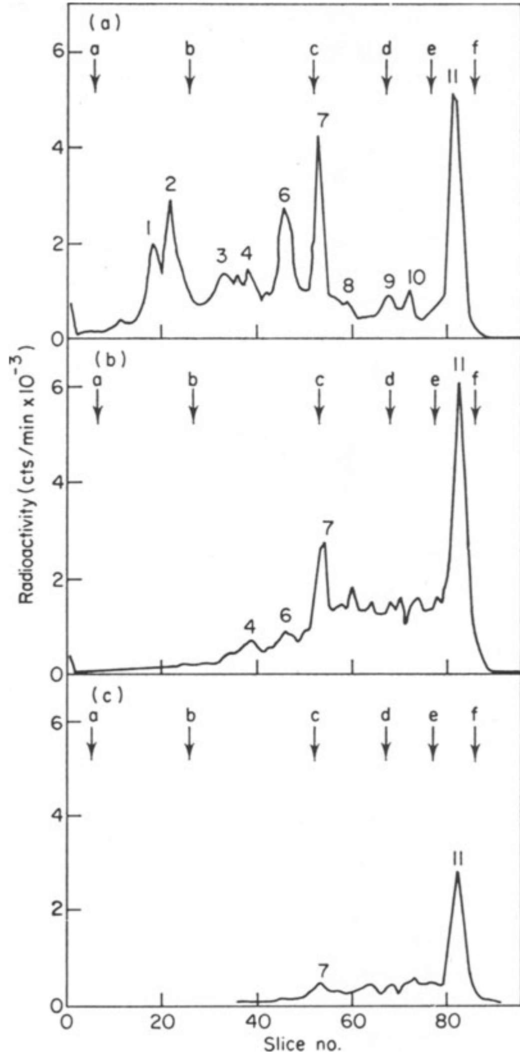



Fig. 6. Effects of puromycin on the biosynthesis of the *E. coli* membrane proteins (Hirashima *et al.*, 1973*a*). The membrane fractions are prepared from cells labeled with [$^3$H]arginine in the absence and presence of puromycin. They are then analyzed by SDS-polyacrylamide gel electrophoresis, as described in Fig. 3. Arrows with letters indicate positions of the internal molecular weight standards (Inouye, 1971). Assignments of peaks and internal standards are the same as in Fig. 2. (a) No addition; (b) 300 $\mu$g/ml puromycin; (c) 600 $\mu$g/ml puromycin.

inhibition of the lipoprotein (peak 11) biosynthesis, whereas the biosynthesis of all the other membrane proteins is severely inhibited. With 600 $\mu$g puromycin/ml, the lipoprotein is still being produced at about 60% of the normal rate, whereas the production of all the other membrane proteins is almost completely suppressed (Fig. 6c). Since puromycin is known to inhibit protein synthesis by prematurely terminating peptide formation, one can argue that peak 11 in Fig. 6b and c may arise from

the prematurely formed peptides derived from the other membrane proteins of higher molecular weights. This possibility is ruled out by the fact that lipoprotein biosynthesis is resistant to puromycin even in histidine-starved cells in which the lipoprotein is exclusively produced as mentioned in the previous section (Hirashima *et al.*, 1973*a*). Furthermore, we found that the lipoprotein produced in the presence of puromycin contains the same amount of fatty acids as the normal lipoprotein.

These results suggest that the lipoprotein biosynthetic machinery may in some way be compartmentalized within the cell so as to be inaccessible to puromycin. Alternatively, the machinery itself may somehow differ from the usual puromycin-sensitive machinery. On the basis of this consideration, we have examined the biosynthesis of the lipoprotein in three different systems in which the cellular accessibility to puromycin was increased by disruption of the cell structure using ethylenediaminetetraacetate (EDTA) or toluene, or finally by preparing polyribosomes (Halegoua *et al.*, 1976*a*). Puromycin sensitivity of overall protein synthesis increases by about tenfold for each method of disruption of the cell structure: 50% inhibitions are obtained at 330, 35, 2.7, and 0.22 μg puromycin/ml for intact cells, EDTA treated cells, toluene-treated cells, and the polyribosome system, respectively. However, lipoprotein biosynthesis remains more resistant to puromycin than biosynthesis of other proteins in all of the systems tested. Therefore the lipoprotein biosynthetic machinery itself seems to be intrinsically resistant to puromycin.

On the basis of our earlier suggestion (Hirashima *et al.*, 1973*a*), Randall and Hardy (1975) compared the ribosomal proteins of polyribosomes engaged in the synthesis of the outer-membrane proteins with those engaged in total protein synthesis. However, they could not find any quantitative difference in ribosomal protein components between membrane-bound polyribisomes and free polyribosomes. Later they showed that outer-membrane proteins are produced on membrane-bound polyribisomes and that the biosynthetic activity of the membrane-bound polyribisomes is more resistant to puromycin than is that of free polyribosomes *in vitro* (Randall and Hardy, 1977). Therefore, the extreme puromycin resistance of lipoprotein biosynthesis may be caused by the way in which the polyribosomes specific for lipoprotein biosynthesis attach to the cytoplasmic membrane, such that puromycin cannot reach its binding site on ribosomes. Thus, the differences in puromycin resistance among the outer-membrane proteins may be due to the different affinities of individual polyribosomes for the membrane. It is also possible that the differential effects caused by puromycin are at least partially due to the base composition of mRNA, since poly(U)-directed polypeptide

synthesis appears to be more resistant than is poly(G, U)- or poly(C, U)-directed polypeptide synthesis (Butler and Maledon, 1976). At any rate, it is very important to understand why the lipoprotein biosynthesis is resistant to puromycin, since it will very likely provide an essential clue to the molecular mechanism of the lipoprotein biosynthesis.

In this regard, it is noteworthy that the biosynthesis of membrane proteins in rabbit erythrocytes is also reported to be more resistant to puromycin than is the biosynthesis of globin, a cytoplasmic protein (Koch *et al.*, 1975). Furthermore, it is interesting to note the possible existence of a subpopulation of ribosomes with different sensitivities for MS2 phage protein synthesis in *E. coli* (Kozak and Nathans, 1972) and for $\alpha$ and $\beta$ chains of hemoglobin in rabbit erythrocytes (Lodish and Nathan, 1972).

### 3. *Effect of Rifampicin: Stable mRNA*

Since rifampicin is known to block initiation of mRNA synthesis, we can estimate the stability of various mRNAs by measuring the capacity for biosynthesis of the proteins they encode after the addition of rifampicin to the culture (Hirashima *et al.*, 1973*a*). We found that the average half-lives of the cytoplasmic proteins and the membrane proteins are 2.1 and 5.5 min, respectively. This indicates that mRNAs for the membrane proteins, on the average, are about 2.5 times more stable than those for the cytoplasmic proteins.

When the stabilities of mRNAs for the individual outer-membrane proteins were measured, we found that the mRNA for the lipoprotein is extraordinarily stable, having a half-life of 11.5 min (Hirashima *et al.*, 1973*a*). These stable mRNAs have also been identified in DNA-less minicells formed during abnormal cell division (Levy, 1975). In these cells the mRNA seems to be much more stable.

The reasons for the high stability of the lipoprotein mRNA are not yet known. However, since its mRNA has been purified, and the nucleotide sequence of the mRNA has been partially determined, as will be discussed later, it will not be long before we fully understand the unusual stability of the lipoprotein mRNA.

## C. Cell-Free Synthesis

### 1. *General Considerations*

Establishment of membrane protein synthesis in a cell-free system would allow specific detailed analysis of the mechanism of biosynthesis

and assembly of the membrane proteins. In this direction, we have established cell-free synthesis of the lipoprotein on polyribosomes (Hirashima and Inouye, 1975; Halegoua *et al.,* 1976*a*). However, it is more desirable to establish a cell-free synthesis of the lipoprotein directed by its mRNA, since this would enable more precise study of the mechanisms of the biosynthesis and assembly of the lipoprotein.

### 2. *Purification of the mRNA*

Since *E. coli* mRNAs are highly unstable in general, it is extremely difficult to purify an mRNA which can direct protein synthesis in a cell-free system. However, purification of the mRNA for the lipoprotein appeared to be possible, since it has a few unique advantages over the purification of other *E. coli* mRNAs, as follows: (1) it is highly stable; (2) it is assumed to exist in large quantities, since the lipoprotein is the most abundant protein in *E. coli* on the basis of molecular numbers; (3) it is assumed to be smaller than almost all of the other *E. coli* mRNAs, since the lipoprotein is a protein of a very small molecular weight.

Taking advantage of these prospects, we successfully isolated the biologically active mRNA for the lipoprotein (Hirashima *et al.,* 1974). Recently, the mRNA has been extensively purified with the use of: (1) phenol extraction; (2) NaCl fractionation; (3) gel filtration on Sephadex G-100 and G-200; and (4) reversed-phase column chromatography on RPC-5 (S. S. Wang, R. L. Pirtle, I. L. Pirtle, M. Small, and M. Inouye, manuscript in preparation). From a 100-liter culture of *E. coli* cells, we can isolate about 60 $\mu$g of the mRNA of about 85% purity.

### 3. *E. Coli Cell-Free System*

When the purified lipoprotein mRNA is added to an *E. coli* cell-free protein synthesizing system, [$^{35}$S]methionine is incorporated into hot trichloroacetic acid (TCA)-precipitable material. This incorporation continues for at least 15 min, and the total incorporation increases linearly with increasing amounts of the mRNA fraction added to the cell-free system (Hirashima *et al.,* 1974). The product can be immunoprecipitated by anti-lipoprotein serum.

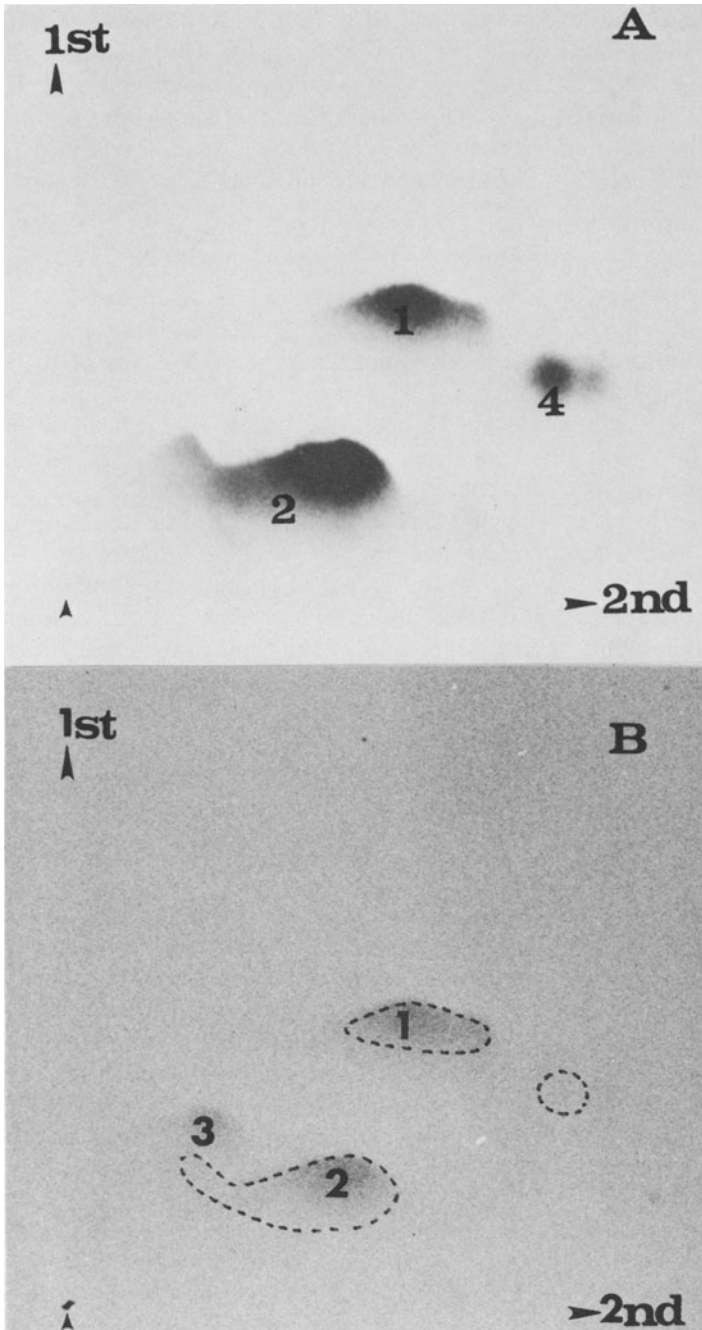
Another definite proof that the purified mRNA is directing the production of the lipoprotein in a cell-free system is provided by peptide mapping (Hirashima *et al.,* 1974). Since the lipoprotein is extremely resistant to proteases, the *in vitro* product labeled with [$^{14}$C]arginine is cleaved by cyanogen bromide. The radioactive peptides derived from

cyanogen bromide cleavage are mixed with nonradioactive cyanogen bromide-cleaved peptides prepared from the purified lipoprotein. The mixture is then subjected to peptide mapping. As shown in Fig. 7, radioactive spots derived from the *in vitro* product (Fig. 7A) are in very good agreement with ninhydrin-stained spots derived from the *in vivo* product (Fig. 7B). These spots have been identified as follows (Inouye *et al.*, 1976): spot 1, $^{53}$Ala-Thr-Lys-Tyr-Arg-$^{58}$Lys; spot 2, $^{32}$Arg-Ser-Asp-Val-Gln-Ala-Ala-Lys-Asp-Asp-Ala-Ala-Arg-Ala-Asn-Glu-Arg-Leu-Asp-Asn-$^{52}$Homoserine; and spot 3, uncleaved peptide from residues 32 to 58 (spot 1 peptide + spot 2 peptide; see also Fig. 2 for the amino acid sequence of the lipoprotein). Spot 3 only appears on peptide maps of the *in vivo* product, possibly because some of the purified lipoprotein contains a methionine residue, at the 52 position, which is oxidized during the purification. Spot 4 has not been completely identified, but judging from data obtained so far, it appears to be the same peptide as spot 1 except that the tryosine residue is modified (possibly brominated during cyanogen bromide cleavage). Since the *in vitro* product is labeled with [$^{14}$C]arginine, and the spot-1 and spot-4 peptides each contain only one arginine residue (as underlined above) in contrast to three arginine residues in the spot-2 peptide, the ratio of the total radioactivity in spots 1 + 4 to that in spot 2 is expected to be 1:3. The experimental ratio was found to be 1:2.5, which is in good agreement with the theoretical value.

These results indicate that the *in vitro* product is being completely synthesized from the amino terminus to the carboxyl terminus in the *E. coli* cell-free system directed by the purified mRNA.

### 4. *Wheat Germ Cell-Free System*

An interesting question to examine is whether the mRNA for the lipoprotein purified from *E. coli* can be translated in the eukaryotic wheat germ cell-free system. We found that the lipoprotein mRNA is quite efficiently translated in the wheat germ cell-free system and that the product is cross-reactive with anti-lipoprotein serum (Wang *et al.*, 1976). Protein synthesis directed by the mRNA is sensitive to cycloheximide (an inhibitor of eukaryotic 80 S ribosomes), but not to chloramphenicol (an inhibitor of 70 S ribosomes). This indicates that the *E. coli* mRNA can bind to eukaryotic 80 S ribosomes to form a correct initiation complex. This initiation complex is then capable of producing proteins which possess common chemical structures with the lipoprotein. It is extremely interesting to determine the mechanism whereby prokaryotic mRNA



1st
A
1
4
2
2nd
1st
B
1
3
2
2nd

forms the correct initiation complex with eukaryotic 80 S ribosomes, since it has been shown that an unusual structure, 7-methyl guanosine in a 5′,5′-triphosphate linkage with ribose-methylated nucleotides, is generally required for the efficient translation of eukaryotic mRNAs (see review by Lodish, 1976).

## D. Prolipoprotein: Precursor of the Lipoprotein

### 1. *Toluene-Treated Cells*

When we studied the effects of puromycin we developed a protein-synthesizing system using cells treated with toluene (see Section IIIB). Since toluene-treated cells are known to become permeable to molecules such as nucleotide triphosphate, toluene treatment is an important and simple method, particularly *in vitro,* for studying DNA synthesis (Moses and Richardson, 1970), RNA synthesis (Peterson *et al.,* 1971), and peptidoglycan synthesis (Schrader and Fan, 1974).

We found that protein synthesis in toluene-treated cells is entirely dependent upon the addition of ATP, and only membrane proteins are produced in these cells (Halegoua *et al.,* 1976*a,b*). When the membrane proteins synthesized in the toluene-treated cells are analyzed by SDS-polyacrylamide gel electrophoresis, a new large peak (peak I) appears at about the position of the internal molecular weight standard e as shown in Fig. 8B, (Halegoua *et al.,* 1977). Peak II protein in Fig. 8B is thought to be the lipoprotein, since it appears at exactly the same position in the gel as that of the lipoprotein (see Fig. 8A). Synthesis of both peak I and peak II proteins is sensitive to chloramphenicol but resistant to puromycin and rifampicin. Furthermore, both proteins are cross-reactive to anti-lipoprotein serum. These results indicate that the protein of the new peak (peak I) is related to the lipoprotein.

How are these two proteins related? The apparent molecular weight of the new protein is estimated to be between 10,000 and 15,000 as determined from its mobility relative to the internal molecular weight standards—a figure about twice as large as that of the lipoprotein. Fur-

←

Fig. 7. Peptide map of cyanogen bromide-cleaved peptides of the product of the cell-free system directed by the purified mRNA for the lipoprotein (Hirashima *et al.,* 1974). The *in vitro* product is labeled with [$^{14}$C]arginine. (A) Autoradiogram, and (B) ninhydrin-stained map. The autoradiogram (A), specified by the dotted lines, is superimposed on the ninhydrinstained map (B). Small arrows indicate the position of the origin. The peptides were separated first by chromatography (from left to right) and then by electrophoresis (from bottom to top).

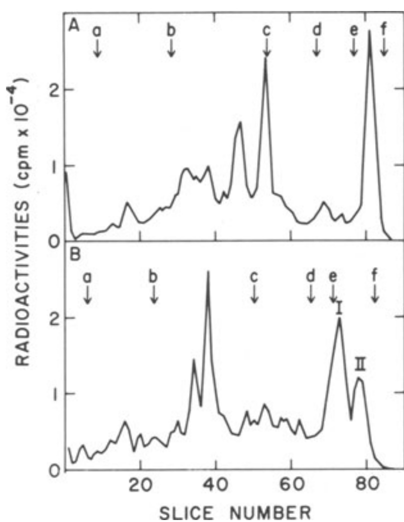



Fig. 8. Biosynthesis of membrane proteins in toluene-treated cells (Halegoua *et al.*, 1977). Proteins synthesized in intact and toluene-treated cells were labeled with [$^3$H]arginine, and the membrane fractions were prepared and analyzed by SDS-gel electrophoresis. (A) Membrane proteins of intact cells; (B) membrane proteins of toluene-treated cells. Peaks labeled I and II indicate the new form of the lipoprotein and the original form of the lipoprotein, respectively, in toluene-treated cells. Arrows with letters indicate the positions of the internal molecular weight standards: a, dimer; b, monomer of DANS-bovine serum albumin; c, dimer; d, monomer of DANS-egg white lysozyme; e, cytochrome c; f, DANS-insulin.

thermore, the peptide mapping of the new protein after cyanogen bromide cleavage gives a similar pattern as shown in Fig. 7 (see Section IIIC.3). The new protein, therefore, has the same carboxyl-terminal structure as that of the lipoprotein. Thus, one can imagine that the new peak might have been formed by dimerization of two nascent lipoproteins through an S–S bridge at unmodified amino-terminal cysteine residues. However, this is not the case, since peak I is not converted to a size comparable to that of the monomer of the lipoprotein (peak II) by treatment with $\beta$-mercaptoethanol (Halegoua *et al.*, 1977).

Another explanation that can accommodate all of the results is that the new protein has an extra sequence at the amino-terminal end of the lipoprotein. We are able to determine the amino acid composition of this extra region by double-labeling experiments as follows (Halegoua *et al.*, 1977): Toluene-treated cells are labeled with [$^3$H]arginine and [$^{14}$C]leucine, and the membrane fraction is then prepared from the cells. Peak I and peak II proteins are immunoprecipitated with anti-lipoprotein serum, and separated by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 9, both [$^3$H]arginine and [$^{14}$C]leucine are incorporated into the new protein (peak I) as well as into the lipoprotein (peak II). The ratio of the leucine to arginine content of the new protein peak is, however, 2.1 times as great as that of the lipoprotein peak. If the arginine contents of the new protein and the lipoprotein are the same, the total number of leucine residues in the new protein is calculated to be 8.4 (= $2.1 \times 4 \times 4/4$), since the lipoprotein contains 4 leucine residues (case 1

in Table I). If the new protein has 1 extra arginine residue, the total number of arginine residues in the new protein is 5, and the total number of leucine residues can be calculated to be 10.5 (= 2.1 × 4 × 5/4) (case 2 in Table I). Similarly, the composition of another 16 amino acids in the new protein are examined, and the results are summarized in Table I.

From Table I, one can conclude the following: (1) 4 of the 5 amino acids (phenylalanine, tryptophan, proline, and histidine; except glycine) which are absent in the lipoprotein are also absent in the new form of the lipoprotein. (2) Since [$^{14}$C]arginine is not detected in the amino-terminal fragment, the new protein most likely contains 4 arginine residues. Thus the new protein would have 18 or 19 extra amino acid residues at the amino-terminal end of the lipoprotein. (3) Among the 18 amino acids examined, the new protein is enriched in hydrophobic amino acids when compared to the lipoprotein. The content of hydrophobic amino acids in

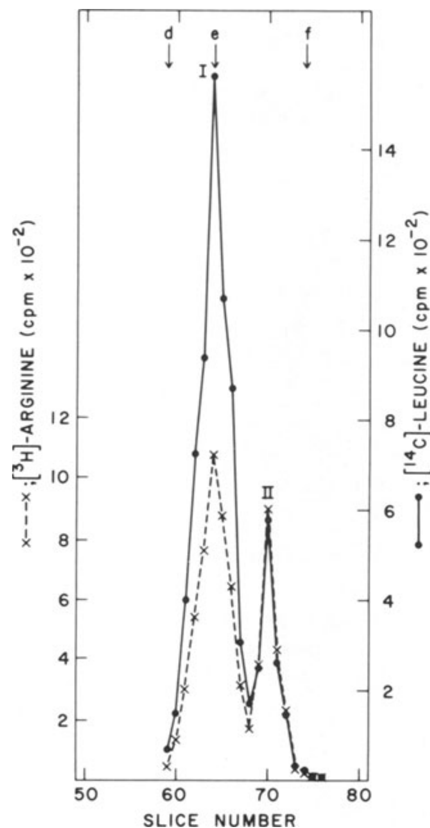



Fig. 9. SDS-gel electrophoresis of the immunoprecipitated forms of the lipoprotein isolated from the membrane fraction of toluene-treated cells double labeled with [$^3$H]arginine and [$^{14}$C]leucine. Protein synthesis in toluene-treated cells was carried out using [$^3$H]arginine and [$^{14}$C]leucine as described by Halegoua *et al.* (1977). ——, [$^{14}$C]leucine; ------, [$^3$H]arginine. Assignment of internal standards is the same as in Fig. 2.

**Table I**
**Amino Acid Composition of the New Protein**

| Amino acid | Number of residues | | |
|---|---|---|---|
| | New protein | | |
| | Case 1 | Case 2 | Lipoprotein |
| Arginine | 4(0) | 5(+1) | 4 |
| Alanine | 11.9(+3) | 14.9(+6) | 9 |
| Asparagine | N.D. | N.D. | 6 |
| Aspartic acid | 7.7(0) | 9.6(+2) | 8 |
| Cysteine | 0.9(0) | 1.2(0) | 1 |
| Glutamic acid | N.D. | N.D. | 3 |
| Glutamine | 1.6(0) | 2.0(0) | 2 |
| Glycine | 2.7(+3) | 3.4(+3) | 0 |
| Histidine | 0(0) | 0(0) | 0 |
| Isoleucine | 1.9(+1) | 2.3(+1) | 1 |
| Leucine | 8.4(+4 – +5) | 10.5(+7) | 4 |
| Lysine | 5.5(+1) | 6.9(+2) | 5 |
| Methionine | 2.5(+1) | 3.3(+1) | 2 |
| Phenylalanine | 0(0) | 0(0) | 0 |
| Proline | 0(0) | 0(0) | 0 |
| Serine | 7.3(+1) | 9.2(+3) | 6 |
| Threonine | 4.2(+2) | 5.3(+3) | 2 |
| Tryptophan | 0(0) | 0(0) | 0 |
| Tyrosine | 0.9(0) | 1.1(0) | 1 |
| Valine | 5.7(+2) | 7.1(+3) | 4 |
| Total | +18 – +19 | +32 | 58 |

the extra region is calculated to be 61–63%, in contrast to 42% in the case of the lipoprotein (excluding aspargine and glutamic acid, since they were not tested).

These results indicate that the lipoprotein is produced from a precursor, prolipoprotein, and toluene-treated cells accumulate the prolipoprotein, possibly because toluene treatment inhibits (or denatures) the enzyme(s) required for processing of the prolipoprotein to the lipoprotein.

### 2. *Amino Acid Sequence of Prolipoprotein*

The peptide extension at the amino-terminal end of the prolipoprotein is thought to have a very important function in the translocation of the lipoprotein through the cytoplasmic membrane to the outer membrane. The amino acid sequence of this region will give essential clues solving the mechanism of translocation and assembly of the lipoprotein.

In order to determine the amino acid sequence of the prolipoprotein, we used the *E. coli* cell-free system of protein synthesis directed by the

purified mRNA for the lipoprotein. As we have seen earlier, the cell-free product also has the same carboxyl-terminal structure as that of the lipoprotein (Fig. 7). Furthermore, the product gives a single band in SDS-gel electrophoresis at exactly the same position as peak I of toluene-treated cells (Inouye *et al.*, 1977*a*), indicating that the prolipoprotein is also produced in the *E. coli* cell-free system of protein synthesis directed by the lipoprotein mRNA.

In order to determine the length of the extended region at the amino-terminal end of the prolipoprotein, the cell-free product directed by the purified mRNA is labeled with [$^3$H]leucine and subjected to 46 consecutive Edman degradations in a sequenator which automatically removes amino acid residues one by one from the amino-terminal end of the protein. Figure 10 shows the release of radioactivity at each cycle of the Edman degradation. As can be seen from Fig. 10, radioactivity peaks appeared at cycles 6, 8, 13, 17, 18, 30, 37, and 44. As expected from sequenator analysis, the recovery of radioactivity decreases with increasing cycles, and there is always some trailing of radioactivity from the previous cycle because of incomplete degradation at the previous cycle. Therefore, the radioactivity at cycle 18 is slightly higher than that at cycle 17 because of the trailing of the radioactivity from cycle 17.

The lipoprotein has leucine residues at the 10th, 17th, 24th, and 49th positions from its amino terminus (see Fig. 2). Thus, one can notice that the distribution of leucine residues in the cell-free product at positions 30, 37, and 44 coincides very well with that of positions 10, 17, and 24 of the lipoprotein, if the prolipoprotein is assumed to have 20 extra amino acid residues at its amino-terminal end. It is also important to point out that there are no leucine residues between position 21 and 29 in Fig. 10. The results in Fig. 10 also indicate that there are five leucine residues in

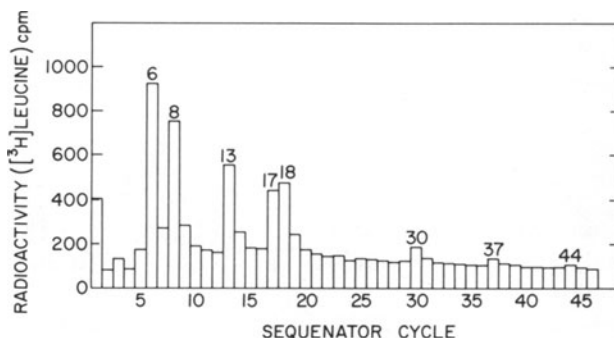



Fig. 10. Forty-six consecutive Edman degradations of the cell-free product labeled with [$^3$H]leucine (Inouye *et al.*, 1977*a*).

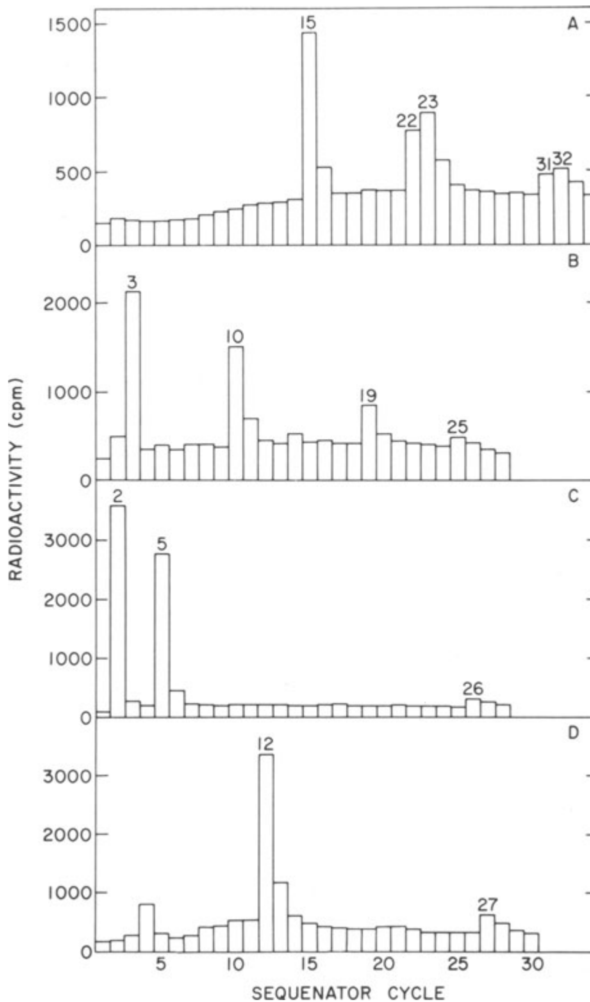



Fig. 11. Sequential Edman degradation of the cell-free products labeled with [$^3$H]serine (A), [$^3$H]alanine (B), [$^3$H]lysine (C), and [$^3$H]isoleucine (D) (Inouye *et al.*, 1977*a*).

the extended region at positions 6, 8, 13, 17, and 18. This is also in very good agreement with the data obtained from toluene-treated cells (Table I), in which 4 to 5 leucine residues are predicted to exist in the extended region of the prolipoprotein.

If the extended region has 20 amino acid residues, one can predict the appearance of amino acids after the twenty-first cycle of the Edman degradation, because the amino acid sequence of the lipoprotein is

known. For example, serine residues are known to be at positions 2, 3, 11, 12, 25, and 33 of the lipoprotein (see Fig. 2). Thus, one can predict that serine residues should appear at the 22nd, 23rd, 31st, 32nd, 45th, and 53rd cycles of the Edman degradation of the cell-free product. Fig. 11A shows the Edman degradation of the cell-free product, labeled with [$^3$H]serine, up to the 34th cycle. The radioactivities clearly appear at the 22nd, 23rd, 31st, and 32nd cycles, as predicted. Fig. 11A also shows that there is one serine residue at the position 15 of the extended region. Figure 11B, C, and D provides further confirmation of the length of the extended region. Alanine residues appear at the 25th cycle of the Edman degradation, as predicted (see Fig. 2), as well as at positions 3, 10, and 19 (Fig. 11B). Lysine residues are detected at the position 26 as predicted (see Fig. 2), as well as at positions 2 and 5 of the extended region (Fig. 11C). Furthermore, isoleucine residues appeared at the 27th cycle of the Edman degradation, as predicted, as well as at position 12 of the extended region of the cell-free product (Fig. 11D).

In order to determine the complete amino acid sequence of the extended region, Edman degradation is carried out with the cell-free product labeled with the following amino acids, which are predicted to exist in the prolipoprotein produced in toluene-treated cells (Table I): methionine, glycine, threonine, and valine. In this way, the final complete amino acid sequence of the prolipoprotein is obtained as shown in Fig. 12. As one can see from the sequence in Fig. 12, the amino acid composition of the peptide extension of the prolipoprotein is almost exactly

```
 1           5              10                  15
Met-Lys-Ala-Thr-Lys-Leu-Val-Leu-Gly-Ala-Val-Ile-Leu-Gly-Ser-

                20           25                  30
Thr-Leu-Leu-Ala-Gly-Cys-Ser-Ser-Asn-Ala-Lys-Ile-Asp-Glu-Leu-
                     1           5                  10
                35              40                  45
Ser-Ser-Asp-Val-Gln-Thr-Leu-Asn-Ala-Lys-Val-Asp-Glu-Leu-Ser-
                15              20                  25
                50              55                  60
Asn-Asp-Val-Asn-Ala-Met-Arg-Ser-Asp-Val-Gln-Ala-Ala-Lys-Asp-
                30              35                  40
                65              70                  75
Asp-Ala-Ala-Arg-Ala-Asn-Glu-Arg-Leu-Asp-Asn-Met-Ala-Thr-Lys-
                45              50                  55
        78
Tyr-Arg-Lys
        58
```

Fig. 12. Amino acid sequence of the proliproprotein. The extended region is framed. The numbers above the amino acids represent the positions of the prolipoprotein amino acid residues counted in sequences from the amino terminus. The numbers under the amino acids represent the positions counted in sequence from the lipoprotein amino terminus.

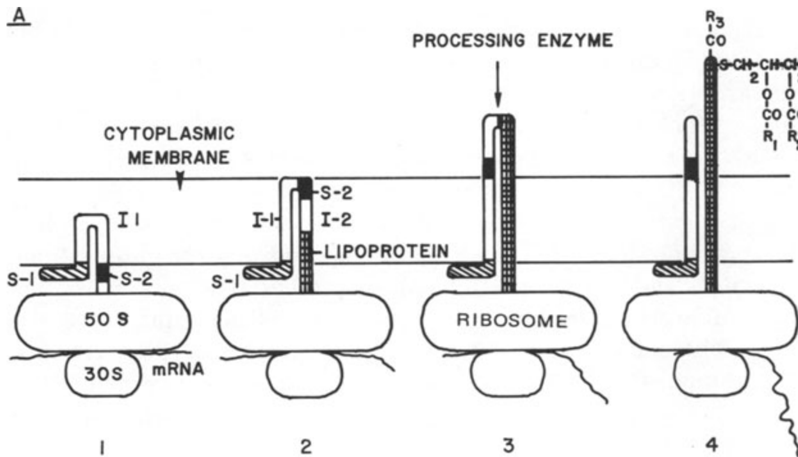
the same as that of the peak I protein of the toluene-treated cells (Table I). This can be further confirmed by sequential Edman degradation of the peak I protein labeled with [$^3$H]leucine and [$^{35}$S]methionine from the toluene-treated cells. We found that peak I protein has a methionine residue at position 1 (Halegoua *et al.,* 1977), and leucine residues at positions 6, 8, 13, 17, and 18 (Inouye *et al.,* 1977*a*). Therefore, we can conclude that the cell-free product is identical to the peak I protein. It should be pointed out that the first methionine seems to be formylated in the cell-free product, since a hot TCA treatment of the product improves the recovery of the radioactivity in the Edman degradation. This indicates that the prolipoprotein shown in Fig. 12 is the primary product in the cell-free system.

### 3. *Functions of the Peptide Extension*

The extended region of the prolipoprotein has several profound features: (1) The extended region is basic and positively charged at neutral pH because it contains two lysine residues but no acidic amino acid residues; (2) this region contains three glycine residues which are not present in the lipoprotein. It is particularly interesting that the last amino acid residue of the extended region is glycine, where a specific enzyme must process the prolipoprotein to produce the lipoprotein; (3) 60% of the amino acid residues in the extended region are hydrophobic, in contrast to 38% in the lipoprotein; (4) the distribution of these hydrophobic amino acids along the peptide chain is completely different from their periodic distribution in the lipoprotein, as will be discussed later.

The extended region can be divided into four separate sections on the basis of the amino acid arrangement. The first section (section S-1), consisting of five amino acid residues, is hydrophilic and positively charged because of two lysine residues and one threonine residue. The second section (section I-1) is formed by nine hydrophobic amino acid residues and contains two very similar hydrophobic sequences: Leu-Val-Leu-Gly and Val-Ile-Leu-Gly. The third section (section S-2) is the second hydrophilic part, consisting of one serine and one threonine residue. The fourth section (section I-2) is the second hydrophobic segment and is formed by four amino acids: Leu-Leu-Ala-Gly—a hydrophobic sequence similar to the sequences found in section I-1. Section I-2 is followed by the lipoprotein. Each section of the extra region may play an important role in translocating the lipoprotein, produced in the cytoplasm, to the outer membrane. For instance, section S-1 is probably involved in leading the initial attachment of the prolipoprotein to the

membrane by forming ionic interactions between the positively charged section S-1 and the negatively charged surface of the membrane. Following section S-1, a long stretch of hydrophobic section I-1 is produced and is most likely inserted inside the membrane. The following hydrophilic section S-2 may play a role in keeping the carboxyl-terminal end of section I-1 on the surface of the membrane or may be inserted into the membrane together with the following hydrophobic section I-2. Figure 13A shows a schematic diagram of the possible first step in prolipoprotein attachment to the cytoplasmic membrane, where section I-1 is bent at its



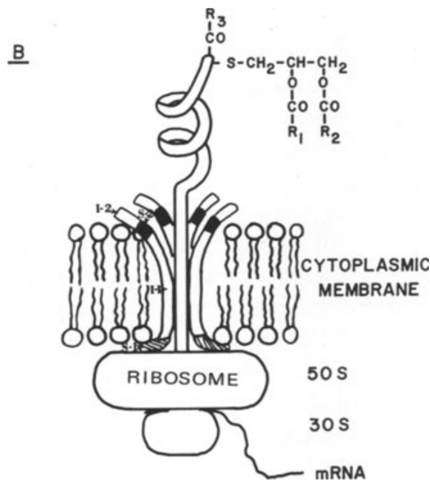




Fig. 13. A possible mechanism of translocation of the prolipoprotein across the cytoplasmic membrane. S-1, Met-Lys-Ala-Thr-Lys; I-1, Leu-Val-Leu-Gly-Ala-Val-Ile-Leu-Gly; S-2, Ser-Thr; I-2, Leu-Leu-Ala-Gly.

center and penetrates halfway through the membrane. This molecular mechanism of secretion of proteins across the cytoplasmic membrane is called a "loop model" and has been extensively discussed in a recent review (Inouye and Halegoua, 1979).

As the peptide chain is further elongated, the translocation of the prolipoprotein across the cytoplasmic membrane may proceed as shown in Fig. 13. In this model, the hydrophilic section S-1 remains on the inside surface of the cytoplasmic membrane, whereas the hydrophilic section S-2 becomes exposed to the outside surface of the cytoplasmic membrane. When the translocation process proceeds to step 3 in Fig. 13A, specific endopeptidase would cleave off the peptide extension of 20 amino acid residues. The newly formed amino-terminal end is then modified immediately and, as the peptide is further elongated, it could begin to form the lipoprotein secondary structure, making the translocation process irreversible. It is interesting to point out that the three glycine residues in the peptide extension are present at positions 9, 14, and 20, which are located at the bending positions at steps 1, 2, and 3, respectively.

What happens with the peptide extensions left in the cytoplasmic membrane is not known. They may be digested quickly into free amino acids, which could then be reutilized for protein synthesis. However, it is conceivable that before digestion, molecules of the peptide extensions assemble with each other in the cytoplasmic membrane to provide a self-supplied tunnel for the translocation of the lipoprotein (Fig. 13B). The loop model would explain very well the existence of the membrane-bound ribosomes for outer membrane proteins (Randall and Hardy, 1977). Moreover, it predicts that: (1) the precursor could not be inserted into the outer membrane without being processed; (2) the prolipoprotein could not be found as an intermediate under normal growth conditions; and (3) the peptide extension would be found in the cytoplasmic membrane.

Recently we have found that two other major outer-membrane proteins of *E. coli*, the matrix protein and the tolG protein, are also synthesized from precursors of higher molecular weights (Sekizawa *et al.*, 1977). The data suggest that these precursors also have a peptide extension consisting of about 20 amino acid residues, as does the prolipoprotein. Furthermore, alkaline phosphatase, a periplasmic enzyme, has been shown to be produced from a precursor of higher molecular weight (Inouye and Beckwith, 1977). The determination of the amino acid sequences of these peptide extensions will provide important information concerning the mechanism of translocation of the outer membrane proteins as well as of the periplasmic proteins.

### 4. *Signal Hypothesis*

It is extremely interesting to point out that many secretory proteins in eukaryotic cells are produced from their precursors. These proteins have also been shown to contain from 16 to 25 extra amino acid residues at their amino-terminal ends. Unfortunately, only a few complete amino acid sequences of these proteins have been determined [as of August, 1977; for recent results, see the review by Inouye and Halegoua (1979)]. However, we can find rather amazing similarities among these amino acid sequences, especially between those of the *E. coli* prolipoprotein and the prelysozyme of chick oviduct (Thibodeau *et al.*, 1977). As shown in Fig. 14: (1) both proteins start from a methionine residue; (2) both contain a basic amino acid at position 2; (3) both have a row of 9 hydrophobic amino acid residues; (4) both have a proline or a glycine residue at the end of the hydrophobic row, both of which are known to be able to bend the peptide chain; and (5) the extended regions of both proteins end either in Ala-Ala-Leu-Gly (for prelysozyme), or Leu-Leu-Ala-Gly (for prolipoprotein), which is possibly recognized by the processing enzyme for the precursor.

Besides the amino acid sequence of chick oviduct prelysozyme, the complete amino acid sequence of the peptide extensions of the precursors of two mouse immunoglobulin L-chains have been determined (Burstein and Schechter, 1977). One L-chain precursor contains 19, and the other 22, extra residues. In these cases, the similarity to the sequence of the prolipoprotein is less evident. However, both sequences start with methionine, and both also have a row of 8 to 9 hydrophobic amino acid residues in the middle of their extension peptides. Furthermore, each of them has a proline or a glycine residue at the end of the hydrophobic stretch. Beside these proteins, the following proteins are shown to be produced from their precursors (the numbers in parentheses indicate the numbers of amino acid residues in the extra regions of the precursors): preovomucoid of chick oviduct (23) (Thibodeau *et al.*, 1977); fish preproinsulin (23 and 25) (Shields and Blobel, 1977); rat preproalbumin (18) (Strauss *et al.*, 1977; Yu and Redman, 1977); bovine preproparathyroid

Prolipoprotein: Met-Lys-Ala-Thr-Lys-Leu-Val-Leu-Gly-Ala-Val-Ile-Leu-Gly-Ser-Thr-Leu-Leu-Ala-Gly-

Prelysozyme: Met-Arg-Ser-Leu-Leu-Ile-Leu-Val-Leu-Cys-Phe-Leu-Pro-Leu-Ala-Ala-Leu-Gly

Fig. 14. Comparison of the amino acid sequence of the extension peptide of prelysozyme from chick oviduct (Thibodeau *et al.*, 1977) with that of the prolipoprotein (Inouye *et al.*, 1977*a*).

hormone (25) (Kemper *et al.,* 1976); rat preproinsulin (23) (Chan *et al.,* 1976); and pretrypsinogen and other pancreatic secretory proteins (16) (Devillers-Thiery *et al.,* 1975).

Blobel has proposed a model called the "signal hypothesis" to explain the mechanism of translocation of secretory proteins across the membrane (Blobel and Dobberstein, 1975). In this hypothesis, the precursors are first synthesized on free ribosomes. However, when the peptide extensions are completed, their unique signal sequences are recognized by specific receptor proteins on the surface of the membrane. This results in firm attachment of the ribosomes to the membrane, as well as induction of the receptor proteins to form tunnels through the membrane. Through these tunnels, the peptide chains are translocated as they are elongated on the ribosomes. This model also explains the very effective translocation of secretory proteins across the membrane without premature folding of nascent peptides inside the cells. Folding of the secretory proteins inside the cells would probably prevent them from being translocated across the membrane.

This model can be applied to the translocation of the *E. coli* lipoprotein across the cytoplasmic membrane. However, it is not certain whether receptor proteins exist in the *E. coli* cytoplasmic membrane. Since there are $7.2 \times 10^5$ molecules of the lipoprotein, about 1500 such receptor proteins for the lipoprotein should exist at any given time, assuming that protein synthesis occurs at the rate of 10 amino acid residues/sec in cells growing at a generation time of 60 min. Since at least two other major outer membrane proteins, the matrix protein (mol. wt. = 36,500; about $1.5 \times 10^5$ molecules), and the tolG protein (mol. wt. = 33,000; about $1 \times 10^5$ molecules), as well as periplasmic proteins, are produced from their precursors, there should be another 3000–4000 receptor proteins in the cytoplasmic membrane. Therefore, at least 5000 receptor proteins are required. If such receptor proteins exist in the *E. coli* cytoplasmic membrane, many questions can be raised concerning their structures and function, such as: (1) How can we find these proteins? (2) Are these receptor proteins specific for individual proteins translocated across the cytoplasmic membrane? If they are specific, one can predict that the production of each outer-membrane protein is controlled not only by its own structural gene but also by its own receptor gene. However, if the receptors are not specific and one receptor protein is shared by many different outer-membrane proteins, periplasmic proteins, or both, one can predict that a single mutation on the structural gene for the receptor protein causes inhibition of the translocation of all outer-membrane proteins and of periplasmic proteins as well. (3) How is the biosynthesis of

these receptor proteins regulated? It should be closely coordinated with the biosynthesis of the outer-membrane proteins.

One important difference between the loop model proposed for the translocation of the prolipoprotein in Fig. 13 and the signal hypothesis is that in the latter model, the peptide extension also passes through the membrane to the outside, and a specific proteolytic enzyme then digests the extension peptide. At any rate, studies on the mechanism of the translocation of the prolipoprotein will also provide many important clues for an understanding of the precise molecular mechanism of the secretion of hormones, enzymes, and immunoglobulin in eukaryotic cells.

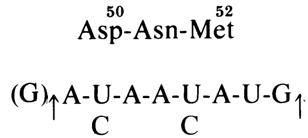
## E. Structure of the Lipoprotein mRNA

### *1. Identification of the mRNA*

As mentioned earlier, the mRNA for the lipoprotein has been purified and shown to be biologically active. It is extremely interesting to determine its chemical structure. This is not only owing to this mRNA being the first to be isolated from *E. coli,* but also for many other reasons: (1) since the mRNA is possibly being produced constitutively in the cells, its structure may differ from that of the mRNAs specific for inducible enzymes such as $\beta$-galactosidase and tryptophan synthetase. (2) As the mRNA for a membrane protein, the mRNA of the lipoprotein may have a unique base sequence. (3) The mRNA is extremely stable, and the determination of its chemical structure may elucidate the reason for this stability.

In order to determine the complete nucleotide sequence of the lipoprotein mRNA, different procedures from those mentioned in Section IIIC.2 were used for purification of the mRNA. The mRNA was isolated from *E. coli* cells labeled with 100 mCi [$^{32}$P]orthophosphate (Takeishi *et al.,* 1976). After phenol extraction, the mRNA was purified by three polyacrylamide gel electrophoreses. The final product was identified as the lipoprotein mRNA from its comigration with nonradioactive lipoprotein mRNA which was active in an *E. coli* cell-free system. However, more conclusive evidence was provided by the fact that $T_1$ ribonuclease digestion of the purified mRNA produces many oligonucleotides that can be assigned to parts of the amino acid sequence of the lipoprotein. Among them, the following octanucleotide (T26) is especially important, since from amino acid sequence of the lipoprotein and the genetic code, the

existence of this octanucleotide is obligatory:




The arrows indicate the positions of $T_1$ ribonuclease cleavage. This obligatory octanucleotide (T26) was found in the spot shown by an arrow in Fig. 15, and the base sequence of T26 has been determined to be A-C-A-A-C-A-U-G (R. M. Pirtle, I. L. Pirtle, and M. Inouye, unpublished data).

### 2. *Base Sequence of the mRNA*

The base sequences of all 33 $T_1$ fragments in Fig. 15, as well as fragments obtained from pancreatic ribonuclease A digestion of the

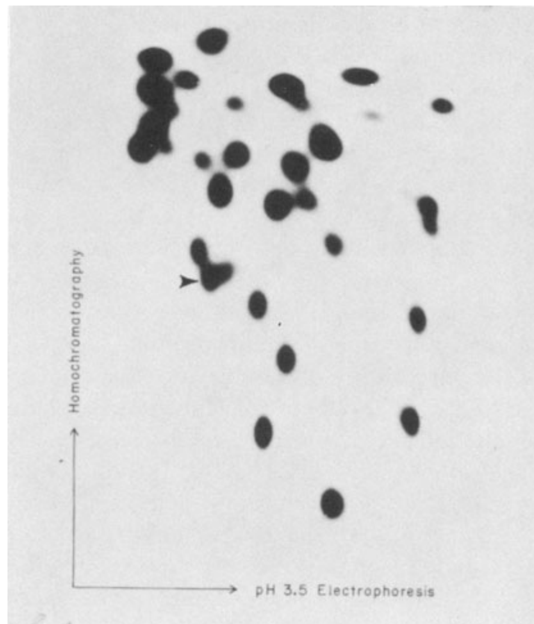



Fig. 15. Autoradiogram of the two-dimensional separation of T1 ribonuclease-digests of the lipoprotein mRNA. An arrow in the figure indicates the spot of T26 obligatory octanucleotide (Takeishi *et al.*, 1976).

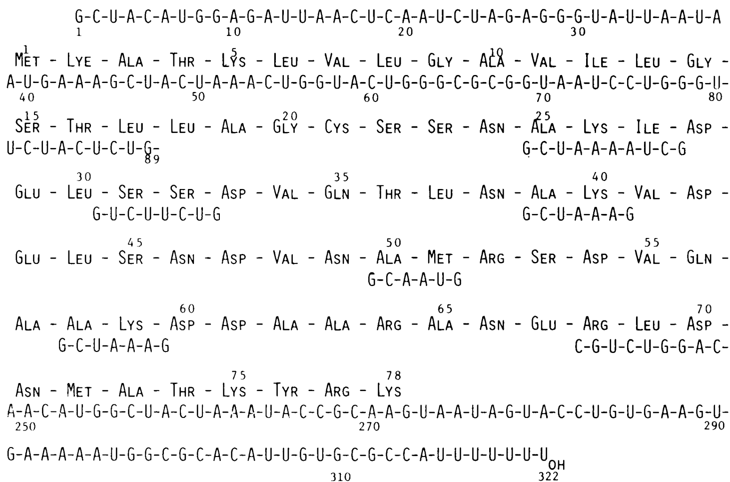



Fig. 16. Nucleotide sequence of the lipoprotein mRNA. The base sequences of the 5′-end (Pirtle *et al.*, 1978), the 3′-end (R. M. Pirtle, I. L. Pirtle, and M. Inouye, unpublished data), and oligonucleotides assigned to the amino acid sequence of the prolipoprotein (R. M. Pirtle, I. L. Pirtle, and M. Inouye, unpublished data) are shown.

mRNA, have been determined. We have also recently determined the nucleotide sequence of the 5′ end of the mRNA, consisting of 89 bases (Pirtle *et al.*, 1978), and the nontranslated region of the 3′ end of the mRNA, consisting of 50 bases (R. M. Pirtle, I. L. Pirtle, and M. Inouye, unpublished data). Figure 16 shows the nucleotide sequence of the mRNA so far determined.

From the data obtained thus far, the following facts summarize the chemical structure of the mRNA: (1) The size of the mRNA is 8.2 S. (2) It consists of 322 bases. (3) It has nontranslated regions of 38 and 50 bases at the 5′ end and the 3′ end of the mRNA, respectively. (4) One of the most unique features of the 5′ end structure is that it has the same sequence of 12 bases ($\overset{30}{\text{G}}$-U-A-U-U-A-A-U-A-A-U-$\overset{41}{\text{G}}$) that contains the 80 S ribosome binding site in brome mosaic virus RNA4, a eukaryotic mRNA (Pirtle *et al.*, 1978). (5) At the end of the coding region, three different termination codons—UAA, UAG, and UGA—are used in the same reading phase. (6) At the 3′ end, there is a very stable stem-and-loop structure between $\overset{292}{\text{A}}$-A-A-A-A-U-G-G-C-G-C-A-$\overset{304}{\text{C}}$ and $\overset{308}{\text{G}}$-U-G-C-G-C-C-A-U-U-U-U-$\overset{320}{\text{U}}$ (R. M. Pirtle, I. L. Pirtle, and M. Inouye, unpublished data). This structure may be important for the stability of the mRNA.

# IV. MODIFICATION AND ASSEMBLY

## A. Posttranslational Modification

### 1. General Considerations

As seen in the previous section, the lipoprotein is produced from its precursor, the prolipoprotein. Knowing the final structure of the lipoprotein in the outer membrane (Fig. 2), one can realize that surprisingly many modification steps are involved in processing the prolipoprotein to its final form. This posttranslational modification is possibly very important for the assembly of the lipoprotein in the outer membrane, as well as for the function of the lipoprotein. In Fig. 17, the steps in this modification are summarized: removal of the extension peptide (step 1),

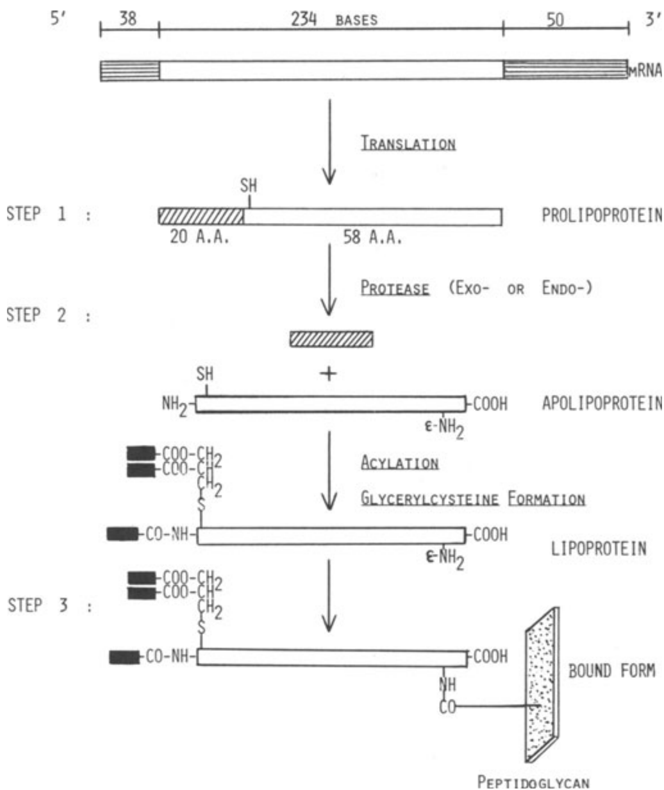



Fig. 17. Posttranslational modification of the prolipoprotein.

amino-terminal modification (step 2); and conversion from the free form to the bound form (step 3). An interesting question is: At which step is it determined that the lipoprotein is to be inserted into the outer membrane, but not into the cytoplasmic membrane?

### 2. *Step 1: Removal of the Peptide Extension*

The peptide extension of the prolipoprotein should be removed by a proteolytic enzyme(s). If the prolipoprotein is processed as proposed in Fig. 13, an endopeptidase recognizes the specific amino acid sequence of section I-2 of the prolipoprotein ($\overset{17}{\text{Leu}}$-Leu-Ala-$\overset{20}{\text{Gly}}$), and cleaves the peptide bond between Gly(20) and Cys (21). On the other hand, if the whole extended region is translocated across the membrane, as proposed in the signal hypothesis (see Section IIID4), an exopeptidase or combination of exopeptidase and endopeptidase removes the peptide extension. In any event, there are many other interesting questions concerning this step: (1) Is this enzyme localized on the outside surface of the cytoplasmic membrane, in the periplasmic space, or on the inner surface of the outer membrane? It has been reported that the proteolytic enzyme that processes the precursor of alkaline phosphatase (a periplasmic enzyme) to the mature protein resides in the outer membrane fraction (Inouye and Beckwith, 1977). (2) Is this proteolytic enzyme specific for each outer-membrane and periplasmic protein? (3) What will happen if the activity of this enzyme is blocked by an inhibitor or by mutation?

### 3. *Step 2: Amino-Terminal Modification*

In this step, there are at least two independent reactions: acylation of the amino group of the first cysteine residue; and addition of a diglyceride group to the SH group of the first cysteine residue. Hantke and Braun (1973) showed that the compositions of the amide-linked fatty acids and the ester-linked fatty acids (see Fig. 2) are different: The amide-linked fatty acids are mainly palmitic acid (65%), palmitoleic acid (11%), and *cis*-vaccenic acid (11%). On the other hand, the composition of the ester-linked fatty acids is similar to the fatty acid composition of the phospholipids of the same cells. This suggests that the diglyceride moiety of the lipoprotein is derived from one stage in the pathway of phospholipid metabolism. Lin and Wu (1976) suggested that the attachment of a diglyceride to the amino-terminal cysteine is independent of the synthesis of the lipoprotein. Recently, Schulman and Kennedy (1977) reported that

the diglyceride of the lipoprotein contains glycerol derived from a metabolic pool with a relatively large half-life. This finding rules out CDP-diglyceride as an immediate precursor for the lipoprotein, as had been originally predicted (Hantke and Braun, 1973). Using an *E. coli* mutant in which one can control the turnover of both phosphatidylglycerol and cardiolipin, Schulman and Kennedy (1977) suggested that the diglyceride moiety of the lipoprotein may derive from the diglyceride moiety of cardiolipin. More recently, however, Chattopadhyay and Wu (1977) presented data which strongly suggest that the donor for the glycerol moiety of the lipoprotein is the nonacylated glycerol moiety of phosphatidylglycerol (see Fig. 18). They showed that the C-1 rather than the C-3 carbon of *sn*-glycerol is involved in the thioether linkage and that in a mutant of cardiolipin synthetase there is no reduction of incorporation of [2-$^3$H]glycerol into the lipoprotein. In view of these results, it is likely that the glyceryl group is first transferred from phosphatidylglycerol to form glycerylcysteine (apolipoprotein II) and that two acyl groups are then transferred to the glyceryl group (apolipoprotein III) as shown in Fig. 18. This suggests the existence of another specific modification enzyme required for the acylation.

In a mutant lipoprotein in which the 57th arginine residue was replaced with a cysteine residue (see Section V), the amino group of the amino-terminal cysteine residue was found to be fully acylated, whereas only 50% of glyceryl OH groups were found to be acylated (Inouye *et al.*, 1977*b*). This result is possibly because the mutation in the lipoprotein causes an inhibitory effect on the acylation of the glyceryl group. If this is true, it indicates that the acylation of the amino group (apolipoprotein I) occurs before or independently of the acylation of the glyceryl group as shown in Fig. 18. Again, many interesting questions remain to be solved for this modification step: (1) How are these modification reactions at the amino-terminal end coordinated? (2) Where is the site of these reactions? (3) How important is this modification step for the assembly of the lipoprotein in the outer membrane?

#### 4. *Step 3: Conversion of the Free Form to the Bound Form*

In Section IIB, a method for identifying both the free and the bound forms of the lipoprotein was discussed (see Fig. 4). Using this technique, we can characterize the biosynthetic relationship between the free and the bound forms. When cells are pulse-labeled with [$^{14}$C]arginine, the

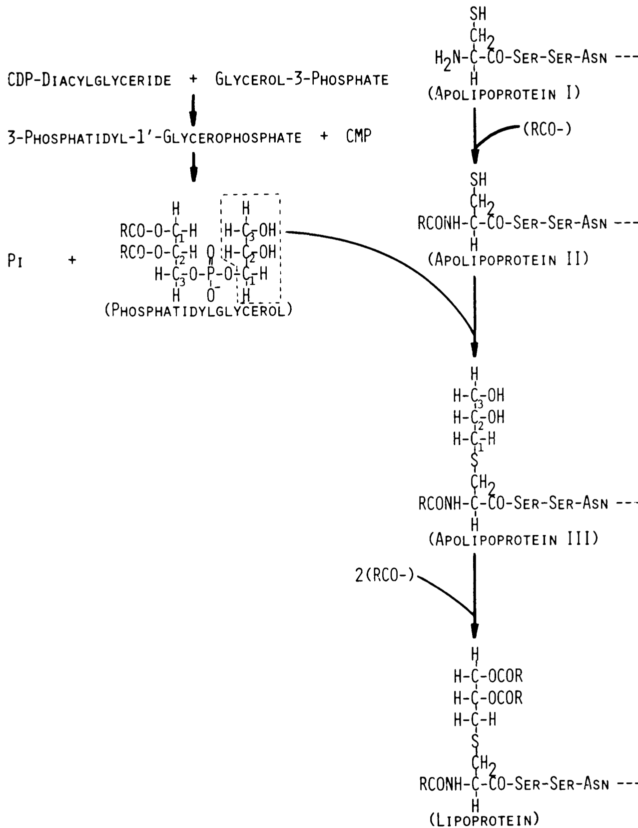
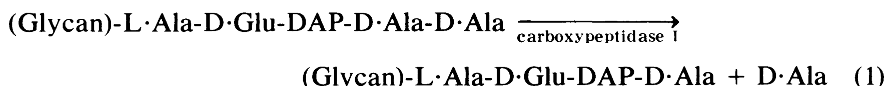


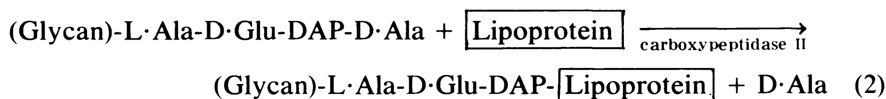

Fig. 18. Modifications of cysteine residue at the first position of the lipoprotein with phosphatidylglycerol (Chattopadhyay and Wu, 1977).

radioactivity is incorporated only into the free form. When the pulse is chased by adding a large excess of nonradioactive arginine, however, the bound form also becomes labeled (Inouye *et al.*, 1972). From these results one can conclude that the free form of the lipoprotein is synthesized first and is then converted to the bound form.

The conversion of the free to the bound form has been shown to be unaffected by inhibiting protein synthesis through amino acid starvation or chloramphenicol, or through inhibiting energy production with carbonyl cyanide *m*-chlorophenylhydrazone (Inouye *et al.*, 1972). Since energy does not appear to be directly required for the conversion reaction, it was proposed that the reaction takes place by transpeptidation (Inouye

*et al.*, 1972). Carboxypeptidase II may work as follows:




In these reactions, carboxypeptidase I is known to be sensitive to penicillin, but carboxypeptidase II is not (Izuki *et al.*, 1966). In fact, it has been reported that penicillin G (Braun *et al.*, 1974) and penicillin FL 1060 (Braun and Wolff, 1975) do not inhibit the conversion reaction. However, it appears that high concentrations of penicillin G result in inhibition of the reaction, probably by inhibiting step (1) (S. Halegoua and M. Inouye, unpublished results).

It has been suggested that the conversion is reversible; about 40% of the pulse-labeled free form is chased into the bound form after one generation, but further chasing does not increase the radioactivity in the bound form (Inouye *et al.*, 1972). The relative amount of radioactivity in the free form remains constant after from one to at least three doubling times. This can be explained as follows: The newly synthesized free form is diluted with the large preexisting pool of the free form. There is, however, a dynamic equilibrium (reversible conversion) between the free and bound forms, and the ratio of free to bound lipoprotein is maintained at 2. If 40% of the free form is irreversibly converted to the bound form for every doubling time, the relative amounts of the radioactive free form should be 60%, 33% and 19%, after one, two, and three doubling times, respectively. Alternatively, the conversion can be irreversible if the newly synthesized and pulse-labeled free form is somehow compartmentalized in the evenlope without being diluted by the large pool of preexisting free form (for instance, if it localizes at the septum-forming site); that is, 40% of the newly synthesized free form is converted to the bound form in one doubling time, and the remaining 60% is transferred to the large pool of preexisting free form, which does not participate in conversion.

It should be pointed out that the conversion reaction occurs after the free form of the lipoprotein is transported to the outer membrane. Thus, the enzyme responsible for the conversion should exist in the periplasmic region or at the inside surface of the outer membrane.

Another intriguing question is how the conversion reaction is coordinated with peptidoglycan biosynthesis. Newly inserted peptidoglycan subunits appear to be linked very slowly to the lipoprotein (Braun and Bosch, 1973; Braun and Wolff, 1975). This suggests that the enlargement of the peptidoglycan layer occurs at the site where the lipoprotein is not attached. In this regard, it is interesting to note that mutants containing very low amounts of the bound form of the lipoprotein have been isolated, in which the outer membrane cannot properly invaginate in spite of normal ingrowth of the cytoplasmic membrane and the peptidoglycan layer (Weigand *et al.*, 1976). Such mutants may be useful not only for the study of the function of the lipoprotein but also for the characterization of the conversion reaction. Furthermore, bicyclomycin, an antibiotic, has been reported to specifically inhibit the biosynthesis of the lipoprotein, particularly the formation of the bound form (Tanaka *et al.*, 1976). It would be very interesting to know how this antibiotic inhibits the conversion reaction.

#### 5. *Protein Folding*

Another important question concerning the posttranslational modification of the lipoprotein is the formation of the proper protein conformation. How does the proper folding of nascent polypeptides of the lipoprotein take place? As discussed earlier (Section III), it is most likely that the folding of the lipoprotein occurs outside the cytoplasmic membrane. The peptide extension of the prolipoprotein triggers the binding of the ribosomes to the membrane, from which the nascent polypeptide is directly forced to go across the cytoplasmic membrane.

Including this step, which process is the most crucial one for the insertion of the lipoprotein into the outer membrane? How does this mechanism prevent the lipoprotein from being inserted into the cytoplasmic membrane? These are intriguing questions in the study of the assembly mechanism of the outer-membrane protein.

### B. Molecular Assembly Models

#### 1. *Channel Model*

As discussed in Section IID, the lipoprotein has very high $\alpha$-helical content. It should also be noted that the lipoprotein lacks proline and glycine residues, suggesting that there are no bends in the $\alpha$-helical

structure. The pattern of the amino acid sequence of the lipoprotein is surprisingly similar to that of the carboxyl-terminal half of the tropomyosin molecule, which also has a high $\alpha$-helical content (Hodges *et al.*, 1972). Namely, a regular pattern of hydrophobic residues occurs in two series, I and II in band H along the length of the helix, as shown in Fig. 19, where the position of each residue in the sequence of the lipoprotein is plotted on a helical net. In this figure, a regular right-handed $\alpha$-helix, with 3.6 residues per turn, is represented as a cylinder which has been opened up and laid flat on the paper (helical net). As can be seen, there are no hydrophobic residues in the whole structure outside of band H, except for alanine residues at positions 5, 19, 30, 37, 41, and 51. As illustrated, the hydrophobic residues in band H are arranged in straight lines (Inouye, 1974). Since the lipoprotein is a membrane component, the $\alpha$-helices are more likely to be arranged in such a way as to allow the hydrophobic residues to face out, while the hydrophilic residues are localized inside the assembly.

From these considerations, Inouye (1974) proposed that the lipoprotein molecules are arranged in a superhelix, in which a number of ionic interactions are formed between adjacent molecules, thus stabilizing the entire assembly. As can be seen in Fig. 19, the hydrophilic bands $P_A$ and $P_B$, which run parallel to the hydrophobic band H, are complementary to each other in terms of ionic properties; that is, when an acidic residue is located on one side, a basic residue is located on the other side. In the superhelical arrangement, as many as seven stable ionic interactions are formed between the $P_A$ band of one $\alpha$-helix and the $P_B$ band of the adjacent $\alpha$-helix. In Fig. 19, those residues forming ionic interactions are encircled by solid lines. Of seven ionic interactions thus formed, two ($\overset{6}{\text{Lys}}$:$\overset{8}{\text{Asp}}$ and $\overset{20}{\text{Lys}}$:$\overset{22}{\text{Asp}}$) are located in the amino-terminal half, and the remaining five ($\overset{34}{\text{Asp}}$:$\overset{32}{\text{Arg}}$, $\overset{41}{\text{Asp}}$:$\overset{39}{\text{Lys}}$, $\overset{44}{\text{Arg}}$:$\overset{40}{\text{Asp}}$, $\overset{48}{\text{Arg}}$:$\overset{47}{\text{Glu}}$, and $\overset{55}{\text{Lys}}$:$\overset{50}{\text{Asp}}$) are located in the carboxyl-terminal half.

The number of molecules in one superhelical assembly may be determined by maximizing the number and stability of the ionic interactions. From such considerations, the number of lipoprotein molecules per assembly could range from 6 to as many as 12. Since both the free and the bound forms of the lipoprotein are located exclusively in the outer membrane, Inouye (1974) proposed that there are two possible ways in which a superhelical assembly could interact with the outer membrane: (1) The interaction could occur through the three fatty acids attached to the amino-terminal amino acid of the lipoprotein, as suggested by Braun (1975). In this case, the hydrocarbon chains of the fatty acids stick out

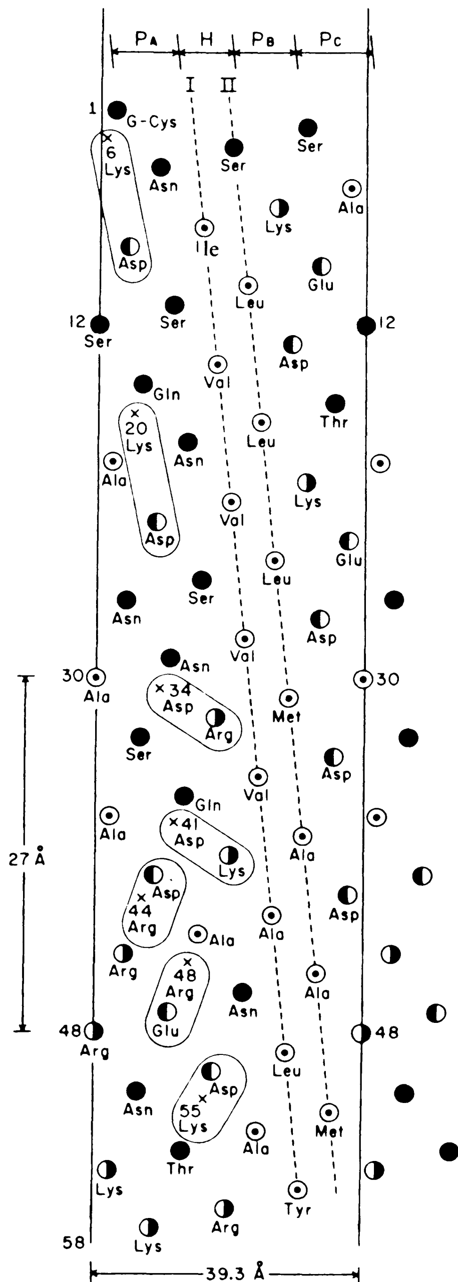



Fig. 19. Amino acid sequence of the lipoprotein (see Fig. 2) plotted on an $\alpha$-helical net (see the text). The diameter of the $\alpha$-helix is taken as 12.5 Å with 3.6 residues per turn, and the height of 5 turns as 27 Å. The numbers correspond to the positions of residues from the amino terminus. Hydrophobic residues in positions of series I and II are shown on dotted lines. Positions of the hydrophobic band, H, and the hydrophilic bands, $P_A$, $P_B$, and $P_C$, are also shown at the top of the $\alpha$-helical net. Some amino acid residues are plotted twice at the right-hand side of the lower-half section in order to show residues in the band $P_B$. For these duplicated residues, names of amino acids are not indicated. Hydrophobic residues, ⊙; basic residues, ◑; acidic residues, ◐; and all other hydrophilic residues, ●. The amino-terminal residue, G-Cys, represents glycerylcysteine. Complementary amino acid residues on the band $P_B$ of an adjacent $\alpha$-helix (helix 2). X: These residues of helix 2 are superimposed on the $\alpha$-helical net of helix 1 to show ionic interactions with residues on the band $P_A$ of helix 1. The ionic interactions are shown by encircling two complementary residues by a solid line. The positions of the superimposed residues are determined by assuming that six $\alpha$-helices are arranged to form a superhelix, as discussed in the text (Inouye, 1974).

of the assembly and penetrate into the phospholipid bilayer of the outer membrane. Therefore, the protein part of the assembly protrudes from the inside surface of the outer membrane. This model would predict that the peptidoglycan layer should be at least 76 Å apart from the outer membrane, which is not likely. (2) Alternatively, the whole assembled structure, with a height of 76 Å, penetrates through the 75-Å-thick outer membrane with hydrophobic interactions between the surface of the assembly and the lipid bilayer of the outer membrane. This arrangement is further stabilized by the three hydrocarbon chains at the amino-terminal end of the individual molecules, which could be flipped back over the helix and inserted into the bilayer (Fig. 20). In order to arrange the hydrocarbon chains as shown in Fig. 20, the side chains of two serine residues at the amino terminus are made to face upward as a part of the surface of the outer membrane, which makes the uppermost part of the assembly hydrophilic.

At the carboxyl-terminal end of the assembly, two of six molecules of the assembly are covalently linked to the peptidoglycan layer. The distance between the outer membrane and the glycan layer of the peptidoglycan could be about 20 Å. Two carboxyl-terminal residues (-Arg-Lys), which are connected to the peptide portion of the peptidoglycan are exposed in this space. Thus the assembly is prevented from moving freely in the lipid bilayer.

As can be seen in Fig. 20, the above assembly model provides a pore through the outer membrane. The size of the pore, or the channel, depends on the number (*n*) of lipoprotein molecules per assembly. The diameter of the channel will change from 12.5–35.8 Å as *n* changes from 6–12. Accordingly, the total number of channels per cell could range from $0.63 \times 10^5$ to $1.25 \times 10^5$, and the area occupied by the assemblies from 35–46% of the total area of the cell surface.

The interior of the channel is hydrophilic and acidic because of four extra acidic residues ($\overset{9}{\mathrm{Glu}}$, $\overset{13}{\mathrm{Asp}}$, $\overset{23}{\mathrm{Glu}}$, and $\overset{27}{\mathrm{Asp}}$), which are localized in the upper half of the channel. The acidic property might confer some specificity for substances which could pass through the channel. Fundamentally, the channel could provide a passive-diffusion pore through the outer membrane, which accounts for the fact that many substances can pass through this surface structure independently of special transport systems.

This channel model is plausible and explains diffusion pores which are supposed to exist in the outer membrane (Decad and Nikaido, 1976). However, Nikaido and his associates have recently reported that another major protein of the outer membrane, the matrix protein, is required for

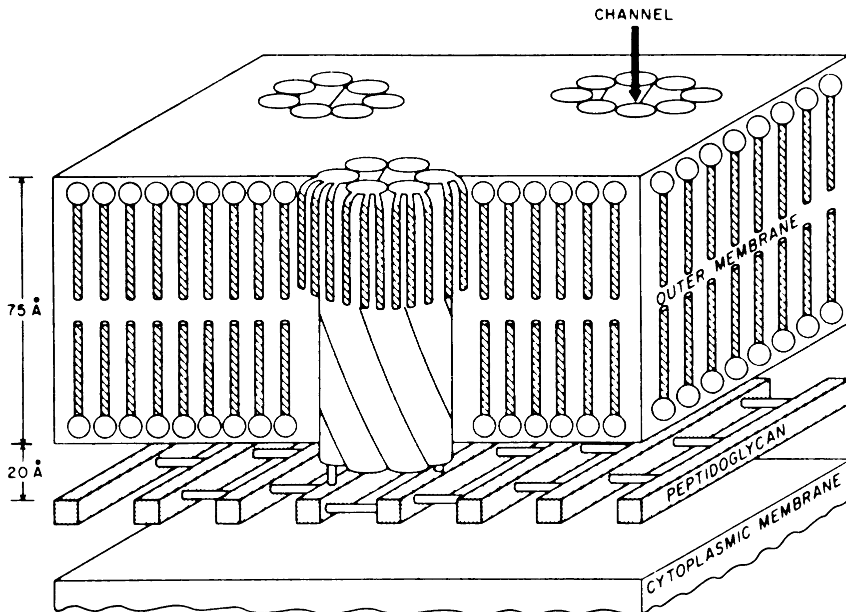



Fig. 20. Schematic illustration of the outer membrane structure (Inouye, 1974). A superhelix made of six $\alpha$-helices is shown to be inserted into the outer membrane and to span the full 75-Å-thick membrane. The three hydrocarbon chains attached at the top of each molecule are flipped over, hanging down from the top, and are anchored in the lipid bilayer of the outer membrane. At the bottom (carboxyl-terminal ends of the lipoproteins) of the assembly, two molecules are linked to the peptidoglycan layer, as shown by small bars. The peptidoglycan layer is illustrated by rectangular blocks (for the glycan chains) and small bars (for the peptide portions) which crosslink the glycan chains. Phospholipids forming the lipid bilayer are shown by hydrophilic, open, circular heads and hydrophobic, hatched, long tails. Channel openings of 7- and 8-membered assemblies are also illustrated on the surface of the outer membrane.

diffusion of a $\beta$-lactam antibiotic, cephaloridine, across the outer membrane of *Salmonella typhimurium* cells (Nikaido *et al.*, 1977). Furthermore, it has been shown that membrane vesicles reconstituted from purified matrix protein, lipopolysaccharide, and phospholipids have the same molecular sieving property as the intact outer membrane, which is permeable to a variety of low-molecular-weight compounds, but not to oligo- and polysaccharides of molecular weight higher than 700 (Nakae, 1976*a*,*b*). It was not possible to reconstitute lipoprotein-containing membrane vesicles having the same molecular sieving property.

In view of these results, the function of the lipoprotein should be reconsidered. However, it is still possible that lipoprotein molecules

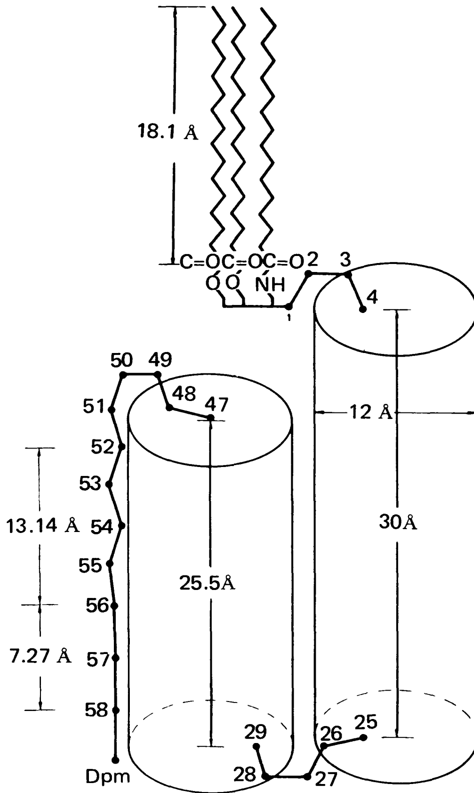



Fig. 21. Three-dimensional structure of the lipoprotein as deduced from the circular dichroism measurements and the amino acid sequence when applying the Chou-Fasman rules (Braun, 1975). Amino acid residues 1-4 form a $\beta$ loop, 5-24 an $\alpha$-helix with the possible exception of 13-17, which also could be arranged in a $\beta$-sheet), 25-29 clearly forms a $\beta$-loop, 30-47 an $\alpha$-helix, 48-51 a $\beta$-loop, 52-56 a $\beta$ sheet, and residues 57 and 58 a coil. The arrangement of the amino acid residues in the helical portions is only symbolized by the two cylinders. The length of the lipoprotein (48 Å) would span just half the thickness of the outer membrane, suggesting that the lipid portion of the lipoprotein immerses into the inner layer of the lipid bilayer of the outer membrane.

serve as channels along with the matrix protein, as will be discussed later. It is also possible that two or three $\alpha$-helices of the lipoprotein may form a coiled-coil structure, as in the case of tropomyosin (Hodges *et al.*, 1972), which may given an important structural function to the *E. coli* envelope. It should be pointed out that the lipoprotein has a very close association with the matrix protein in the intact membrane, as will be discussed in Section IVC.

## 2. *Other Models*

As described in Section IID, Braun and co-workers (1976*a,b*) estimated that the conformation of the bound form of the lipoprotein consists of about 15% $\beta$-structure and 80% $\alpha$-helical structure. An examination of these data according to the Chou and Fasman empirical rules, for predicting conformation of a protein from its amino acid sequence (Chou

and Fasman, 1974), leads to a molecular model shown in Fig. 21 (Braun, 1975; Braun *et al.*, 1976*a*). In this model, the bound-form lipoprotein may serve as an anchor to connect the outer membrane with the peptidoglycan layer. However, the possible functions of the free form of the lipoprotein—which exists in double the amount of the bound form—are not clear.

## C. Interactions with Other Proteins

### *1. Lipoprotein-Lipoprotein*

In view of the molecular assembly models discussed in the previous section, it is interesting to examine the interactions between the lipoprotein molecules themselves and between the lipoprotein and other outer-membrane proteins. When the outer-membrane proteins are cross-linked with a reversible cross-linking agent, a dimer and possibly a trimer of the lipoprotein are formed (Reithemeier and Bragg, 1977). The *in vivo* formation of dimers between the free and the bound forms and between the free forms themselves has been detected using a mutant of the lipoprotein which has an extra cysteine residue (see Section VA) (M. DeMartini and M. Inouye, unpublished results). These results suggest that the lipoprotein molecules are closely associated with each other in the outer membrane.

### *2. Lipoprotein-Matrix Protein*

We have found that both the bound and the free form of the lipoprotein are also closely associated with the matrix protein (DeMartini and Inouye, 1978). This protein is another major outer-membrane protein, of molecular weight 36,500, and is known to be firmly bound to the peptidoglycan layer (Rosenbusch, 1974). Thus, the matrix protein cannot be dissociated from the peptidoglycan even in 2% SDS at 55°C unless NaCl is added to the solution, as seen in Fig. 22. However, it was found that in a mutant lacking the lipoprotein (*lpp*$^-$; Section VA), the matrix protein could be more easily extracted from the peptidoglycan than the matrix protein of the wild type (*lpp*$^+$) cells (see Fig. 22). One can see that in the case of the *lpp*$^+$ cells, about 70% of the matrix protein is retained on the peptidoglycan in the absence of NaCl, whereas only 20% is retained in the case of *lpp*$^-$ cells. When up to 0.025 M NaCl is added to the SDS solution, the percent retention values of the matrix protein of the *lpp*$^+$ cells remained almost constant at 60%. On the other hand, the

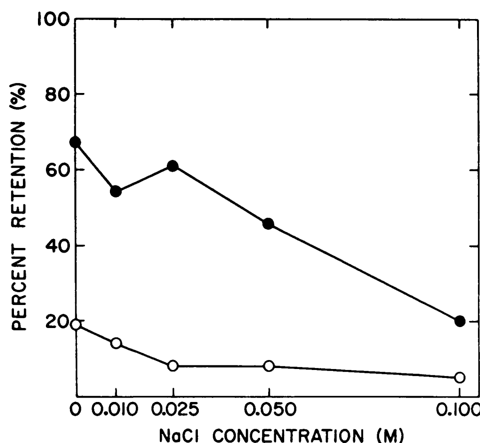



Fig. 22. Retention of the matrix protein bound to the peptidoglycan of *lpp*$^+$ and *lpp*$^-$ cells after the SDS extraction at different NaCl concentrations (DeMartini and Inouye, 1978). The amount of the matrix protein in the whole cell envelope is expressed as 100%.

percent retention values dropped to about 10% in the case of the *lpp*$^-$ cells.

When the bound form of the lipoprotein is cleaved from the peptidoglycan by trypsin treatment, the affinity of the matrix protein for the peptidoglycan decreases to the same level as the affinity of the matrix protein for the peptidoglycan from the mutant strain. Moreover, the free form of the lipoprotein appears to bind to the matrix protein/bound-form lipoprotein/peptidoglycan complex. These results suggest that the bound form of the lipoprotein is playing an important role in the association of the matrix protein with the peptidoglycan. On the other hand, Mizushima and co-workers showed that purified matrix protein can bind to the peptidoglycan in SDS solution, and that the bound-form lipoprotein is not required for this binding (Hasegawa *et al.*, 1976; Yu and Mizushima, 1977). However, in order to reconstitute membranous vesicles resembling the outer-membrane structure from the SDS-solubilized membrane fraction, the bound-form lipoprotein seems to be required (Yamada and Mizushima, 1977). In view of our finding mentioned above, it is possible that the reconstituted binding between the peptidoglycan and purified matrix protein in SDS solution is different from that in the intact cells.

### 3. *Assembly Models of the Matrix Protein and the Lipoprotein*

The matrix protein has been shown to be arranged in a periodic monolayer which covers most of the outer surface of the peptidoglycan (Steven *et al.*, 1977). The electron microscopic observations reveal that protein molecules are arranged according to a threefold symmetry on a

hexagonal lattice whose repeat interval is 7.7 nm. Furthermore, three molecules seem to be arranged in a unit cell which shows a triplet of indentations, each approximately 2 nm in diameter. How is the lipoprotein arranged in this structure?

Let us consider the facts concerning the lipoprotein and the matrix protein.

1. Both proteins are closely associated with each other by ionic interactions (DeMartini and Inouye, 1978).

2. The matrix protein covers almost the entire surface of the peptidoglycan (Steven *et al.*, 1977).

3. It is most likely that the lipoprotein is also distributed evenly on the surface of the peptidoglycan (Braun, 1975).

4. The number of the free-form molecules of the lipoprotein is 2 to 3 times greater than that of the matrix protein molecules (Inouye, unpublished data). This indicates that there are almost equal numbers of molecules of the bound-form lipoprotein and the matrix protein.

5. The lipoprotein has a very high $\alpha$-helical structure (Section IID), whereas the matrix protein has very high $\beta$-structure (Rosenbusch, 1974).

6. The matrix protein forms a trimeric assembly (Steven *et al.*, 1977), whereas the lipoprotein molecules are cross-linked *in vivo* not only between the free form and the free form but also between the free form and the bound form (DeMartini and Inouye, 1978).

7. The matrix protein serves as a phage receptor, suggesting that a part of the protein is exposed to the outside surface of the outer membrane (Schnaitman *et al.*, 1975). Recently, Kamio and Nikaido (1977) showed more definite evidence that the matrix protein is exposed on the outer surface of the outer membrane: When a mutant producing a lipopolysaccharide with a very short carbohydrate chain was treated with CNBr-activated dextran, the matrix protein was modified with the reagent. In the case of the lipoprotein, it remains inconclusive whether the lipoprotein is exposed to the outside surface of the outer membrane or not. However, at least in the case of *E. coli* strains which have defects in the polysaccharide chains of the lipopolysaccharides (with large molecules of the anti-lipoprotein immunoglobulin thus somehow being able to contact the outer membrane surface), the lipoprotein seems to react with the anti-lipoprotein serum (Braun, 1975; Braun *et al.*, 1976*b*). This result indicates that a part of the lipoprotein may also be exposed to the outside surface of the outer membrane.

8. The matrix protein molecules can form diffusion pores incorporated into liposomes consisting of lipids and lipopolysaccharides (Nakae, 1976*a,b*).

To accommodate all of these facts, a possible assembly model for the matrix protein and the lipoprotein can be constructed, as shown in Fig. 23. This model utilizes the unique features of the lipoprotein structure emphasized in the three-dimensional molecular assembly model (Inouye, 1974). In Fig. 23, three molecules of the matrix protein composed mainly of $\beta$-structures are forming a hydrophilic diffusion pore with a diameter of 1.5–2.0 nm. Each of the matrix-protein molecules is fixed or stabilized with a triple coiled-coil structure of the lipoprotein, which consists of one molecule of the bound-form and two molecules of the free form.

## D. Effects of Lipid Fluidity

The question of how lipid fluidity of the membrane affects the assembly of the membrane proteins is extremely interesting. Using an unsaturated fatty acid auxotroph, the fatty acid composition of membrane lipid can be controlled by growing the cells with elaidate (*trans*-$\Delta^9$-octadecenoate) or with oleate (*cis*-$\Delta^9$-octadecenoate). It has been shown that induction of alkaline phosphatase, a periplasmic enzyme, is arrested at 25°C in the elaidate-supplemented cells but not in the oleate-supplemented cells (Kimura and Izui, 1976). This arrest in the elaidate-supplemented cells is released when lipid fluidity is allowed to return to normal at 38°C. However, there seems to be no accumulation of the precursor of alkaline phosphatase while the induction is arrested at 25°C. Similar experiments have been performed to examine the effects of lipid fluidity on the assembly of the outer- and the inner-membrane proteins (Ito *et*

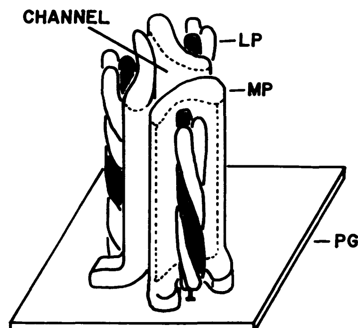



Fig. 23. Assembly model of the matrix protein and lipoprotein. MP, matrix protein; LP, lipoprotein. The bound forms are darkened. PG, peptidoglycan.

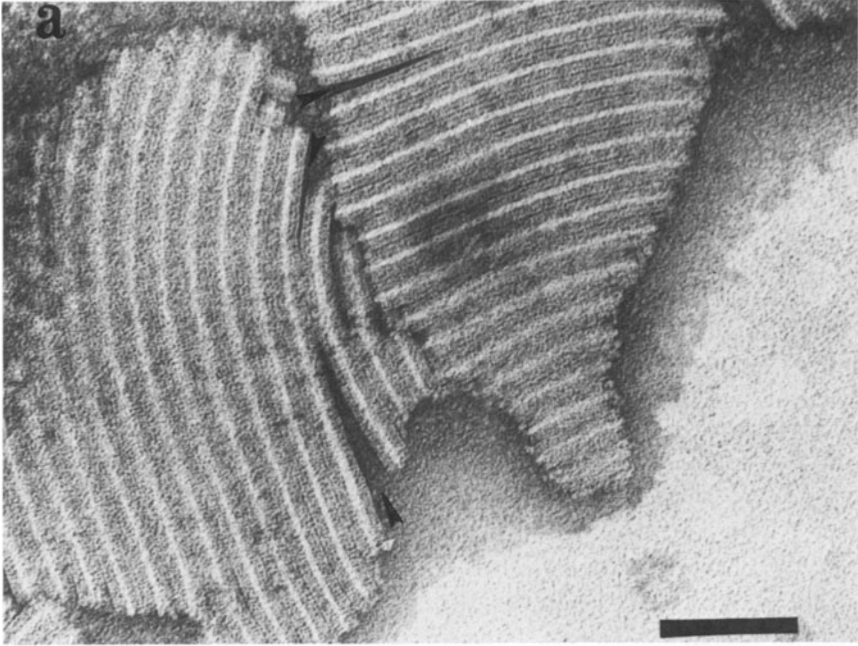
*al.*, 1977). Their results suggest that membrane fluidity is required for normal assembly of the membrane proteins and that this requirement is more stringent for the outer-membrane proteins than for the cytoplasmic-membrane proteins.

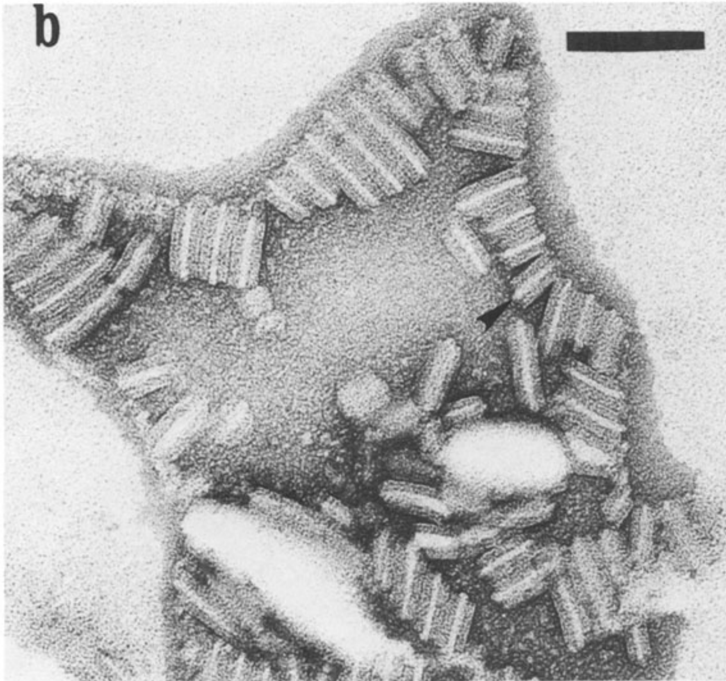
However, when the effects of the lipid fluidity on the assemblies of the individual outer-membrane proteins are examined more precisely, there are, surprisingly, remarkable differences between them (DiRienzo and Inouye, 1979). The assembly of the lipoprotein appears to be hardly affected, whereas the assembly of the matrix protein is severely inhibited. The reasons for this difference are not presently understood. However, it is of great interest to find out at which step the assembly of the matrix protein is inhibited: at the step of translation of the mRNA; at translocation across the cytoplasmic membrane; at processing of the precursor; or at insertion of the matrix protein into the outer membrane. At any rate, this will provide an important clue for solving the molecular-assembly mechanism of the outer-membrane proteins.

## E. *In Vitro* Assembly

### *1. Ionic Interactions in Lipoprotein Aggregates*

When the purified lipoprotein (free form) is solubilized in 0.1% SDS, the lipoprotein forms aggregates, and only one of five lysine residues can be modified with fluorescamine, a modifying reagent of free amino groups. However, if 0.5 M NaCl is added to the solution, at least three additional lysine residues become susceptible to fluorescamine (Lee *et al.*, 1977*c*). The lysine residue modified in the absence of NaCl is identified as the carboxyl-terminal lysine, since very low labeling is observed when this lysine residue is removed by carboxypeptidase B before the addition of fluorescamine. The modification rate of arginine residues by 2,3-butanedione is also enhanced by the presence of NaCl. Furthermore, gel filtration of the lipoprotein in 0.1% SDS shows a broad distribution of SDS-protein micelles having larger effective radii. However, in the presence of 0.5M NaCl, only aggregates with smaller radii, equivalent to those of dimers or monomers of the lipoprotein molecules, are found (Lee *et al.*, 1977*c*). These results indicate that ionic interactions play a major role in forming the lipoprotein aggregates in the presence of SDS. Although from these *in vitro* experiments, one cannot infer the same mechanism of lipoprotein assembly in the intact outer membrane, these results are consistent with the proposed three-dimensional molecular assembly models shown in Figs. 20 and 23.


a


b

### 2. *Ultrastructure of Paracrystals*

As mentioned earlier, the free form of the lipoprotein was purified and paracrystallized (Inouye *et al.*, 1976). The paracrystals are usually needle-shaped and show highly ordered ultrastructures when observed by electron microscopy (DeMartini *et al.*, 1976). Fig. 24 shows the ultrastructure of negatively stained paracrystals. Relatively electron-transparent bands of 4.7 nm thickness are regularly spaced in the crystals, with a repeat interval of 22 nm.

How do the lipoprotein molecules fit into this periodic assembly? Since the lipoprotein has a very high $\alpha$-helical content even in SDS (Section IID), the main part of the protein moiety of the lipoprotein in the paracrystal forms a rigid $\alpha$-helix of 8–9 nm in length. At one end of this helix, three fatty acids are covalently attached and their total length is 2.1 nm long. This part forms an extremely hydrophobic moiety. On the basis of these facts and our observations from the micrographs, we assigned the fatty acid moieties to the light bands and the protein moieties to the dark bands. The assembly of the lipoprotein in the paracrystals is most probably explained as illustrated in Fig. 25 (DeMartini *et al.*, 1976). The basic unit of the paracrystals consists of a number of lipoprotein molecules arranged with their 2.3-nm-long lipid heads side by side and their 8.7-nm-long protein tails projecting from the lipid region (Fig. 25A). These basic units appear to be held together in the paracrystals by head-head and tail-tail interactions of the lipoprotein molecules (Fig. 25B). This interaction forms a lipid bilayer, producing a light band of 7.4 nm after negative staining. At the end of the paracrystals the lipid bilayer is formed between the lipid portion of the lipoprotein on one side and the hydrocarbon chains of the detergent on the other side (Fig. 25B). Thus, this arrangement would account for the major repeat pattern seen in the electrom micrographs after negative staining of the paracrystals (Fig. 25C).

In this model, the conformation of the lipoprotein molecules inside the major repeating units is not established. However, the dark regions between the light bands do yield minor repeating patterns (Fig. 24) that can be interpreted as either the superhelical assemblies proposed previously (Fig. 20) (Inouye, 1974) or as simple coiled-coil structures.

In the light of these ultrastructural studies of the lipoprotein para-

←

Fig. 24. Ultrastructure of negatively-stained lipoprotein paracrystals (DeMartini *et al.*, 1976). Bar, 100 nm. (a) Paracrystals are dissociating at the centers of the dark bands, as shown by arrows. (b) More advanced dissociation of paracrystals. Arrow shows dissociating unit.

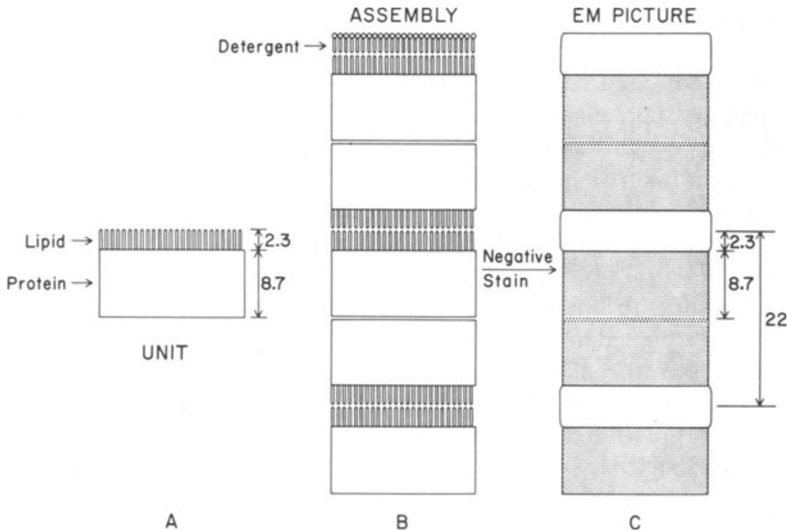



Fig. 25. Proposed model for lipoprotein paracrystals in Fig. 24 with a 22-nm repeat (DeMartini *et al.*, 1976). (A) Assembly subunit consisting of lipid moieties (2.3 nm) and protein moieties (8.7 nm). (B) Arrangement of subunits in 22-nm-repeat paracrystals. The assembly can end with the protein moiety as its terminus or it can end with a lipid moiety, where a lipid bilayer is formed with detergent (or phospholipid) molecules as its outer monolayer. (C) Schematic representation of how this structure would appear after negative staining with uranyl acetate. Shading reflects penetration by the stain. The dimensions shown on the figures are given in nanometers.

crystals, it is extremely interesting to attempt to examine the ultrastructures of more intact assemblies of the lipoprotein molecules by themselves or of assemblies between the lipoprotein and the matrix protein.

## V. GENETIC APPROACHES

### A. Isolation of Mutants of the Lipoprotein

#### *1. General Considerations*

One of the great advantages in using *E. coli* for the study of membrane proteins is its genetics. The chromosome map of *E. coli* has been very well established (Bachman *et al.*, 1976). In addition, many excellent techniques for the isolation of mutants have been developed. However, isolation of mutants of proteins, such as the lipoprotein—which has no

known function as yet—is extremely difficult. In this case one has to either apply brute-force selection procedures to isolate a mutant or depend on a fortuitous, chance isolation.

In this section, I would like to describe all of the mutants of the lipoprotein so far reported. Needless to say, isolation and characterization of mutants of the outer-membrane proteins are extremely important and valuable. However, one should bear in mind that the phenotype of a mutant of a particular membrane protein does not necessarily reflect the direct effect of the mutation in the membrane protein. Furthermore, isolation of a mutant lacking a particular outer-membrane protein does not necessarily mean that the protein is dispensable under many different growth conditions.

### 2. *Structural Gene for the Lipoprotein*

A mutant which has a mutation in the structural gene for the lipoprotein was found fortuitously by Hirota from a collection of temperature-sensitive cell division mutants. In this mutant the lipoprotein has a free thiol group which is susceptible to modification by monoiodoacetic acid (Suzuki *et al.*, 1976). Because of the free thiol group of the mutant lipoprotein, it easily forms a dimer when the membrane fraction is analyzed by gel electrophoresis without β-mercaptoethanol (Fig. 26). This mutant was extensively characterized and the following facts have been established (Suzuki *et al.*, 1976; Inouye *et al.*, 1977*b*): (1) From the analysis of the primary structure of the mutant lipoprotein, it was found that the arginine residue at position 57 (see Fig. 2) is replaced by a cysteine residue. This mutation can be explained by a single-base change from U to C. (2) Therefore, the mutation occurred in the structural gene (*lpp*) for the lipoprotein. The mutation (*lpp*-1) was found to map at 36.5 min on the *E. coli* chromosome. (3) The mutation was found to have nothing to do with the temperature-sensitive cell division and showed no special phenotype except that the mutant carrying *lpp*-1 is sensitive to mercury compounds.

### 3. *Deletion Mutant of the Lipoprotein*

Another lipoprotein mutant was again found fortuitously by Hirota and associates (1977). In this mutant, both the free and the bound forms of the lipoprotein were found to be completely missing. This mutation leading to the loss of the lipoprotein was originally assigned as *lpo*, and appears not to produce the mRNA for the lipoprotein. We have also

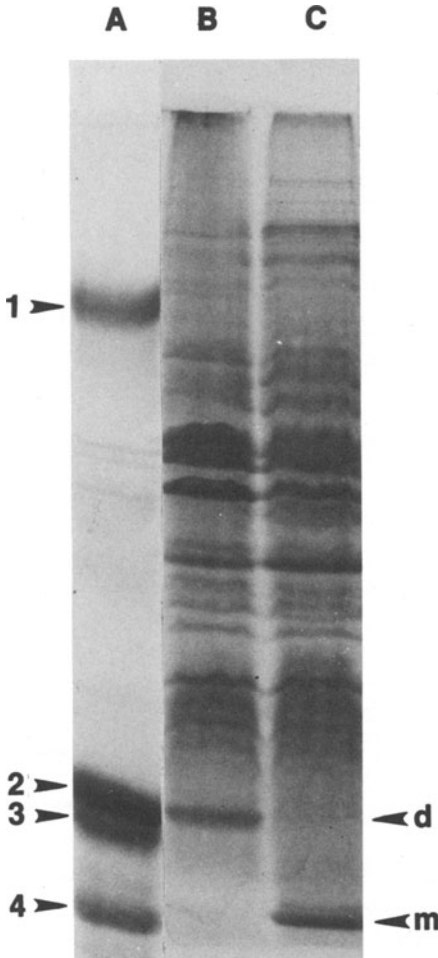



Fig. 26. Slab-gel electrophoresis of the membrane fraction of the lipoprotein mutant (Inouye *et al.*, 1977*b*). (A) Standard proteins: 1, bovine serum albumin; 2, hen egg-white lysozyme; 3, cytochrome C; 4, purified wild-type lipoprotein. (B) The membrane fraction of the mutant strain, *E. coli* JE 5525 *lpp*-1. (C) The same membrane fraction as B except that the membrane fraction was treated with β-mercaptoethanol before gel electrophoresis. The gel was stained with Coomassie Blue. Arrows with letters d and m indicate the positions of the dimer and the monomer of the lipoprotein, respectively.

found that DNA from this mutant does not have the restriction fragment which can hybridize with the radioactive mRNA for the lipoprotein (Nakamura and Inouye, unpublished data). These data suggest that this mutation (*lpp*-2) is most likely a deletion of the structural gene (*lpp*) for the lipoprotein. The mutation is also mapped at 36.5 min on the *E. coli* chromosome.

The *lpp*-2 mutant grows and divides normally. However, the mutant is extremely sensitive to EDTA and leaks considerable amounts of periplasmic enzymes into the medium (Hirota *et al.*, 1977). The leakage of cytoplasmic proteins such as β-galactosidase seems to be unchanged.

From these results one is tempted to conclude that the lipoprotein is dispensable in *E. coli*. However, this conclusion may not necessarily be correct, simply because one cannot conclude that tryptophan synthetase is dispensable even if the tryptophan-synthetase-negative mutants can grow in a culture medium supplemented with tryptophan. This mutation is not only extremely important in the identification of the functions of the lipoprotein but is also useful for genetic manipulation of the lipoprotein gene, as will be discussed later.

#### *4. Other Lipoprotein Mutants*

As described in Section IVA4, Weigand *et al.* (1976) found a new class of mutants of *Salmonella typhimurium* (*lkyD* mutants), in which the amount of the bound-form lipoprotein is very low. These mutants were originally isolated as mutants that leak periplasmic enzymes. When these mutant cells are examined under light and electron microscopes, they show a defect in invagination of the outer membrane during formation of the cell-division septum. However, the cytoplasmic membrane and the peptidoglycan layer at the septum seem to be formed normally. As a result, the outer membrane begins to bulge outward over the septum region, leading to the formation of large "blebs" over the septum region. When the contents of the lipoprotein in these mutants were analyzed, it was found that the amount of the free-form lipoprotein increases severalfold in the mutant strains, with a concomitant decrease in the amount of the bound-form lipoprotein. The ratio of the amount of the free form to that of the bound form was found to be 1.8 and 7.3 for the wild-type and the mutant cells, respectively (Wiegand *et al.,* 1976). Furthermore, Wiegand *et al.* (1976) showed that genetic reversion of the septal defect is associated with a loss of the periplasmic leaky phenotype. Together with the fact that the *lpp*-2 mutant is extremely EDTA sensitive, the results from the *lkyD* mutants suggest that one of the functions of the bound-form lipoprotein is in maintenance of the normal outer-membrane structure.

A similar mutant has been isolated by Torti and Park (1976). They isolated this mutant by the so-called suicide selection procedure on the basis of the exclusive biosynthesis of the lipoprotein in the absence of histidine, proline, and tryptophan (see Section IIIA). All normal cells which can incorporate [$^3$H]arginine into the lipoprotein during the above starvation will be killed by the radiation from [$^3$H], whereas the mutants which are defective in the biosynthesis of the lipoprotein can survive. A mutant thus isolated was found to be temperature sensitive in cell division

and to form filamentous cells at 42°C. It was also found that this mutant contains very low amounts of the bound-form lipoprotein as well as the free-form lipoprotein at 42°C, but not at permissive temperatures. Since the revertants of this mutant can grow normally at 42°C, it was concluded that the lipoprotein may serve a vital function(s) in cellular activities (Torti and Park, 1976). However, in view of the finding that the mutant that lacks the lipoprotein (*lpp*$^-$) is viable, the temperature-sensitive mutant should be reexamined genetically. The mutation may not be located in the structural gene of the lipoprotein. At any rate, this mutant is still very interesting, since the temperature-sensitive growth of the mutant may be caused by a mutation which also causes a pleiotropic effect on the modification process during the lipoprotein biosynthesis.

Another group of mutants was isolated by Wu and Lin (1976) using the suicide selection method. One of the mutants was further characterized and found to have a structurally altered lipoprotein on the basis of the following (Wu *et al.*, 1977): (1) the mutant lipoprotein exists in an appreciable amount in the soluble fraction; (2) it lacks the covalently linked diglyceride; (3) it contains an unmodified cysteine; (4) it undergoes dimerization and the dimer can be converted into the monomeric form by $\beta$-mercaptoethanol treatment; (5) its molecular weight appears to be larger than that of the wild-type lipoprotein, judging from its mobility in SDS-gel electrophoresis; and (6) the amount of the bound-form lipoprotein is greatly reduced. These results suggest that the mutation causes an alteration of the primary structure of the lipoprotein in such a way that the modification reactions of the lipoprotein or the prolipoprotein (see Section IVA) cannot proceed. The fact that this mutation was mapped at 36.4 min (*mlpA*) (Yem and Wu, 1978) is consistent with the alteration of the lipoprotein primary structure, since the structural gene of the lipoprotein (*lpp*) is located at 36.5 min in the *E. coli* chromosome map (see Section V). In fact, it has recently been shown that the glycine residue at position 14 of the prolipoprotein was replaced with aspartic acid in the mutant (Lin *et al.*, 1978).

### B. Gene-Dosage Effects

Another intriguing question is how the gene expression of membrane protein is controlled. It has been suggested that the tolG protein, one of the major proteins in the outer membrane, is synthesized under a simple feedback mechanism (Datta *et al.*, 1976). On the other hand, in the case of the vitamin $B_{12}$ receptor in the *E. coli* outer membrane, a distinct gene-

dosage effect has been observed in a merodiploid strain (Bradbeer *et al.*, 1976).

In order to examine the gene-dosage effects of the structural gene for the lipoprotein (*lpp*), a new F-prime factor containing the *lpp* gene was isolated (Movva *et al.*, 1978). The merodiploid strain carrying this F-prime factor was found to have about two times as much free-form lipoprotein as did the corresponding haploid strain. On the other hand, the amount of the bound-form lipoprotein in the merodiploid strain was almost the same as in the haploid strain (Movva *et al.*, 1978). These results are contrary to the results obtained with the tolG protein, and raise an interesting question concerning the gene expression of the outer-membrane proteins.

In view of this finding, the report that the rate of production of the free-form lipoprotein is highest at the time of cell division (James and Gudas, 1976) can be interpreted as a result of the gene-dosage effect of the *lpp* gene. The *lpp* gene (36.5 min) (Suzuki *et al.*, 1976) is located near the termination site of DNA replication (32 min) (Bachman *et al.*, 1976), and a round of DNA replication is completed before cell division (Helmstetter and Cooper, 1968). Therefore, the number of copies of the *lpp* gene is doubled just before cell division. Thus the rate of lipoprotein production increases as a result of gene dosage. The reported cell-cycle-specific synthesis of other membrane proteins can also be interpreted as a result of the gene-dosage effects of individual genes (Churchward and Holland, 1976).

### C. Genetic Engineering

Another important approach in the study of the biosynthesis and assembly of membrane proteins is by genetic engineering. It has been shown that $\beta$-galactosidase, a cytoplasmic (soluble) enzyme, can be inserted into the cytoplasmic membrane when the *lacZ* gene ($\beta$-galactosidase) is fused to one of the maltose operons: the gene for a maltose transport protein (*malF*) (Silhavy *et al.*, 1976). In the new strain, the $\beta$-galactosidase production becomes inducible by maltose. It was assumed that a hybrid protein molecule is produced, which is composed of an amino-terminal part from the maltose transport protein and a carboxyl-terminal part from $\beta$-galactosidase. These results suggest that the component from the *malF* gene is essential for the incorporation of the hybrid protein into the cytoplasmic membrane. This gene fusion technique is applicable for many different purposes in membrane research. It would

be especially useful in the study of the functions of the peptide extension of precursors of the outer-membrane protein.

In this direction, a DNA fragment which can hybridize with the lipoprotein mRNA has been isolated after digestion of *E. coli* DNA by restriction nucleases (Nakamura *et al.*, 1979). The cloning of the *lpp* gene; further fragmentation of the DNA fragment; isolation of the promoter fragment, as well as the DNA fragment corresponding to the peptide extension of the proliproprotein; and the determination of the base sequences of these fragments will be extremely fruitful approaches and are now in progress in our laboratory.

### D. Other Gram-Negative Bacteria

Braun and co-workers found that the bound-form lipoprotein also exists in three different strains, E. coli, *Salmonella typhimurium*, and *Serratia marcescens*, but not in *Proteus mirabilis, Proteus vulgaris*, or *Pseudomonas fluorescens* (Braun *et al.*, 1970). These results were also confirmed immunologically (Braun, 1975).

The existence of the free-form lipoprotein in other gram-negative bacteria has also been examined, by the use of anti-*E. coli* lipoprotein serum (Halegoua *et al.*, 1974). We found that *Salmonella typhimurium* and *Serratia marcescens* have the free form of the lipoprotein as well, which can react with anti-*E. coli* liproprotein serum. *Pseudomonas aeruginosa* contains a protein of the same size as the lipoprotein, but this protein is not cross-reactive with anti-*E. coli* liproprotein serum. Recently we found that this protein has biosynthetic properties similar to those of the *E. coli* lipoprotein: (1) it does not contain histidine; (2) its biosynthesis is resistant to puromycin; and (3) the mRNA for this protein is very stable (M. Yasumura and M. Inouye, unpublished results). These results indicate that this protein is probably the lipoprotein. The lipoprotein was recently purified from *Pseudomonas aeruginosa* and was reported to lack proline, valine, isoleucine, phenylalanine, tryptophan, and cysteine (Mizuno and Kageyama, 1979). The lipoprotein of *Pseudomonas aeruginosa* contained only 0.89 mol of fatty acid (mainly palmitic acid), although it appeared to have a glycerol group, suggesting that the lipoprotein lacks ester-linked fatty acids, as in the case of the mutant lipoprotein from the *lpp*-1 strain (see Section VA) (Mizuno and Kageyama, 1979).

In the case of *Proteus mirabilis*, there is also no membrane protein which cross-reacts against anti-*E. coli* lipoprotein (Halegoua *et al.*, 1974). However, there is again a protein of the approximate size of the *E. coli*

lipoprotein, whose biosynthesis is resistant to puromycin as well as rifampicin (Katz *et al.*, 1978). The existence of the lipoprotein in *Proteus mirabilis* was indicated by a comparison of the amino acid compositions of the purified free and bound forms of this protein with those of the *E. coli* free and bound lipoproteins (Katz *et al.*, 1978). It was also shown that the *Proteus mirabilis* peptidoglycan contains five to six times less bound-form lipoprotein than does the *E. coli* peptidoglycan (Katz *et al.*, 1978). Similar results have been obtained by Martin and co-workers (Gruss *et al.*, 1975; Gmeiner *et al.*, 1978). They have showed that *Proteus mirabilis* lipoprotein contains 1.71 mol of ester-linked and 1.14 mol of amide-linked fatty acids (mainly palmitic acid) per mole of lipoprotein (Gmeiner *et al.*, 1978). These results suggest that the lipoprotein exists widely in many different genera of gram-negative bacteria.

Another approach is to examine whether DNAs from other bacteria have homologous sequence to the lipoprotein gene of *E. coli*. This was done by hybridization of $^{32}$P-labeled mRNA for the *E. coli* lipoprotein with restriction endonuclease fragments of total DNA from various gram-negative bacteria (Nakamura *et al.*, 1979). It was found that the *E. coli* lipoprotein mRNA hybridized with DNAs from nine bacteria in the family *Enterobacteriaceae: E. coli, Shigella dysenteriae, Salmonella typhimurium, Citrobacter freundii, Klebsiella aerogenes, Enterobacter aerogenes, Edwardsiella tarda, Serratia marcescens,* and *Erwinia amylovora*. However, among the *Enterobacteriaceae*, DNA from two species of *Proteus* (*P. mirabilis* and *P. morganii*) did not hybridize with the *E. coli* lipoprotein mRNA. DNA from *Pseudomonas aeruginosa, Acinetobacter* sp. HO1-N, *Caulobacter crescentus,* and *Myxococcus xanthus* also did not hybridize with the *E. coli* mRNA.

## VI. OTHER APPROACHES

### A. Electron Spin Resonance (ESR); Nuclear Magnetic Resonance (NMR)

We have recently developed a method for attaching a spin label to a specific site on the lipoprotein in its membrane environment (Lee *et al.*, 1978). Using a lipoprotein mutant in which the second amino acid from the carboxyl terminus is changed from arginine to cysteine (see Section VA2), an SH-specific spin label [*N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl) maleimide] was incorporated into the cysteine residue with no significant disruption of the membrane structure. Similarly, we are

able to incorporate NMR probes into the lipoprotein without disrupting the membrane structure (Lee *et al.*, 1977*b*). In this case, *m*-fluorotyrosine or [2-$^{13}$C]tyrosine was exclusively incorporated into the position of amino acid 56 of the lipoprotein using the histidine starvation technique (see Section IIIA). Both ESR and NMR spectra indicate that the carboxyl-terminal region of the lipoprotein is relatively mobile. It is possible, by the same method, to incorporate ESR or NMR probes into other portions of the lipoprotein molecules *in situ*. Analysis of their spectra should yield important information concerning the conformation of the lipoprotein in the outer membrane, and on the interaction of the lipoprotein with other components in the outer membrane.

### B. Mitogenic Activity

The lipoprotein has been found to be a very potent and specific B-lymphocyte mitogen (Melchers *et al.*, 1975). The lipoprotein stimulates resting B lymphocytes to proliferate and to produce IgM immunoglobulins. It appears that the mitogenic activity of the lipoprotein is specific to B lymphocytes, since the lipoprotein does not stimulate T lymphocytes. It was shown that the ester-linked fatty acids at the amino terminus are essential for the mitogenic activity of the lipoprotein (Melchers *et al.*, 1975), whereas the carboxyl-terminal end has little influence on the mitogenic activity (Bessler and Ottenbreit, 1977). These results suggest that the fatty acids may be required for the proper anchoring of the lipoprotein in the lipid bilayer of the B-lymphocyte membrane (Braun, 1975). This unique property of the lipoprotein may have medical importance as well, since it might be used clinically as an agent to protect against bacterial infection (Braun, 1975).

### C. Identification of Lysozyme Specificity

When the peptidoglycan is hydrolyzed by an *N*-acetylmuramidase such as T4 phage lysozyme or hen egg-white lysozyme, the bound-form lipoprotein is cleaved from the peptidoglycan and becomes soluble in SDS (see Fig. 4, Section II). If the peptidoglycan is labeled with both [$^{3}$H]arginine (for the bound-form lipoprotein) and [$^{14}$C]-*N*-acetylglucosamine (for the peptidoglycan), the bound-form lipoprotein released from the peptidoglycan by T4 phage lysozyme is labeled with both the $^{3}$H and $^{14}$C isotopes, since it contains a fragment of the peptidoglycan. The bound-form lipoprotein migrates to a position of molecular weight 10,000

during SDS-gel electrophoresis (Inouye *et al.*, 1973). However, when the same peptidoglycan is hydrolyzed with *N*-acetylmuramyl-L-alanine amidase, the bound-form lipoprotein released is labeled only with [$^3$H]arginine, and migrates to a position of molecular weight 7500 during SDS-gel electrophoresis. This is because *N*-acetylmuramyl-L-alanine amidase cleaves the bound-form lipoprotein from the peptidoglycan at the linkage between the glycan and the peptide of the peptidoglycan (Inouye *et al.*, 1973).

This method provides a simple, easy assay system for determining the specificity of lytic enzymes. The specificity of "lysozyme" from bacteriophage T7—as well as from bacteriophages T3 and T5 (DeMartini *et al.*, 1976)—has been determined as an *N*-acetylmuramyl-L-alanine amidase by this method (Inouye *et al.*, 1973).

## VII. CONCLUSIONS

In the past decade, a substantial amount of work has been performed regarding the lipoprotein of the *E. coli* outer membrane. As we have seen in this chapter, the lipoprotein is now probably one of the most extensively investigated membrane proteins in prokaryotic as well as eukaryotic cells.

In the foreseeable future, the complete base sequence of the mRNA for the lipoprotein will be determined. This will shed additional light on the precise mechanism of the biosynthesis of the lipoprotein, as well as help to understand the reasons for the unusual properties of the mRNA. Furthermore, since we have isolated a DNA fragment which can hybridize with the lipoprotein mRNA, we are able to clone the *lpp* gene and can thus determine the base sequence of the promoter region of the gene. This may lead us to understand how gene expression of the outer-membrane proteins is controlled. The problem of attempting to elucidate the regulatory mechanism of gene expression of the major outer-membrane proteins is intriguing, since production of some outer-membrane proteins seems to be closely controlled in such a way that the loss of one protein is compensated for by an increase in one or several other major outer-membrane proteins (Chai and Foulds, 1977).

We have learned that the lipoprotein of *E. coli* is probably translocated across the cytoplasmic membrane in a similar manner to secretory proteins in animal cells. Thus, lipoprotein research will provide important insight into understanding of the secretory mechanism of hormones,

immunoglobulins, and many other proteins in animal cells. It also provides insight as to the mechanism for the differentiation of membrane structures. It is of great interest to find out whether there are limited numbers of insertion sites for the outer-membrane proteins on the cytoplasmic membrane, and whether these sites are localized in certain areas of the envelope. In the case of lipopolysaccharides, it has been reported that newly synthesized lipopolysaccharide is translocated, within a few minutes, through localized sites (220/cell) in the cell envelope and is then evenly distributed over the entire cell surface (Mühlradt *et al.*, 1973).

It is also of great interest to elucidate the precise interactions among the major outer-membrane proteins. For this purpose, many different techniques, such as ESR and NMR, are now available. It may also be possible to crystallize the outer-membrane proteins. Even crystallization of the matrix protein–lipoprotein complex may be possible in the near future. Determination of the primary structures of the other major outer-membrane proteins is also feasible, since these proteins exist in large quantities and are easy to purify. I believe that it will not be long before not only the functions and architectures of the major outer-membrane proteins will be understood but also the precise molecular mechanism of their biosyntheses and assembly.

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