JOURNAL OF BACTERIOLOGY, Apr. 1976, p. 183-191
Vol. 126, No. 1

Printed in U.S.A.

Puromycin-Resistant Biosynthesis of a Specific Outer-Membrane Lipoprotein of *Escherichia coli*

SIMON HALEGOUA, AKIKAZU HIRASHIMA, AND MASAYORI INOUYE*

Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794

Received for publication 3 November 1975

The reported puromycin resistance of the in vivo biosynthesis of a specific outer-membrane lipoprotein of *Escherichia coli* was further investigated. The biosynthetic machinery making the lipoprotein was made more accessible to puromycin by disruption of the cell structure using ethylenediaminetetraacetate or toluene, and finally in an in vitro protein biosynthesis system using polyribosomes. Puromycin sensitivity of overall protein sythesis increased by about 10-fold for each method of disruption of the cell structure; 50% inhibitions were obtained at 330, 35, 2.7, and 0.22 μg of puromycin per ml for intact cells, ethylenediaminetetraacetate-treated cells, toluene-treated cells, and the polyribosome system, respectively. However, the lipoprotein biosynthesis remained more resistant to puromycin than the biosynthesis of other proteins in all systems tested. These results strongly suggest that puromycin resistance of the lipoprotein biosynthesis is due to an intrinsic property of the lipoprotein biosynthetic machinery.

Although the mechanism of biosynthesis and assembly of membrane proteins is still obscure, we have shown that in vivo, membrane proteins of *Escherichia coli* are biosynthesized in a somewhat different manner than are cytoplasmic proteins (6). In the course of these studies we found that the biosynthesis of one of the membrane proteins was unusually resistant to puromycin (6). This is a specific lipoprotein that has been investigated most extensively thus far among the *E. coli* membrane proteins; the entire chemical structure of this lipoprotein has been determined (2, 5), the mechanism of biosynthesis and assembly of the lipoprotein has been extensively investigated (7, 8, 10, 16), and it has been shown to exist in the outer membrane (1, 18); a three-dimensional molecular model has been proposed (13). Recently, we have also demonstrated cell-free biosynthesis of the lipoprotein directed by the purified messenger ribonucleic acid (mRNA) (9).

As previously discussed (6), resistance of the biosynthesis of the lipoprotein to puromycin may be explained by two different mechanisms. First, the lipoprotein biosynthetic machinery may in some way be compartmentalized within the cell, for instance by way of the cell membrane, making the lipoprotein biosynthetic machinery inaccessible to puromycin. Alternatively, the lipoprotein biosynthetic machinery itself may somehow differ from the usual puromycin-sensitive one. In the present paper, biosynthesis of the lipoprotein was examined in three different systems in which the cell's permeability to puromycin was increased by ethylenediaminetetraacetate (EDTA) or toluene treatment and in an in vitro system of protein synthesis using isolated polyribosomes. In every case, lipoprotein biosynthesis remained relatively resistant to puromycin, showing that puromycin resistance is an intrinsic property of the lipoprotein biosynthetic machinery.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strains MX74T2 (11) and CP78 (9) were used.

Media and growth conditions. M9 medium supplemented with glucose (4 mg/ml), thiamine (2 μg/ml), and thymidine (4 μg/ml) was used in all experiments (11) with additional supplements of arginine, histidine, threonine, and leucine (20 μg/ml each) for strain CP78. All cultures were grown at 37 C.

EDTA treatment. *E. coli* strain MX74T2 was treated with EDTA according to the method of Lieve et al. (19) with slight modifications. Cell cultures were grown to a cell concentration of about 2×10^8 cells/ml. The cells were harvested by centrifugation at 4 C and concentrated to one-tenth the original volume in tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (10 mM), pH 8.0, containing 1 mM EDTA. This suspension was allowed to stand at room temperature for 5 min. The cells were then used immediately for protein synthesis as described below.

Toluene treatment. *E. coli* strain MX74T2 was treated with toluene according to the method of Levin et al. (20) with modification. Cell cultures

were grown to a cell concentration of about 2×10^8 cells/ml. The cells were then harvested at 4 C and concentrated to about 3.5×10^9 cells/ml in a buffer containing 10 mM Tris-hydrochloride, pH 7.8, 50 mM NH_4CL, 10 mM $Mg(CH_3COO)_2$, and 7 mM β-mercaptoethanol. Toluene was added to 1%, and the suspension was briefly mixed and shaken for 10 min in an ice-water bath. The toluene-treated cells were then used for protein synthesis immediately after toluene treatment, as described below.

Label experiments. Intact cells were labeled with L-[^{3}H]arginine with or without puromycin as previously described (6). After EDTA treatment, the cell suspension described above was diluted 10-fold with M9 growth medium described above lacking Mg^{2+} and supplemented with L-arginine (5 μg/ml). The culture was then incubated at 37 C with 0.2 mM $MgSO_4$ with or without puromycin, as described in Fig. 2. After 5 min the cells were labeled with 25 μCi of L-[^{3}H]arginine for 2 min, at which time cold arginine (500 μg/ml) and formaldehyde (1%) were added to stop label incorporation. Toluene-treated cells were used in in vitro-type reaction mixtures for protein synthesis. A typical reaction mixture consisted of 14.5 mM adenosine triphosphate, 0.2 mM guanosine triphosphate, 17.4 mM $Mg(CH_3COO)_2$, 60 mM NH_4Cl, 75 mM Tris-hydrochloride, pH 7.4, a mixture of 19 amino acids (all 20 amino acids minus arginine, 0.2 mM each), L-[^{3}H]arginine (10 μCi/ml, 66 Ci/mmol; Schwarz/Mann), and toluene-treated cells (10^9 cells/ml). Varying amounts of puromycin as specified in Fig. 3 were also added to the reaction mixtures. Reaction mixtures were incubated at 37 C for 30 min. In all cases (intact, EDTA-treated and tolune-treated cells), total protein synthesis was measured by incorporation of the label into hot trichloroacetic acid-insoluble material. Envelope fractions were prepared by differential centrifugation and solubilized in 1% sodium dodecyl sulfate (SDS) as previously described (14). One-half of the solubilized envelope was subjected to polyacrylamide gel electrophoresis, and the other half was subjected to immunoprecipitation.

In vitro protein synthesis. In vitro protein synthesis using polyribosomes isolated from *E. coli* cells was carried out as described previously (8), using 480 μg of *E. coli* soluble enzyme fraction (S-150) per ml, 8 μCi of [^{35}S]methionine (397 Ci/mmol; New England Nuclear Corp.) per ml, and polyribosome fraction. Amounts of polyribosomes used are described in Fig. 4.

Immunological assay. Solubilized envelope fraction or solubilized in vitro products were subjected to immunoprecipitation, using antiserum against highly purified *E. coli* lipoprotein serum as previously described (4, 9).

Gel electrophoresis. SDS-polyacrylamide gel electrophoresis in 0.5% SDS was carried out on 7.5% acrylamide gels as previously described (15). After gel electrophoresis, the gels were sliced with a razor blade slicer and the radioactivity of each slice was counted as previously described (15). All gel electrophoreses were run by using internal molecular weight standards made of fluorescent proteins (12).

RESULTS

Sensitivity of total protein synthesis to puromycin. To study puromycin sensitivity of the lipoprotein biosynthesis in the various protein-synthesizing systems, we first examined puromycin sensitivity of total protein synthesis in these systems. In intact cells, 50% inhibition of total protein synthesis required 330 μg of puromycin per ml (Fig. 1). In cells treated with EDTA which is known to increase cellular permeability (19), total protein synthesis became about 10 times more sensitive to puromycin than with intact cells; 35 μg of puromycin per ml was enough to effect 50% inhibition of total protein synthesis (Fig. 1). Further disruption of the cell structure by toluene caused another 10-fold increase of puromycin sensitivity, requiring 2.7 μg of puromycin per ml for 50% inhibition of total protein synthesis (Fig. 1). Finally, the cell-free protein-synthesizing system consisting of polyribosomes was about 10 times more sensitive to puromycin than the toluene-treated cells, requiring 0.22 μg of puromycin per ml for 50% inhibition of total protein synthesis (Fig. 1).

Lipoprotein synthesis in EDTA-treated cells. In the previous study (6), we showed that the biosynthesis of the lipoprotein in intact cells was not inhibited at all at the puromycin concentration (300 μg/ml) that inhibited 50% of total protein synthesis. We examined whether the lipoprotein biosynthesis in EDTA-treated cells remains resistant to puromycin in spite of the disruption caused by the chemical. Figure 2 shows SDS-gel electrophoresis of total envelope proteins synthesized in EDTA-treated cells in the absence and presence of puromycin. The puromycin concentration tested was chosed to be 80 μg/ml, which inhibited 80% of total protein synthesis in this system (see Fig. 1). EDTA treatment did not cause significant effects on envelope protein biosynthesis (solid line in Fig. 2); the pattern was identical to that of intact cells. When 80 μg of puromycin per ml was added, the biosynthesis of all membrane proteins, whose molecular weights were larger than internal standard c, was severely inhibited; the biosynthesis of peak 4, 5, and 7 proteins was inhibited by about 80, 60, and 70%, respectively. However, as in intact cells (6), the biosynthesis of the lipoprotein, the peak between internal standards e and f, was not inhibited at all. This result indicates that the lipoprotein biosynthesis is still resistant to puromycin in the EDTA-treated cells.

Lipoprotein synthesis in toluene-treated cells. If the lipoprotein biosynthetic machinery

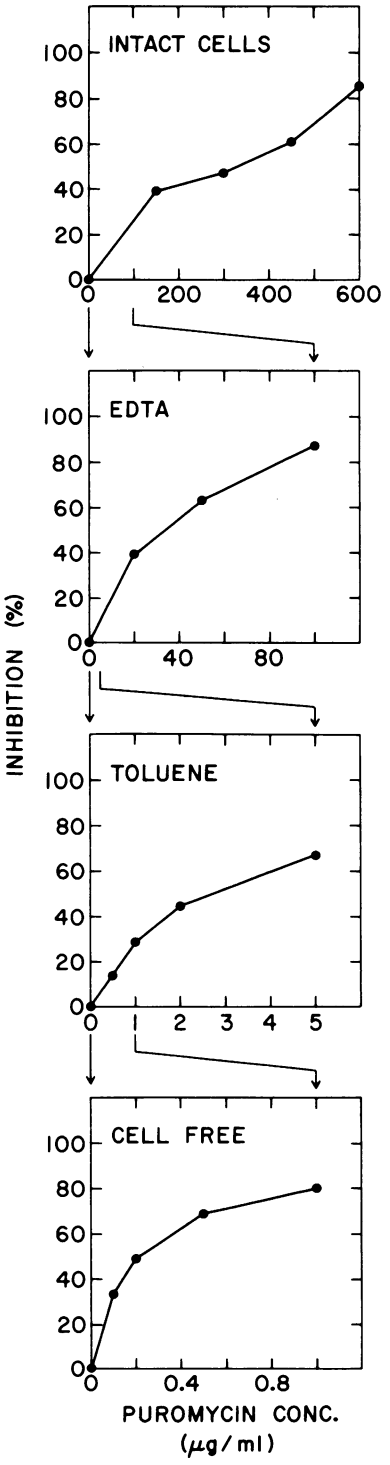

FIG. 1. *Increased cell permeability to puromycin after disruption of the cell structure. Sensitivity of protein synthesis to puromycin was tested in the four systems indicated: intact cells, EDTA-treated cells, toluene-treated cells, and in an in vitro system using polyribosomes as described in the text. The cells or polyribosome mixture were labeled with* L-*[^{3}H]arginine or [^{35}S]methionine, respectively, in the presence of varying amounts of puromycin as indicated in the figure. After labeling, the protein was precipitated by hot trichloroacetic acid and the radioactivity was counted in a scintillation counter. The data are expressed as percentage of inhibition of protein synthesis at various puromycin concentrations.*

is compartmentalized in the cell so that puromycin has no access to the machinery, the above result can be interpreted as showing that EDTA treatment is not sufficient for disrupting the compartment. Thus, by using toluene rather than EDTA treatment, we applied a more severe method to disrupt the cellular structure. Toluene treatment of cells is known to disrupt the cell structure, making cells permeable not only to phosphate compounds such as adenosine triphosphate (21) but also to macromolecules (3, 17). Toluene-treated cells have been reported to be unable to synthesize proteins (17, 22). However, we have developed a system in which cells are treated with toluene and maintain the ability to synthesize protein (Halegoua, Hirashima, and Inouye, manuscript in preparation). The present system was completely dependent upon the addition of an energy source.

Total protein synthesis in the toluene-treated cells became about 100-fold more sensitive to puromycin than the intact cells (Fig. 1). Cell viability after the toluene treatment was about 10^{-6} as measured by ability to form colonies on nutrient broth agar plates.

Membrane proteins produced in the toluene-treated cells in the absence and presence of 2.73 μg of puromycin per ml were examined by SDS-polyacrylamide gel electrophoresis (Fig. 3A). Although the gel pattern of membrane proteins produced in the toluene-treated cells was somewhat different from that of intact or EDTA-treated cells (Fig. 2), the biosynthesis of the lipoprotein (the peak between internal standards e and f) was again hardly affected by 2.73 μg of puromycin per ml, which was enough to cause 50% inhibition of total protein synthesis (see Fig. 1). One striking feature of the membrane proteins in the toluene-treated cells was the appearance of a large peak between internal standards d and e (Fig. 3A). Furthermore, the biosynthesis of this new protein was not inhibited by puromycin at all. The molecular weight of this new protein was about twice that of the lipoprotein, and the protein could cross-

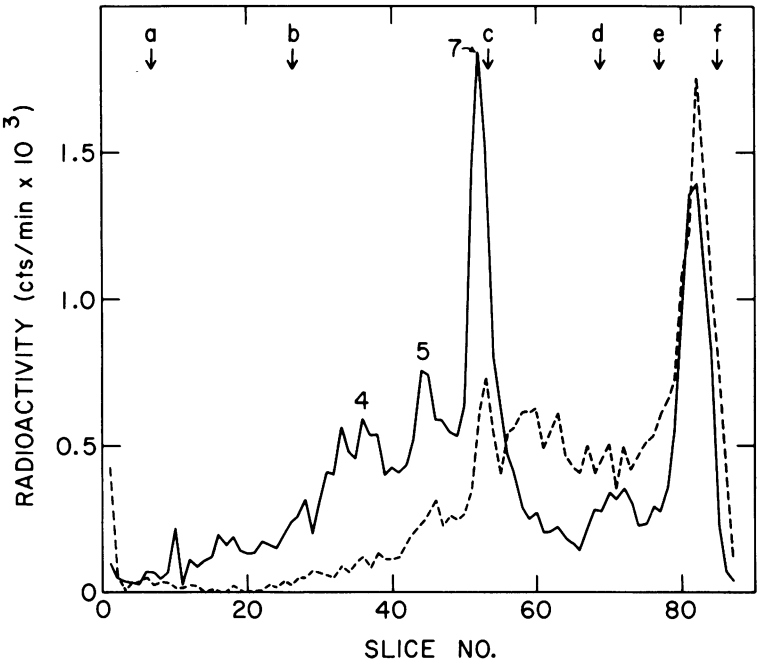

FIG. 2. *Effect of puromycin on the biosynthesis of the envelope proteins of EDTA-treated E. coli MX74T2 labeled with L-[3H]arginine. The cells were treated with EDTA and labeled with L-[3H]arginine in the presence and absence of 80 μg of puromycin per ml, and the envelope fraction was prepared as described previously (14). The envelope fraction was solubilized and subjected to polyacrylamide gel electrophoresis in 0.5% SDS as described previously (14). After gel electrophoresis, the gel was sliced with a razor blade slicer and the radioactivity of each slice was counted in a scintillation counter (15). The gel pattern of the envelope proteins synthesized in the presence of puromycin was superimposed on the gel pattern of the envelope proteins produced in the absence of puromycin with the aid of the positions of internal molecular weight standards. Symbols: ——, No puromycin; - - -, 80 μg of puromycin per ml added. Small arrows with letters indicates the positions of the internal molecular weight standards (12); (a) dimer; (b) monomer of dansylated bovine serum albumin; (c) dimer; (d) monomer of dansylated hen egg white lysozyme; (e) cytochrome c; (f), dansylated insulin. The number on specific peaks corresponds to those in a previous paper (16).*

react with antiserum against the lipoprotein to form the immunoprecipitate (Fig. 3B). Although the new protein shares a common structure with the lipoprotein, it is not simply a dimer of the newly synthesized lipoprotein (Halegoua, Sekizawa, and Inouye, unpublished data). It is possibly a precursor or a byproduct of the lipoprotein that was accumulated in the membrane after toluene treatment. Regardless, it is evident that the biosynthesis of both these proteins is puromycin resistant in the toluene-treated cells. Gel patterns of the immunoprecipitates from envelopes of toluene-treated cells labeled in the presence and absence of puromycin verified the puromycin resistance of both proteins (data not shown).

Lipoprotein synthesis in the polyribosome system. Using a system developed in our laboratory for in vitro biosynthesis of lipoprotein by isolated polyribosomes (8), sensitivity of lipoprotein biosynthesis to puromycin was further investigated. Total protein synthesis in this system was found to become extremely sensitive to puromycin, about 1,000 times more sensitive than intact cells (see Fig. 1). Proteins synthesized in this system labeled with L-[3H]arginine were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4A). With increasing concentrations of puromycin in the reaction mixtures, the biosynthesis of the proteins were inhibited to greater extents. However, the biosynthesis of the protein appearing in the area of lipoprotein molecular weight (between internal standards e and f) was again relatively resistant to puromycin inhibition.

For example, in the presence of 1 μg of puromycin per ml, synthesis of proteins with molecular weights larger than internal standard e was inhibited by about 90%, whereas the synthesis of the proteins appearing between internal standards e and f was still 40% that of the control (in the absence of puromycin; Fig. 4A).

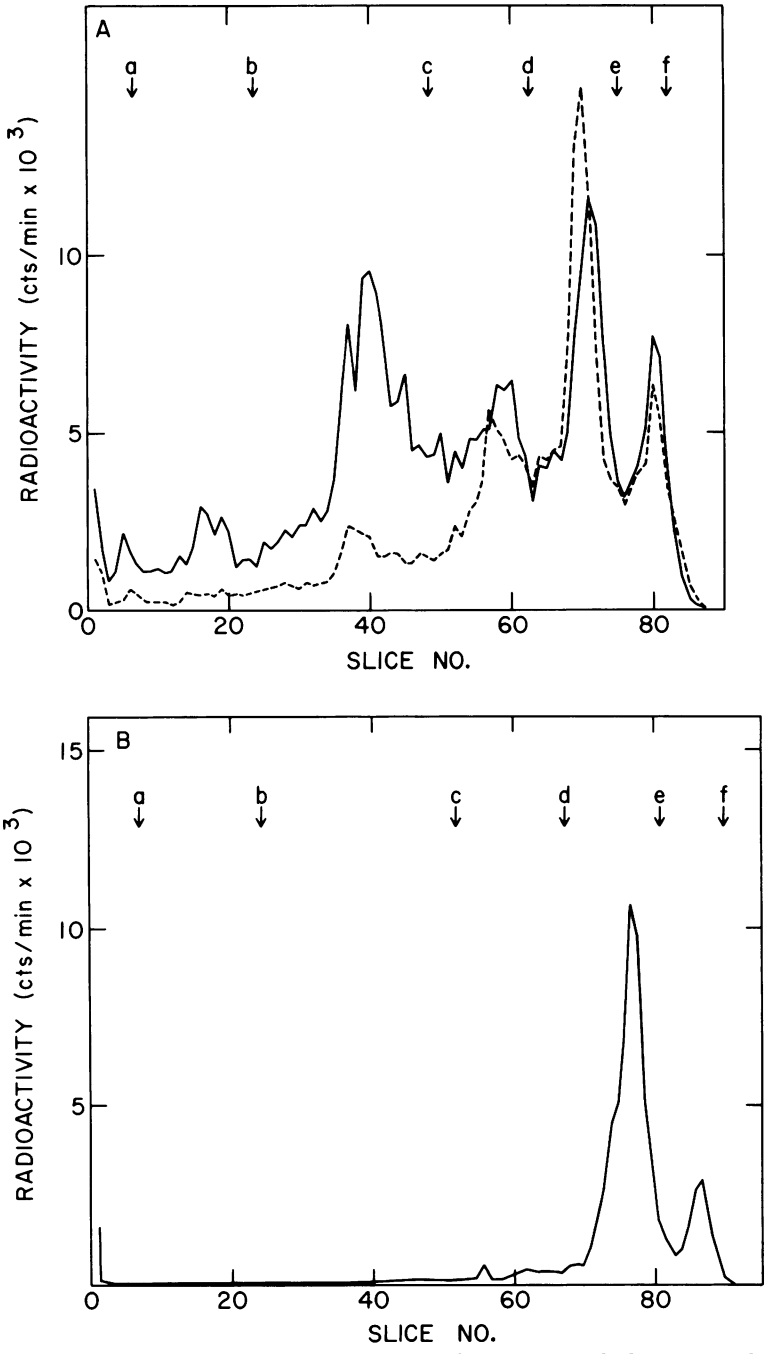

FIG. 3. *Effect of puromycin on the biosynthesis of the envelope proteins of toluene-treated E. coli MX74T2 and gel electrophoresis of the immunoprecipitate with antiserum against E. coli lipoprotein. Cells were treated with toluene and labeled with* L-[^{3}H]*arginine in the presence and absence of 2.73 μg of puromycin per ml. (A) Gel patterns of the envelope proteins obtained individually were superimposed as described in Fig. 2. Symbols: ——, No puromycin; - - -, 2.73 μg of puromycin per ml added (B) Part of the envelope proteins synthesized in the absence of puromycin was treated with antiserum against the lipoprotein, and the immunoprecipitate thus formed was subjected to gel electrophoresis. The preparation of the envelope proteins and gel electrophoresis were carried out as described in Fig. 2. The assignments of the internal molecular weight standards are as described in Fig. 2.*

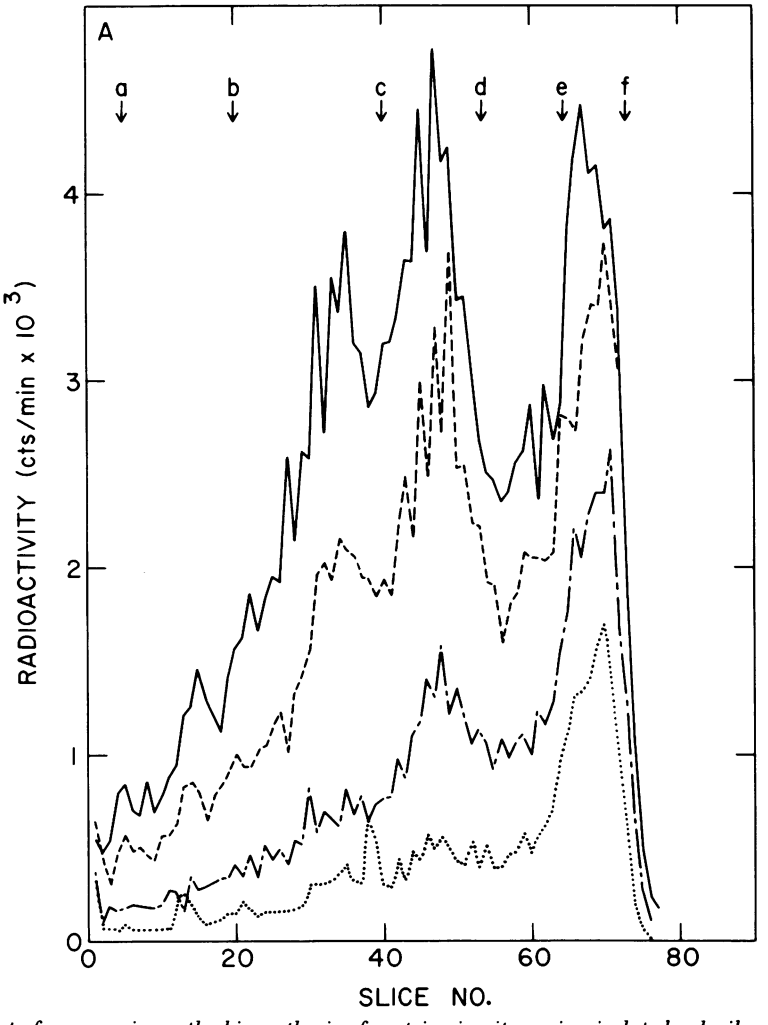

FIG. 4. *Effect of puromycin on the biosynthesis of proteins in vitro using isolated polyribosomes. Proteins were labeled with [^{35}S]methionine in the presence of varying amounts of puromycin as described in the text. (A) Products formed from a reaction mixture containing 8.2 absorbancy units at 260 nm per ml of polyribosomes were subjected to polyacrylamide gel electrophoresis, and the gel patterns for individual puromycin concentrations were superimposed with the aid of the positions of the internal molecular weight standards. Symbols: ——, No puromycin; - - -, 0.06 μg of puromycin per ml added; —·—, 0.2 μg of puromycin per ml added; ···, 1 μg of puromycin per ml added. (B) Products from a reaction mixture containing 5.4 absorbancy units at 260 nm per ml of polyribosomes in the absence and presence of 0.2 μg of puromycin per ml were treated with antiserum against the lipoprotein, and the immunoprecipitates were subjected to gel electrophoresis. The gel patterns were superimposed as described above. Symbols: ——, No puromycin; - - -, 0.2 μg of puromycin per ml added. The assignments of the internal standards are as described in Fig. 2.*

To examine whether the puromycin-resistant proteins in the polyribosome system are related to the lipoprotein, in vitro products in the absence and presence of 0.2 μg of puromycin per ml were reacted with antiserum against the lipoprotein. This puromycin concentration was enough to effect 67% inhibition of total protein synthesis, since the polyribosome concentration here was less than in the experiment shown in Fig. 4. Figure 4B shows the gel patterns of the immunoprecipitates. The gel patterns very closely resembled that of the immunoprecipitate obtained for toluene-treated cells (Fig. 3B); two peaks were present, corresponding to the lipoprotein and the new form of the lipoprotein, whose molecular weight is about twice that of the lipoprotein. In this polyribosome system, however, the relative amounts of the new lipo-

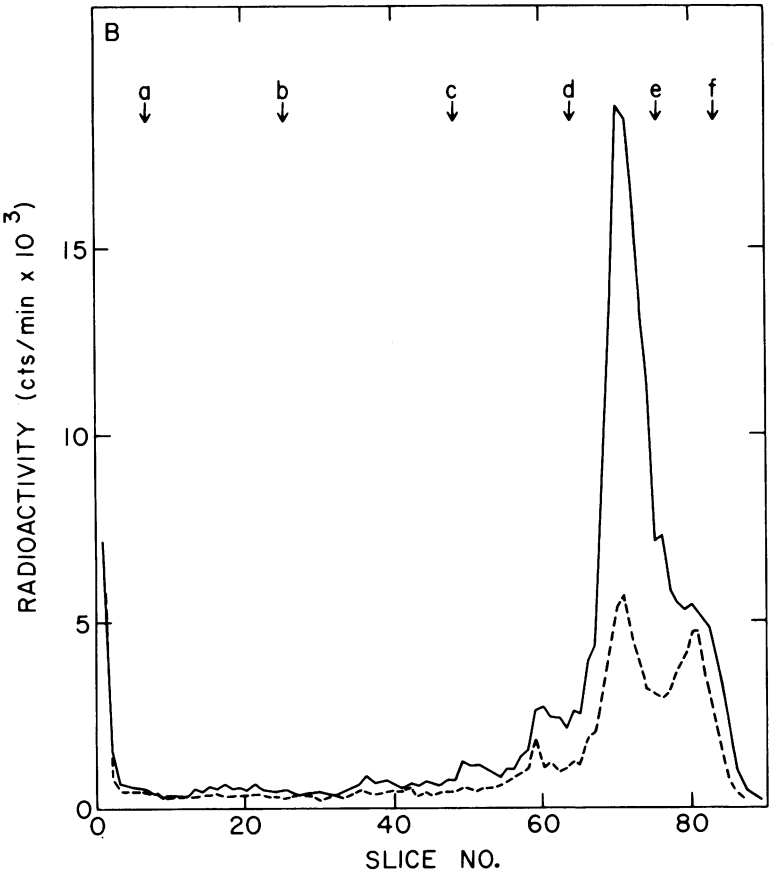

FIG. 4B

protein peak and lipoprotein produced varied with different polyribosome preparations. Lipoprotein synthesis was clearly resistant to puromycin, whereas synthesis of the new form of the lipoprotein was found to be very sensitive to puromycin, in contrast to toluene-treated cells.

DISCUSSION

The present results indicate that the resistance of the lipoprotein biosynthesis to puromycin is not due to compartmentalization of the lipoprotein biosynthetic machinery in the cell, since the lipoprotein biosynthesis remained puromycin resistant regardless of the extent of disruption of the cellular structure. Thus the puromycin resistance appears to be an intrinsic property of the lipoprotein synthetic machinery. Since the lipoprotein biosynthetic machinery consists of mRNA, ribosomes, tRNA's, and many necessary protein factors such as initiation and elongation factors, one of these components (possibly ribosome or protein factor) may be differentiated to be specific for the lipoprotein biosynthesis as discussed previously (6). Such a specific factor may contribute to the puromycin resistance of the machinery. However, since protein synthesis using polyribosomes requires addition of a soluble enzyme fraction (S-150), specificity of soluble enzymes such as elongation factors for lipoprotein biosynthesis is unlikely, leaving mRNA and ribosome-bound proteins as candidates for effecting the puromycin resistance. In addition, it has been reported that there is no significant difference between proteins of ribosomes involved in outer-membrane and cytoplasmic protein synthesis (23).

mRNA for the lipoprotein could be responsible for the puromycin resistance because of its primary or secondary structures. In a preliminary experiment, we found a difference in puromycin sensitivity in a cell-free protein-synthesizing system using different synthetic polynucleotides. Phenylalanine incorporation directed by poly(U) was about twice as sensitive as proline incorporation directed by poly(C)

(Hirashima and Inouye, unpublished data). Although further investigation is needed, the above results can be interpreted as showing that the higher guanine plus cytosine content in mRNAs results in stronger affinity of transfer RNAs to the mRNA's, which results in puromycin resistance. It should also be noted that lipoprotein and/or membrane fractions may be possible candidates for incurring puromycin resistance. Either the growing peptide chain or perhaps an aggregation of lipoprotein and/or membrane with the growing peptide chain may in some way block puromycin accessibility to the ribosome. Although the mRNA for the lipoprotein is abundant in the cell and the size of the mRNA is relatively smaller than that of other mRNA's (9), these facts are probably not the reason for the puromycin resistance of the lipoprotein biosynthesis for the following reasons: (i) the lipoprotein synthesis is as sensitive to other ribosome-directed antibiotics, such as tetracycline, kasugamycin, and sparsomycin, as the biosynthesis of many other membrane proteins (6); and (ii) the lipoprotein biosynthesis is more sensitive to chloramphenicol than the synthesis of other membrane proteins (6).

The appearance of a new form of lipoprotein in both toluene-treated cells and the cell-free system using polyribosomes is quite interesting. We have found that this protein shares many properties with the lipoprotein in addition to the cross-reactivity of the new form with antiserum against the lipoprotein (Halegoua, Sekizawa, and Inouye, unpublished data), although the molecular weight of the new form is about twice that of the lipoprotein. It is not yet certain whether the new form biosynthesized in the polyribosome system is the same as the new form in toluene-treated cells. However, it should be noticed that the biosynthesis of the new form in the polyribosome system appears to be sensitive to puromycin in contrast to the biosynthesis of the new form in toluene-treated cells. The reason for this is unknown at present, but further investigation of this new form of lipoprotein in both toluene-treated cells and the polyribosome system may provide an important clue in elucidating not only the reason for puromycin resistance but also the mechanism of the biosynthesis and assembly of the lipoprotein.

ACKNOWLEDGMENTS

We thank K. Takeishi for valuable discussion and critical reading of the manuscript, and E. Katz for her able technical assistance.

This work was supported by Public Health Service grant GM 19043 from the National Institute of General Medical Sciences and by American Cancer Society grant BC-67B.

LITERATURE CITED

1. **Bosch, V., and V. Braun.** 1973. Distribution of murein lipoprotein between the cytoplasmic and outer membrane of *Escherichia coli*. FEBS Lett. 34:307-310.
2. **Braun, V., and V. Bosch.** 1972. Repetitive sequences in the murein-lipoprotein of the cell wall of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 69:970-974.
3. **Deutscher, M. P.** 1974. Preparation of cells permeable to macromolecules by treatment with toluene: studies of transfer ribonucleic acid nucleotidyl transferase. J. Bacteriol. 118:633-639.
4. **Halegoua, S., A. Hirashima, and M. Inouye.** 1974. Existence of a free form of a specific membrane lipoprotein in gram-negative bacteria. J. Bacteriol. 120:1204-1208.
5. **Hantke, K., and V. Braun.** 1973. Covalent binding of lipid to protein. Diglyceride and amide-linked fatty acid at the N-terminal end of the murein-lipoprotein of the *Escherichia coli* outer membrane. Eur. J. Biochem. 34:284-296.
6. **Hirashima, A., G. Childs, and M. Inouye.** 1973. Differential inhibitory effects of antibiotics on the biosynthesis of envelope proteins of *Escherichia coli*. J. Mol. Biol. 79:373-389.
7. **Hirashima, A., and M. Inouye.** 1973. Specific biosynthesis of an envelope protein of *Escherichia coli*. Nature (London) 242:405-407.
8. **Hirashima, A., and M. Inouye.** 1975. Biosynthesis of a membrane lipoprotein on polysomes. Eur. J. Biochem. 60:395-398.
9. **Hirashima, A., S. Wang, and M. Inouye.** 1974. Cell-free synthesis of a specific lipoprotein of the *Escherichia coli* outer membrane directed by purified messenger RNA. Proc. Natl. Acad. Sci. U.S.A. 71:4149-4153.
10. **Hirashima, A., H. C. Wu, P. C. Vankateswaran, and M. Inouye.** 1973. Two forms of a structural lipoprotein in the envelope of *Escherichia coli*. Further characterization of the free form. J. Biol. Chem. 248:5654-5659.
11. **Inouye, M.** 1969. Unlinking of cell division from deoxyribonucleic acid replication in a temperature-sensitive deoxyribonucleic acid synthesis mutant of *Escherichia coli*. J. Bacteriol. 99:842-850.
12. **Inouye, M.** 1971. Internal standards for molecular weight determinations of proteins by polyacrylamide gel electrophoresis. J. Biol. Chem. 246:4834-4838.
13. **Inouye, M.** 1974. A three-dimensional molecular assembly model of a lipoprotein from the *Escherichia coli* outer membrane. Proc. Natl. Acad. Sci. U.S.A. 71:2396-2400.
14. **Inouye, M., and J. P. Guthrie.** 1969. A mutant which changes a membrane protein of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. 64:957-961.
15. **Inouye, M., and A. B. Pardee.** 1970. Changes of membrane proteins and their relation to DNA synthesis and cell division of *Escherichia coli*. J. Biol. Chem. 245:5813-5819.
16. **Inouye, M., J. Shaw, and C. Shen.** 1972. The assembly of a structural lipoprotein in the envelope of *Escherichia coli*. J. Biol. Chem. 247:8154-8159.
17. **Jackson, R. W., and J. A. DeMoss.** 1965. Effects of toluene on *Escherichia coli*. J. Bacteriol. 90:1420-1425.
18. **Lee, N., and M. Inouye.** 1974. Outer membrane proteins of *Escherichia coli*: biosynthesis and assembly. FEBS Lett. 39:167-170.
19. **Leive, L., V. K. Shovlin, and S. E. Mergenhagen.** 1968. Physical, chemical and immunological properties of lipopolysaccharide released from *Escherichia coli* by

ethylenediaminetetraacetate. J. Biol. Chem. 243:6384-6391.

20. **Levin, D. H., M. N. Thang, and M. Grunberg-Manago.** 1963. Synthesis *in vivo* of polynucleotide phosphorylase in *E. coli*. I. Effect of amino acids of polynucleotide phosphorylase activity in a chloramphenicol-inhibited system. Biochim. Biophys. Acta 76:558-571.
21. **Moses, R. E., and C. C. Richardson.** 1970. Replication and repair of DNA in cells of *Escherichia coli* treated with toluene. Proc. Natl. Acad. Sci. U.S.A. 67:674-681.
22. **Peterson, R. L., C. W. Radcliffe, and N. R. Pace.** 1971. Ribonucleic acid synthesis in bacteria treated with toluene. J. Bacteriol. 107:585-588.
23. **Randall, L. L., and S. J. S. Hardy.** 1975. Analysis of the ribosomes engaged in the synthesis of the outer membrane proteins of *Escherichia coli*. Mol. Gen. Genet. 137:151-160.