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Biosynthesis and Assembly of Envelope Lipoprotein in a Glycerol-Requiring Mutant of *Salmonella typhimurium*

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A glycerol-requiring mutant of *Salmonella typhimurium* was used in a study of the biosynthesis and assembly of a structural lipoprotein in the cell envelope of gram-negative bacteria. Upon removal of glycerol from the growth medium, the biosynthesis of lipoprotein, as measured by radioactive arginine incorporation, was reduced by the same extent as that of other envelope proteins, the cumulative incorporation of arginine being 20% of that of the unstarved control cells. However, the incorporation of radioactive palmitate into lipoprotein was more severely curtailed after glycerol starvation, the cumulative rate of which was 8% of that observed in the unstarved cells. It was further observed that the lipoprotein synthesized in the glycerol-starved cells was more enriched in unmodified cysteine, which is known to be the N-terminal amino acid of lipoprotein, than that synthesized in the unstarved cells. We conclude that the synthesis of the apoprotein portion of Braun's lipoprotein proceeds independently of the attachment of diglyceride to the sulfhydryl group of the N-terminal cysteine and may, in fact, precede the incorporation of the diglyceride moiety.

The cell envelope of *Escherichia coli* and other gram-negative bacteria contains a so-called rigid layer that is in essence a giant macromolecule composed of murein and a covalently attached lipoprotein (1, 4, 5). This lipoprotein is present in two forms, free and bound (2, 15), the latter form being attached to the murein sacculus through the ϵ-NH_2 group of its C-terminal lysine. The primary structure of this unique lipoprotein has been determined by Braun and co-workers (2, 3). The N-terminus of the lipoprotein has been shown to be a novel lipoamino acid with three fatty acyl residues covalently attached to glycerylcysteine [2-amino-3-(2,3-dihydroxypropylthio)-propionic acid] (10). Two of the fatty acids are linked to the hydroxyl groups of glycerylcysteine by ester linkages, and the third is attached through amide linkage to the α-NH_2 group of glycerylcysteine. The similarity between the composition of ester-linked fatty acids in lipoprotein and that of the phospholipids from the same cells, as well as the in vivo labeling of lipoprotein with [2-^{3}H]glycerol, has led Hantke and Braun (10) to postulate that the diglyceride moiety of this lipoamino acid might be derived biosynthetically from a glycerophosphatide.

We have studied the biosynthesis and assembly of this lipoprotein in a glycerol-requiring mutant of *Salmonella typhimurium*. The effects of glycerol starvation on the biosynthesis of this lipoprotein and its subsequent insertion into murein sacculus are described.

MATERIALS AND METHODS

Bacterial strains and media. *S. typhimurium* strain GGD27R2 (*glpD gpsA ilv*) was kindly provided by M. J. Osborn (University of Connecticut Health Center). *E. coli* K-12 strains 7 (*glpR*) and 8 (*glpR glpD*) were gifts of E. C. C. Lin (Harvard Medical School, Boston, Mass.). Media used in the present study included M9 mineral salt medium and proteose peptone beef extract (PPBE) broth medium (19, 21).

Labeling experiments. M9 minimal medium supplemented with 1% DL-sodium lactate, 50 μg each of isoleucine and valine per ml, and 40 μg of glycerol per ml was used for the growth and labeling of strain GGD27R2 with various radioactive precursors. It is referred to as the complete medium. In general, a 500-ml culture of this strain was grown in a 2-liter Erlenmeyer flask at 37 C with vigorous shaking. At the early exponential phase of growth (cell density about 1.5×10^8/ml), radioactive precursors (palmitic acid, glycerol, or arginine) were added and the growth of the bacteria was continued for about three to four generations. The labeled cells were harvested by centrifugation at $7,000 \times g$ for 10 min, washed twice with saline, and processed for the isolation of the cell envelope and subsequent biochemical fractionation by the procedures detailed below.

To investigate the effect of glycerol starvation on the biosynthesis and assembly of murein-lipoprotein, the cells were prelabeled with ^{14}C precursor (arginine or palmitate) for three to four generations, harvested, and washed, and the culture was split into two portions. One portion of the culture, designated the unstarved culture, was resuspended in the complete medium. The remaining portion, desig-

nated the starved culture, was resuspended in complete medium without glycerol. ^3H precursor (arginine or palmitate) was added to the unstarved culture immediately after the resumption of exponential growth. Growth ceased within 2 h after removal of glycerol from the growth medium of the starved culture, and the starved cells were then labeled for an additional 2 h with ^3H precursor (arginine or palmitate). In a separate series of experiments, labeling of the starved culture with ^3H precursor (arginine or palmitate) began 30 min after removal of glycerol from the growth medium and was allowed to proceed for 2 h.

Preparation of cell envelope and isolation of phospholipids, lipopolysaccharide, and lipoprotein from the cell envelope. Washed cells were suspended in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.5) and treated for 1 to 3 min with a Branson Sonifier with intermittent cooling at 0 C. The crude extract was then centrifuged at 250,000 × *g* for 2 h. The pellet was washed once with the same buffer and centrifuged again at 250,000 × *g* for 2 h. The washed pellet was kept frozen at −20 C.

Isolation and identification of phospholipids. The washed cell envelope was lyophilized and extracted with chloroform-methanol (2:1, vol/vol) repeatedly (11). Over 95% of the phospholipid was rendered soluble in chloroform-methanol mixture during the first three extractions, as monitored by both [2-^3H]glycerol- and radioactive palmitate-labeled cell envelope. The combined chloroform-methanol extract was extensively washed with a salt solution containing chloroform-methanol-water-KCl (3:48:47:0.05 M) (7). The chloroform layer was dried under a stream of nitrogen at room temperature. Individual phospholipid species were isolated and identified by one of three methods: diethylaminoethyl-cellulose chromatography in 99% methanol (6), chromatography on silica gel-loaded paper in chloroform-methanol-acetic acid (7:3:1), and high-voltage paper electrophoresis of deacylated glycerophosphoryl derivatives at 70 V/cm for 30 min in pyridine-acetic acid-water (1:10:89), pH 3.5.

Isolation of the free and bound forms of cell envelope lipoprotein. Murein sacculi were isolated by treatment of delipidated cell envelope with 4% sodium dodecyl sulfate (SDS) (pH 7.0) at 100 C for 20 min, and the SDS-insoluble murein sacculi were collected by centrifugation at 250,000 × *g* for 2 h at room temperature. Extractions with 4% SDS were repeated once or twice, depending on the amount of the starting material. The murein sacculi (or murein-lipoprotein complex) were washed extensively with 80% acetone in water to remove the detergent. The washed murein-lipoprotein complex was digested with 50 μg of egg white lysozyme or T4 phage lysozyme per ml of 0.01 M sodium phosphate (pH 7.0) for at least 2 h at 37 C. The free form of lipoprotein was isolated by a procedure described previously (11). The free form of lipoprotein, isolated by this procedure, was relatively pure owing to is solubility in 10% trichloroacetic acid and 1% SDS, and was found to consist of a single major band labeled with radioactive arginine or [2-^3H]glycerol by SDS-gel electrophoresis. Nevertheless, it was found to be contaminated with lipopolysaccharide, as evidenced by SDS-gel electrophoresis of a palmitate-labeled sample. To purify lipoprotein from lipopolysaccharide further, we used the hot-phenol procedure of Westphal et al. (20). The free-form lipoprotein was quantitatively recovered in the phenol phase and interphase. Ether extraction and extensive dialysis against water were used to remove phenol. Lipoprotein was also separated from lipopolysaccharide by preparative paper chromatography in isobutyric acid- 1 M NH_4OH (5:3). Lipopolysaccharide was also isolated from the delipidated cell envelope by the hot-phenol extraction. The aqueous phase was extracted with ether and dialyzed to remove residual phenol.

To check the purity of these lipid-containing membrane components, we used paper chromatography employing both isobutyric acid-1 M NH_4OH (5:3) and 70% phenol as solvents. Chromatography was also carried out on silica gel-loaded paper in chloroform-methanol-acetic acid (7:3:1). The purity of the preparations was also evaluated by SDS-polyacrylamide gel electrophoresis with dansylated internal standards (13, 15). The relative migrations of various components in these systems are given in Table 1.

For chemical analysis of lipoprotein, the free form of lipoprotein was also purified from the cell envelope by a procedure kindly made available to us by Masayori Inouye (S. Inouye, K. Takeishi, N. Lee, M. Demartini, A. Hirashima, and M. Inouye, manuscript in preparation). This procedure avoids the use of trichloroacetic acid, and thereby reduces the loss of ester-linked fatty acids from the lipoprotein.

Isolation and identification of fatty acids. Ester-

TABLE 1. *Identification of phospholipid, fatty acid, lipopolysaccharide, and lipoprotein by paper chromatography and by SDS-gel electrophoresis*

Compounds	R_f in solvents[a]				Relative mobility to cytochrome *c*[b]
	I	II	III	IV	
Phospholipids					1.17
Phosphatidylethanolamine	0.95	0.41	1.0	0.77	
Phosphatidylglycerol	0.95	0.57	1.0	0.77	
Cardiolipin	0.95	0.75	1.0	0.77	
Fatty acids	0.98	0.84	1.0	0.95	1.17
Lipoprotein					
Bound form	0.89	0	0.44	0.38	1.00
Free form	0.89	0	0.63	0.53	1.08
Lipopolysaccharide	0.59	0	0	0.29	0.93

[a] Solvent I: Isobutyric acid-1 M NH_4OH (5:3, vol/vol), descending on Whatman 3MM cellulose paper. Solvent II: Chloroform-methanol-acetic acid (7:3:1, vol/vol), descending on silica gel-loaded paper. Solvent III: 70% phenol, ascending on Whatman 3MM paper. Solvent IV: *n*-Butanol-isobutyric acid-1 M NH_4OH (10:28:15, vol/vol), ascending on Whatman 3MM paper.

[b] Determined by SDS-gel electrophoresis.

linked fatty acids were released from phospholipid, lipopolysaccharide, or lipoprotein by treatment either with 0.125 N LiOH in methanol at room temperature for 30 min or with 0.1 N NaOH at 37 C for 2 h. At the end of incubation, the reaction mixture was acidified to pH 2 with dilute HCl and extracted with *n*-hexane. The hexane layer was reduced to dryness under a stream of nitrogen, and the residues were esterified with a BF_3-methanol mixture (16). The aqueous layer was made 6 N with respect to HCl and hydrolyzed in vacuo at 100 C for 20 h for the liberation of amide-linked fatty acids, which were then extracted with hexane and esterified with BF_3-methanol mixtures. The identification of fatty acid methyl esters by gas-liquid chromatography and the measurement of radioactive palmitate were carried out as described previously (11). Methyl stearate was used as the internal standard for quantitative determination of fatty acid composition in phospholipid and lipoproteins.

Amino acid analysis. Protein samples (0.5 to 2 mg) were hydrolyzed in 6 N HCl at 105 C in vacuo for 20 h. The hydrolysates were dried in a vacuum desiccator over KOH and P_2O_5, lyophilized, and then dissolved in citrate buffer. Amino acid compositions were determined with a Beckman model 120C amino acid analyzer equipped for determination of amino acids in the range of 7.5 to 30 nmol (17). The half-cysteine and methionine contents were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation of the protein prior to acid hydrolysis (12).

Other analytical techniques. Gel electrophoresis was carried out in 0.5% SDS as described by Inouye and Guthrie (14). Gels containing 7.5% acrylamide were used routinely. Occasionally, we used gels containing 10, 12.5, or 15% acrylamide to assure better separation of lipoproteins from other envelope proteins. Internal molecular weight standards (dansyl proteins) were the generous gift of M. Inouye of State University of New York at Stony Brook. Performic acid oxidation of lipoprotein was carried out according to Hirs (12). Cysteic acid and glycerylcysteine sulfone were identified and quantitated by high-voltage paper electrophoresis at pH 3.5 (pyridine-acetic acid-water, 1:10:89) and at pH 1.9 (0.47 M formic acid and 1.4 M acetic acid), 50 V/cm for 40 min. Measurements of radioactivities were made in a Beckman scintillation counter with toluene-based scintillation solution containing 33% Triton X-100.

Chemicals and radiochemicals. All chemicals were of reagent or analytical grade and were purchased from commercial sources. Radioactive chemicals used in the present study include [2-^{3}H(N)]glycerol, 200 mCi/mmol; [U-^{14}C]glycerol, 121 mCi/mol; [9,10-^{3}H(N)]palmitic acid, 520 mCi/mmol; [1-^{14}C]palmitic acid, 56 mCi/mmol; L-[3-^{3}H(N)]-arginine, 27 Ci/mmol; and L-[U-^{14}C]arginine, 309 mCi/mmol. All of the above were purchased from New England Nuclear Corp. [^{35}S]cysteine hydrochloride (56 mCi/mmol) was purchased from Amersham/Searle and was purified by descending paper chromatography (methanol-water-pyridine, 8:2:1) before use.

RESULTS

Specific labeling of lipoprotein by [2-^{3}H]glycerol. *S. typhimurium* strain GGD27R2 *(glpD gpsA ilv)* is defective in both the biosynthetic and catabolic L-α-glycerophosphate dehydrogenases (M. J. Osborn, personal communication). Both [2-^{3}H]glycerol and [U-^{14}C]glycerol (at final concentrations of 0.6 μCi of ^{3}H radioactivity and 0.2 μCi of ^{14}C radioactivity per ml) were added to an exponentially growing culture of this mutant (cell density, 10^8/ml), grown in complete medium. Double labeling was continued for three to four generations. The cells were then harvested, washed, and processed for the preparation of the total cell envelope and the subsequent isolation of phospholipids, lipopolysaccharide, and lipoprotein as described in detail in Materials and Methods. The purity of the purified lipoprotein was checked by gel electrophoresis (Fig. 1). The component migrating between internal standards c and d in Fig. 1B most likely represented a mixture of mucopeptide monomer and dimers, resulting from leakiness in the catabolic α-glycerophosphate dehydrogenase in this mutant. Thus the greatly reduced ^{3}H/^{14}C ratio was due to the loss of the ^{3}H label at the 2-position of L-α-glycerophosphate during its conversion into dihydroxyacetone phosphate.

The ^{3}H/^{14}C ratio in the lipoprotein fraction was similar to that of the phospholipids and much higher than that of either the delipidated cell envelope or lipopolysaccharide (Table 2). Similar results were obtained in experiments with *E. coli* strain 8, which is defective in the catabolic L-α-glycerophosphate dehydrogenase *(glpR glpD)*. These results were taken as a strong evidence that the glycerol moiety in the glycerylcysteine located at the N-terminus of murein lipoprotein is derived directly from L-α-glycerophosphate or its acylated derivatives rather than from any metabolic intermediates derived from dihydroxyacetone phosphate. Furthermore, we labeled strain GGD27R2 with [2-^{3}H]glycerol (2 μCi/ml, 40 μg/ml) and [^{35}S]cysteine (0.15 μCi/ml, 56 μCi/μmol) in the presence of L-methionine (200 μg/ml). Mureinlipoprotein was isolated from the doubly labeled cells, oxidized with performic acid, and hydrolyzed in 6 N HCl at 100 C for 20 h in vacuo. A major component was labeled with both [2-H^3]glycerol and [^{35}S]cysteine, and this component migrated as a neutral amino acid at pH 3.5 and as a cationic compound at pH 1.9 (Fig. 2). The acid hydrolysate also contained a trace amount of cysteic acid that was not labeled with [2-^{3}H]glycerol.

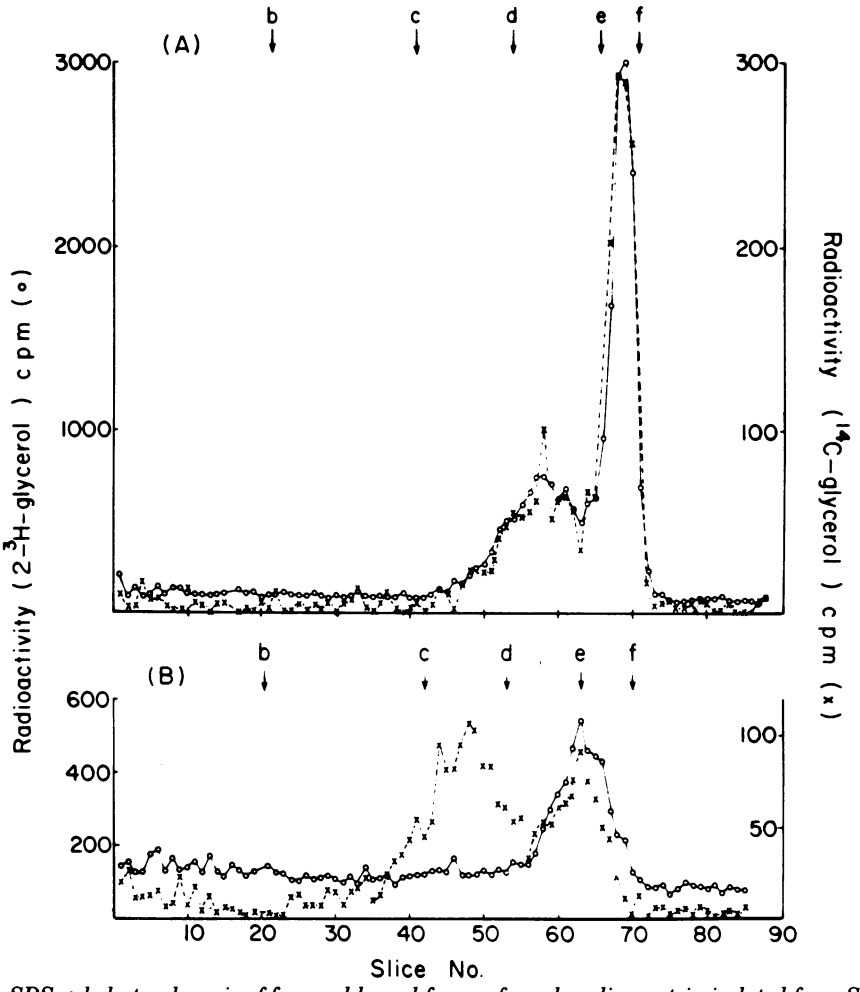

FIG. 1. *SDS-gel electrophoresis of free and bound forms of envelope lipoprotein isolated from S. typhimurium strain GGD27R2 doubly labeled with [2-^{3}H]-glycerol and [U-^{14}C]glycerol. Details of the growth, labeling, and isolation of lipoproteins are given in the text. (A) Free-form lipoprotein; (B) bound-form lipoprotein isolated by lysozyme treatment of murein-lipoprotein complex. Arrows with letters in both panels indicate the positions of the internal molecular weight standards (13): b, monomer of dansyl bovine serum albumin; c, dimer of dansyl egg white lysozyme; d, monomer of dansyl egg white lysozyme; e, cytochrome c; f, dansyl insulin.*

Turnover of the lipid moiety of murein lipoprotein in control and glycerol-starved cells. The mutant strain GGD27R2 was labeled with [2-^{3}H]glycerol (0.16 μCi/ml, 40 μg/ml) and [1-^{14}C]palmitate (0.04 μCi/ml, 25 nmol/ml) for three generations in order to determine whether the glycerol moiety and the fatty acyl residues of *N*-acyl diglyceride cysteine preferentially turn over during growth and during glycerol starvation. The labeled cells were harvested and washed, and one-third of the washed cells were saved (steady-state-labeled cells). The remaining two-thirds were divided into two portions, one in M9 lactate isoleucine-valine medium supplemented with 100 μg of glycerol per ml and the other in the same medium free of glycerol. To both cultures, sodium palmitate was added to a final concentration of 0.25 mM. Both cultures were incubated for three generations (control chase) or for 3 h (starved chase), harvested, and washed. Various lipid-containing components were isolated from the cell envelopes from these three samples, and the ratios of [2-^{3}H]glycerol to [1-^{14}C]palmitate were determined. Whereas phosphatidylethanolamine remained metabolically stable, there was significant turnover of the glycerol moiety in both phosphatidylglycerol and cardiolipin, relative to that of palmitate, in growing cells (Table 3). This turnover was less pronounced in

cells starved for glycerol. On the other hand, the $^3H/^{14}C$ ratio in both free and bound forms of lipoprotein increased slightly during chase in the presence or absence of glycerol, approaching half the value found in phosphatidylethanolamine. Since this increase in $^3H/^{14}C$ ratio was seen in cells chased both in the presence of 100 μg of glycerol per ml and in its absence, it is unlikely that this increase in $^3H/^{14}C$ ratio was due to reutilization of [2-^{3}H]glycerol, derived from turnover of phosphatidylglycerol and cardiolipin, for lipoprotein synthesis. Rather, it may indicate a difference in the precursor pools of glycerol and palmitate utilized for the synthesis of the diglyceride moiety and the amide-linked palmitate, respectively. On the other hand, there may be a turnover of the amide-linked fatty acid in lipoprotein.

TABLE 2. *Comparison of the incorporation of [2-^{3}H]glycerol and [U-^{14}C]glycerol into lipoprotein and phospholipids*

Fraction	$^3H/^{14}C$ ratio
Total envelope	1.00[a]
Phospholipids	
Phosphatidylethanolamine	1.04
Phosphatidylglycerol	1.18
Cardiolipin	1.27
Deacylated phospholipids	
Glycerophosphorylethanolamine	1.19
Glycerophosphorylglycerol	1.54
Bis-(glycerophosphoryl)glycerol	1.33
SDS-soluble fraction	0.15
Purified free-form lipoprotein	1.35
SDS-insoluble murein-lipoprotein	0.85

[a] The $^3H/^{14}C$ ratio of the total cell envelope was arbitrarily set at 1.00.

Synthesis of lipoprotein in control and glycerol-starved cells. Since it appears that the diglyceride moiety of the lipoprotein is derived from glycerophosphatides, we proceeded to investigate the effect of glycerol starvation on the biosynthesis and assembly of lipoprotein in the

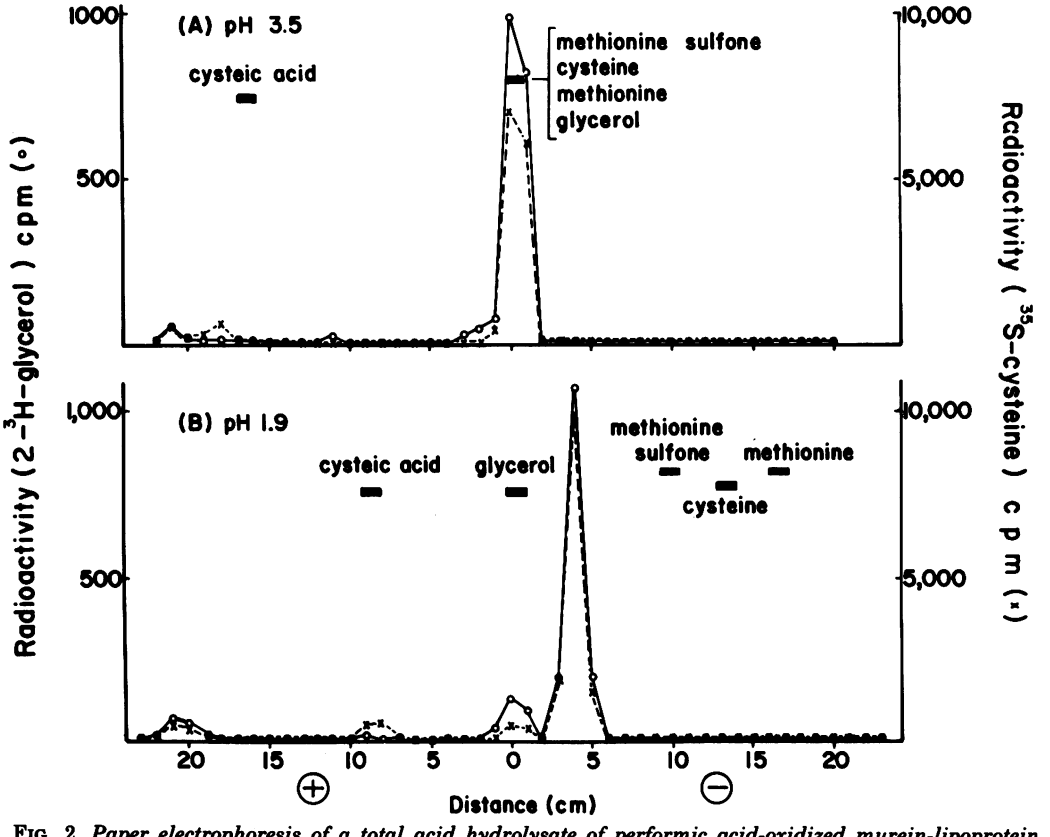

FIG. 2. *Paper electrophoresis of a total acid hydrolysate of performic acid-oxidized murein-lipoprotein doubly labeled with [2-^{3}H]glycerol and [^{35}S]cysteine. (A) pH 3.5 buffer (pyridine-acetic acid-water, 1:10:89, vol/vol); (B) pH 1.9 buffer (0.47 M formic acid and 1.4 M acetic acid). Paper electrophoresis was carried out on a Whatman no. 1 paper, with 50 V/cm for 40 min at 18 to 20 C. Mobilities shown by amino acid standards are indicated by horizontal bars.*

TABLE 3. *Turnover studies of [2-^{3}H]glycerol and [1-^{14}C]palmitate doubly labeled cells grown under permissive (with glycerol) and nonpermissive (without glycerol) conditions*

Fraction	^{3}H/^{14}C ratio		
	Steady-state-labeled cells	Chase with glycerol	Chase without glycerol
Total cell envelope ..	1.00[a]	0.95	1.05
Phospholipids	1.67	1.57	1.76
Phosphatidylethanolamine	1.29	1.33	1.33
Phosphatidylglycerol	2.52	1.48	2.05
Cardiolipin	2.38	1.76	2.24
Delipidated cell envelope			
SDS-soluble fraction	0.10	0.24	0.14
Lipopolysaccharide	0.10	0.19	0.1
Free lipoprotein	0.43	0.62	0.62
Murein-bound lipoprotein	0.48	0.52	0.67

[a] The ^{3}H/^{14}C ratio of the total cell envelope was arbitrarily set at 1.00.

glycerol-requiring mutant of *S. typhimurium*. Cells were grown in complete medium and labeled with L-[U-^{14}C]arginine (10 μg/ml, 0.1 μCi/ml) for three generations (Fig. 3). The cells were harvested and washed twice with saline and divided into two aliquots. Five hundred milliliters of fresh complete medium was added to the control culture, and to the starved culture was added 500 ml of complete medium without glycerol supplement. At the time indicated by the first arrows in Fig. 3, L-[3-^{3}H]arginine was added to both cultures at final concentration of 10 μg/ml and a specific activity of 10 μCi/mg. Two hours after the additions of ^{3}H label (second arrows, Fig. 3), the cells were harvested and washed, and cell envelopes were prepared from both samples. Murein-lipoprotein complexes were isolated by SDS (4%) extraction at 100 C for 20 min, washed extensively with 80% acetone, and digested with lysozyme. The bound form of lipoprotein was identified both by SDS-gel electrophoresis and by paper chromatography in isobutyric acid-1 M NH_4OH (5:3). The ^{3}H/^{14}C ratios determined by these two methods were nearly identical. The SDS-soluble fraction was analyzed by SDS-gel electrophoresis with dansyl proteins as the internal standards. The ^{3}H/^{14}C ratio of the major envelope proteins was determined, together with that of the free-form lipoprotein that migrated between the markers cytochrome *c* and dansylated insulin. The free form lipoprotein was also isolated by the SDS-trichloroacetic acid procedure described previously, and the ^{3}H/^{14}C ratio was confirmed. The cumulative rate of arginine incorporation into total envelope protein in the glycerol-starved cells was about 20% of that in the control cell (Table 4). This reduction by 80% in total envelope protein synthesis represented an average of many envelope proteins. Whereas some of the major outer membrane proteins were synthesized at a rate 18 to 23% of those in the control cells, the relative rates for the free-form and bound-form lipoprotein were 43 and 17%, respectively. Two points can be made with respect to these observations. The rate of lipoprotein synthesis was not preferentially more curtailed by the cessation of net phospholipid synthesis than those of other envelope proteins in general, as one might have anticipated owing to the unique structural feature of this protein. Secondly, the significant increase in the ^{3}H/^{14}C ratio of the free form of lipoprotein and the corresponding decrease in that of the bound form reflects an inhibition of the assembly of lipoprotein into the murein sacculus.

Similar studies were carried out using palmitic acid as the label to compare the relative rates of synthesis and assembly of lipoprotein and other lipid-containing components in control and glycerol-starved cells. Prelabeling was carried out with 50 μCi of [1-^{14}C]palmitic acid (56 mCi/mmol) in 500 ml of complete medium. The ^{14}C-labeled cells were harvested, washed, and reinoculated into two 500-ml cultures, one of which was supplemented with glycerol (40 μg/ml). To each of these two cultures 50 μCi of [9,10-^{3}H]palmitic acid (520 mCi/mmol) was added, and the labeling was continued for about 2 h, following a protocol essentially the same as shown in Fig. 3. The rate of phospholipid synthesis in glycerol-starved cells was about 10% of that in the control cells (Table 5), as one might have expected for a glycerol auxotroph. This reduction in phospholipid synthesis owing to glycerol starvation was accompanied by a severe curtailment of incorporation of palmitate into both free and bound forms of lipoprotein.

It may be argued that by allowing the growth of the glycerol-starved culture to cease completely before the addition of [^{3}H]-labeled precursor, one may be investigating some indirect and secondary effects of glycerol starvation on the biosynthesis and assembly of lipoprotein. Therefore, the experiments described in Tables 4 and 5 were repeated with one modification. The [^{14}C]-prelabeled starved cells were allowed to grow for 30 min in the absence of glycerol before [^{3}H]-labeled precursor (arginine or palmitate) was added, and the labeling proceeded

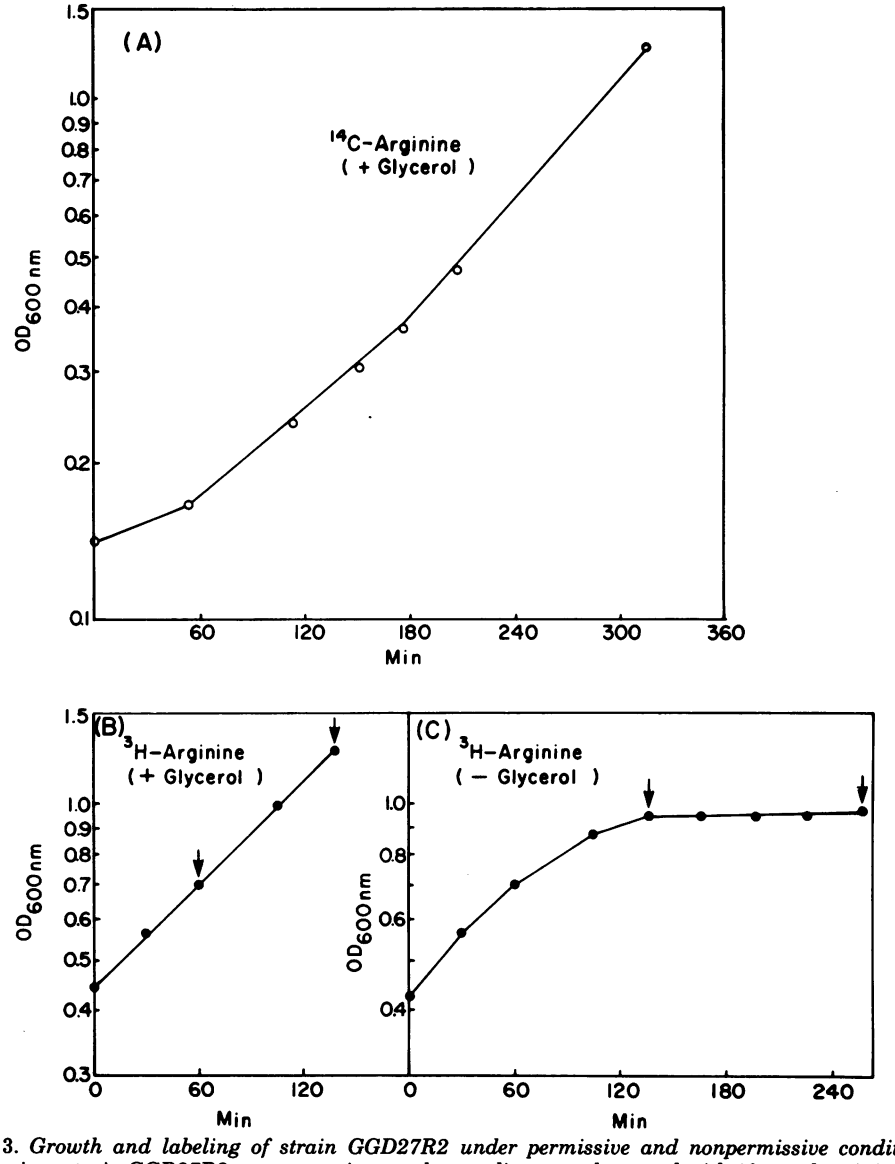

FIG. 3. *Growth and labeling of strain GGD27R2 under permissive and nonpermissive conditions. S. typhimurium strain GGD27R2 was grown in complete medium supplemented with 10 μg of arginine per ml. (A) [^{14}C]arginine (0.1 μCi/ml) was added to this growing culture throughout this prelabeling period; (B) half of the [^{14}C]arginine-labeled cells were harvested, washed, and resuspended in the same complete minimal medium and labeled with [^{3}H]arginine (0.1 μCi/ml, 10 μg/ml) for the period indicated by two arrows; (C) the remaining half of [^{14}C]arginine-labeled cells was harvested, washed, resuspended in minimal medium without glycerol, and labeled with [^{3}H]arginine (0.1 μCi/ml, 10 μg/ml) for the period indicated by two arrows.*

for 2 h. Again there was a stronger inhibition, by glycerol starvation, of incorporation of radioactive palmitate into lipoprotein than of radioactive arginine incorporation (Table 6). The cumulative results of Tables 4, 5, and 6 suggest a preferential inhibition of the synthesis of the lipid moiety of the lipoprotein to that of the protein portion. At least two explanations can be offered to account for this observation. The biosynthesis and assembly of the apoprotein precede and are independent of the incorporation of lipid precursor to the nascent polypeptide chain. Alternatively, the synthesis and assembly of lipoprotein requires the prior covalent attachment of the lipid moiety, which in turn may be derived from the preexisting glyc-

TABLE 4. *Relative rate of arginine incorporation into cell envelope proteins and into lipoprotein in control and glycerol-starved cells*

Fraction	[^{3}H]arginine/[^{14}C]arginine ratio		Relative rate[a]
	Control (with glycerol)	Starved (without glycerol)	
Total cell envelope ..	6.5	1.3	0.20
SDS-soluble envelope proteins	6.5	1.5	0.23
Protein bands in SDS-gel with apparent mol wt of:			
40,000	5.2	1.2	0.23
35,000	6.5	1.2	0.18
15,000	7.0	2.0	0.29
Free-form lipoprotein	6.5	2.8	0.43
SDS-insoluble murein-bound lipoprotein	6.3	1.1	0.17

[a] Relative rate was obtained as the ratio of [^{3}H]arginine/[^{14}C]arginine in a particular fraction isolated from the starved cells to that of the same fraction from the unstarved cells. It is taken as a measure of the cumulative rate of synthesis of this given component in the starved cells relative to that in the unstarved cells.

erophosphatides or from turnover of preexisting lipoproteins.

Comparison of the structure of lipoproteins synthesized in the control and glycerol-starved cells. If the apoprotein moiety can be synthesized and assembled into the murein sacculus as a polypeptide with the N-terminal amino acid sequence of cys-ser-ser-asn-ala-lys-ile, one would expect that the lipoprotein synthesized in glycerol-starved cells would be structurally different from that made in the control cells in two respects. First, there would be an increase in cysteine content of the murein-lipoprotein with a concomitant decrease in glycerylcysteine sulfone. Secondly, assuming that the amide-linked fatty acid is derived biosynthetically from activated fatty acids in the form of thioesters (coenzyme A or acyl carrier protein linked) rather than phospholipids, one would expect a preferential decrease in ester-linked radioactive palmitate, relative to the amide-linked palmitate, in lipoproteins isolated from glycerol-starved cells in the case where the label was added after glycerol starvation. Experiments designed to verify these two predictions are described below.

[^{35}S]cysteine was used to label two 500-ml cultures of *S. typhimurium* strain GGD27R2 under both permissive (with glycerol) and non-permissive (without glycerol) conditions. The starved culture was allowed to grow for 30 min in the absence of glycerol before [^{35}S]cysteine and unlabeled methionine were added. In the experiment shown in Table 7, both unstarved and starved cells were labeled with both L-[3-^{3}H]arginine (0.2 μCi/ml, 10 μg/ml) and [^{35}S]-cysteine (0.4 μCi/ml, 56 mCi/mmol) plus methionine (100 μg/ml). Free and bound forms of lipoprotein were isolated from both starved and unstarved cultures (Fig. 4). The ^{3}H/^{35}S ratios of free and bound forms of lipoprotein in the unstarved cells were 0.9 and 0.7, respectively, whereas the corresponding ratios in the starved cells were 0.87 and 0.8, respectively. Murein-lipoproteins were oxidized with performic acid, and hydrolyzed with 6 N HCl at 100 C for 20 h. The acid hydrolysate was analyzed by high-voltage paper electrophoresis at pH 1.9, 3.5, and 6.8. The ratio of cysteic acid to glycerylcysteine sulfone was then determined. No significant radioactivity was seen in areas corresponding to methionine, methionine sulfone, or cysteine. There was a significant increase in the ratio of cysteic acid/glycerylcysteine sulfone in the bound form of lipoprotein synthesized in glycerol-starved cells as compared with the control cells (Table 7). These data are con-

TABLE 5. *Relative rate of palmitate incorporation into phospholipids, lipopolysaccharide and lipoprotein in control and glycerol-starved cells*

Fraction	[^{3}H]palmitate/[^{14}C]palmitate ratio		Relative rate[a]
	Control (with glycerol)	Starved (without glycerol)	
Total cell envelope ..	1.16	0.17	0.14
Phospholipids			
Phosphatidylethanolamine	1.03	0.10	0.10
Phosphatidylglycerol	1.65	0.20	0.12
Cardiolipin	1.72	0.12	0.07
Lipopolysaccharide ..	0.19	0.04	0.21
Lipoproteins			
Free form	0.52	0.04	0.08
Bound form	0.44	0.03	0.07
Palmitate methyl ester derived from total phospholipids[b]	1.86	0.19	0.10

[a] See footnote *a* Table 4.

[b] Palmitic acid was released from phospholipid by mild alkaline hydrolysis and methylated. The ^{3}H/^{14}C ratio of methyl palmitate was determined by gas-liquid chromatography, using a stream-splitting device and a fraction collector.

TABLE 6. *Relative rates of arginine and palmitate incorporations into membrane components in control and glycerol-starved cells*

Fraction	Relative rate[a] of arginine incorporation	Fraction	Relative rate[a] of palmitate incorporation
Total cell envelope	0.55	Total cell envelope	0.30
SDS-soluble envelope proteins	0.56	Phospholipid	
Free-form lipoprotein	0.65	Phosphatidylethanolamine	0.23
Bound-form lipoprotein	0.44	Phosphatidylglycerol	0.24
		Cardiolipin	0.28
		Lipopolysaccharide	1.68
		Free-form lipoprotein	0.27
		Bound-form lipoprotein	0.15
		Palmitate methyl ester derived from total phospholipids[b]	0.16

[a] Relative rates were obtained as the ratios of [^{3}H]arginine/[^{14}C]arginine (and [^{3}H]palmitate/[^{14}C]palmitate) in a particular fraction isolated from the starved cells to those of the same fraction from the unstarved cells. They are taken as measures of the cumulative rates of synthesis of this given component in the starved cells relative to that in the unstarved cells.

[b] See footnote *b* of Table 5.

TABLE 7. *Cysteine and glycerylcysteine content in lipoprotein synthesized in control and glycerol-starved cells*[a]

Source of lipoprotein	[^{35}S]cysteic acid/[^{35}S]glycerylcysteine sulfone ratio[b]
Bound form from control unstarved cells	0.28
Bound form from glycerol-starved cells	0.75

[a] The purity of lipoprotein preparations was evaluated with SDS-polyacrylamide gel electrophoresis, as shown in Fig. 4.

[b] Determined in total acid hydrolysate of performic acid-oxidized lipoprotein.

sistent with the working hypothesis that lipoprotein synthesized in the starved cells contained a normal equivalent of N-terminal cysteine, and the increased cysteic acid/glycerylcysteine sulfone ratio in the bound form from the starved cell is most likely not due to excessive contamination of murein-lipoprotein with cysteine-containing proteins. It is unlikely that the [^{35}S]cysteic acid we detected in the acid hydrolysate of performic acid oxidized murein-lipoprotein isolated from the control unstarved cells represents a hydrolysis product of glycerylcysteine. Should the latter be the case, one might expect to find [^{35}S]cysteine sulfonic acid instead.

All attempts to separate lipoproteins synthesized in glycerol-starved cells from those synthesized in unstarved cells were unsuccessful. The analytical procedures we used include SDS-polyacrylamide gel electrophoresis (with varying gel concentrations of 7.5 through 15%), paper chromatography using isobutyric acid-1 M NH_4OH (5:3, vol/vol) and 70% phenol as separate solvent systems, and affinity chromatography using both activated thiol-Sepharose 4B (Pharmacia) and Affi-Gel 501 containing organomercuric chloride as the covalent ligand for the SH group.

Chemical analysis of lipoprotein purified from control and glycerol-starved cells. Two 12-liter cultures of strain GGD27R2 in PPBE broth containing 40 μg of glycerol per ml and 5% in M9 minimal salts were grown at 37 C with vigorous aeration (6 liters of air/min) in a New Brunswick microferm, with the agitation control at 400 rpm. The pH of the culture was maintained at 7 with a radiometer model TTT-1 automatic titrator. The growth curve of one of the two cultures is shown in Fig. 5. Two similar cultures of this strain were grown in 12 liters of PPBE broth containing 5% M9 minimal salts but no glycerol (Fig. 5), and the starvation for glycerol was allowed to proceed for 3 h before harvesting by centrifugation. Amounts of 43.4 g (wet weight) of control cells and 16 g (wet weight) of starved cells were obtained.

The free form of lipoprotein was isolated from these two cultures by a procedure kindly made available to us by Masayori Inouye of State University of New York at Stony Brook (Inouye et al., manuscript in preparation). The lipoprotein purified by this procedure was further separated from minor contaminating proteins by

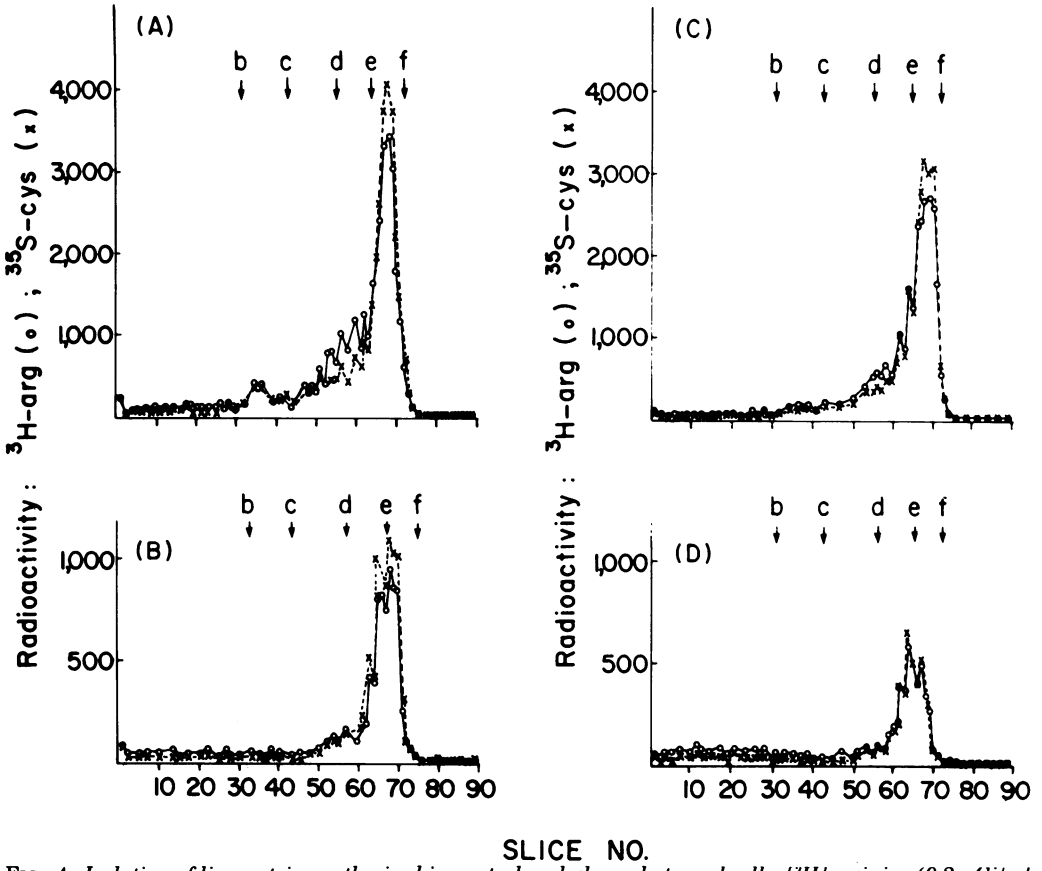

FIG. 4. *Isolation of lipoprotein synthesized in control and glycerol-starved cells. [^{3}H]arginine (0.2 μCi/ml, 10 μg/ml), [^{35}S]cysteine (0.4 μCi/ml, 56 mCi/mmol), and L-methionine (100 μg/ml) were added to the unstarved culture, and the labeling was continued for 2.5 h. The starved culture was allowed to grow in the absence of glycerol for 30 min before the same amounts of [^{3}H]arginine, [^{35}S]cysteine, and L-methionine were added. The labeling of the starved culture was continued for 3 h. Lipoprotein was analyzed by SDS-gel electrophoresis in 7.5% polyacrylamide gels. (A) and (C): Free-form lipoproteins, isolated and purified by the 10% trichloroacetic acid-1% SDS procedure (11), from the unstarved and starved cells, respectively. (B) and (D): Bound-form lipoproteins isolated by lysozyme digestion of murein-lipoprotein complexes from the unstarved and starved cells, respectively.*

Sephadex G-100 gel filtration (2.5 by 90 cm) in 1% SDS and 20 mM ammonium acetate (pH 7.0). A lipoprotein preparation was obtained showing a single band in 7.5% polyacrylamide gel electrophoresis in the presence of 0.5% SDS. The amino acid compositions of performic acid-oxidized lipoprotein from the control and starved cells are shown in Table 8. The fatty acid compositions of the same preparations are given in Table 9. There indeed appeared to be a lipid-deficient lipoprotein species in glycerol-starved cells as compared with that from the control cells (Tables 8 and 9). Likewise, there was a significant amount of unmodified cysteine in lipoprotein isolated from the starved cells.

DISCUSSION

A unique lipoprotein has been found to be present in extremely large quantities in the cell envelope of many gram-negative bacteria (5, 15). The N-terminal amino acid of this lipoprotein has been shown to be a novel lipoamino acid, consisting of a diglyceride moiety covalently linked to the sulfhydryl residue of cysteine through a thioether linkage. In addition, the α-amino group of this amino acid is substituted preferentially with palmitic acid through an amide linkage (10).

The data presented in this paper confirm the results obtained by Hantke and Braun (10) that the diglyceride moiety of this lipoamino acid is

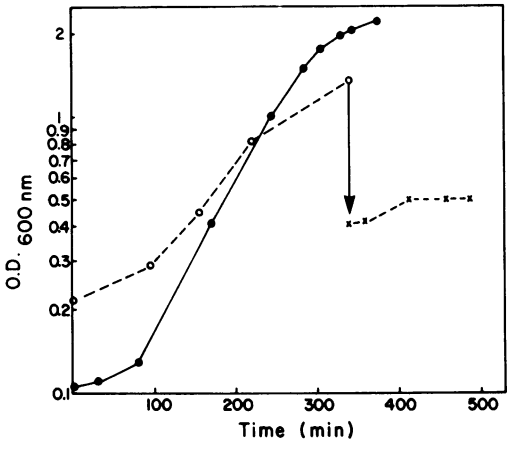

FIG. 5. *Large-scale growth of strain GGD27R2 under permissive and nonpermissive conditions. Two 12-liter cultures of strain GGD27R2 in PPBE broth containing 5% M9 mineral salts were grown at 37 C in a New Brunswick microferm with (○) or without (×) 40 μg of glycerol per ml. Details were given in the text. The starved culture was diluted twofold with fresh medium before the starvation was allowed to proceed.*

derived from L-α-glycerophosphate or its phospholipid derivatives. Had this diglyceride been formed via acylation of dihydroxyacetone phosphate followed by reduction (8, 9), we would have observed a greatly reduced [2-^{3}H]glycerol/[U-^{14}C]glycerol ratio in the lipoprotein as compared with that found in glycerophosphatides. The data given in Table 2 virtually preclude this possibility.

We have shown that after cessation of net phospholipid synthesis due to glycerol deprivation in a *glpD gpsA* double mutant of *S. typhimurium*, the rate of total envelope protein synthesis is reduced to 20% of that in the unstarved cells. However, under these conditions, the rate of [^{3}H]arginine incorporation into murein-lipoprotein is not inhibited to a greater extent than was observed for the remainder of the envelope proteins. The assembly of lipoprotein into the murein sacculus seemed to be inhibited in glycerol-starved cells, and this inhibition was accompanied by an accumulation of the free-form lipoprotein that was synthesized after glycerol starvation.

However, the rate of palmitate incorporation into lipoprotein in glycerol-starved cells was reduced to 7 to 8% of that of the control cells. This differential effect of glycerol starvation on the synthesis of the apoprotein and that of the lipid moiety can be interpreted in at least two ways. It is possible, as suggested by Hantke and Braun (10), that a nucleotide-activated diglyceride is transferred to the mercapto group of the N-terminal cysteine of the polypeptide chain. This possibility is consistent with the

TABLE 8. *Amino acid composition of free form of lipoprotein purified from control and starved cells of strain GGD27R2*[a]

Amino acid	Control	Starved	Lipoprotein[b]
Lysine	5	5	5
Histidine	0	0	0
Arginine	4	3.2	4
Aspartic acid	16.8	15	14
Threonine	2	1.8	2
Serine	6.4	4.7	6
Glutamic acid	6.8	7	5
Proline	0	0	0
Glycine	0	1.4	0
Alanine	10.8	10.3	9
Valine	4	4.1	4
Isoleucine	0.8	0.6	1
Leucine	4.6	3.3	4
Tyrosine	—[c]	—[c]	1
Phenylalanine	0	0	0
Cysteic acid	0	1.1	0
Oxidized cysteinylglycerol	0.5[d]	0.25[d]	(1)
Methionine sulfone	1.4	1.3	2

[a] Amino acid compositions given were averages of a duplicated analysis of each sample.

[b] Based on amino acid sequence of the protein as determined by Braun and Bosch (2).

[c] Tyrosine content in the lipoprotein could not be determined due to the destruction during performic acid oxidation.

[d] An acidic amino acid migrating behind cysteic acid was assumed to be cysteinylglycerol sulfone. The calculation was arbitrarily based on the constant of methionine sulfone standard used in the amino acid analysis.

TABLE 9. *Fatty acid composition of free form of lipoprotein purified from control and starved cells of strain GGD27R2*

Fatty acid	Fatty acid composition (%)			
	Control		Starved	
	Ester linked	Total	Ester linked	Total
C_{14}	2.5	6.6	3	5.4
C_{16}	50.9	78.3	54.9	65
$C_{16:1}$	25.3	6.2	13.4	15.2
C_{17} cyclopropane	3.5		5.2	
$C_{18:1}$	17.8	8.9	23.5	14.4
Total (nmol/nmol of lipoprotein)	1.71	2.48	0.70	1.55

finding that the composition of the ester-linked fatty acids in the lipoprotein closely resembles that of the phospholipids (10). If this is the case, it would follow that the apoprotein lacking the diglyceride moiety might be assembled into the murein sacculus. That this may indeed be the case is shown by the amino acid analysis of performic acid-oxidized bound-form lipoprotein isolated from wild-type strains of *E. coli*. In our studies concerning the characterization of the murein-lipoprotein complexes isolated from *E. coli* K-12 (strain AB1157), *E. coli* B (strain S/6), and various mutants derived from these strains (18), we have repeatedly found varying amounts of cysteic acid in addition to glyceryl-cysteine sulfone (C. V. Sundar Raj and H. C. Wu, unpublished observation). These results were obtained in samples that were virtually devoid of proline, phenylalanine, and histidine, as judged by amino acid analysis, and thus were not due to contaminating proteins. Our finding that the cysteine content in murein-lipoprotein synthesized in glycerol-starved cells was significantly higher than that in the unstarved cells (Table 7) also supports this hypothesis.

Alternatively, one may postulate that either the synthesis of the apoprotein or its assembly into the murein sacculus is preceded by or dependent upon the prior covalent attachment of diglyceride to the sulfhydryl group of cysteine. In both cases, the lesser inhibition of apoprotein synthesis than that of the lipid moiety in the glycerol-starved cells may in turn suggest that preexisting glycerophosphatides may be utilized for the de novo synthesis of murein-lipoprotein.

The results shown in Table 9 may suggest that *N*-acylation of the N-terminal cysteine utilizes, as a source of fatty acid, a different biosynthetic precursor than that utilized as a donor of the diglyceride moiety. The absence of C_{17} or C_{19} cyclopropane fatty acids as amide-linked fatty acid components of lipoprotein (10) does not constitute in itself sufficient evidence against the possibility that both the diglyceride and the amide-linked fatty acids are biosynthetically derived from glycerophosphatides. It is conceivable that the absence of amide-linked cyclopropane fatty acids in the lipoprotein is a consequence of the specificity of the *N*-acyl transferase as well as that of cyclopropane phospholipid synthetase, rather than due to a difference in biosynthetic precursors. Studies aimed at answering these questions are in progress.

Although the present study concerns murein-lipoprotein synthesis in *S. typhimurium*, for which the primary structure has yet to be determined, previous work of Braun et al. (5) has shown that the amino acid composition of the murein-lipoprotein complex of *S. typhimurium* LT-2 is almost identical to that of *E. coli*.

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