JOURNAL OF BACTERIOLOGY, Nov. 1973, p. 1029-1053
Vol. 116, No. 2

Printed in U.S.A.

Mode of Action of Glycine on the Biosynthesis of Peptidoglycan

W. HAMMES, K. H. SCHLEIFER, AND O. KANDLER
Botanical Institute of the University, Munich 19, Menzinger Strasse 67, Germany

Received for publication 9 August 1973

The mechanism of glycine action in growth inhibition was studied on eight different species of bacteria of various genera representing the four most common peptidoglycan types. To inhibit the growth of the different organisms to 80%, glycine concentrations from 0.05 to 1.33 M had to be applied. The inhibited cells showed morphological aberrations. It has been demonstrated that glycine is incorporated into the nucleotide-activated peptidoglycan precursors. The amount of incorporated glycine was equivalent to the decrease in the amount of alanine. With one exception glycine is also incorporated into the peptidoglycan. Studies on the primary structure of both the peptidoglycan precursors and the corresponding peptidoglycan have revealed that glycine can replace L-alanine in position 1 and D-alanine residues in positions 4 and 5 of the peptide subunit. Replacement of L-alanine in position 1 of the peptide subunit together with an accumulation of uridine diphosphate-muramic acid (UDP-MurNAc), indicating an inhibition of the UDP-MurNAc:L-Ala ligase, has been found in three bacteria (*Staphylococcus aureus*, *Lactobacillus cellobiosus* and *L. plantarum*). However, discrimination against precursors with glycine in position 1 in peptidoglycan synthesis has been observed only in *S. aureus*. Replacement of D-alanine residues was most common. It occurred in the peptidoglycan with one exception in all strains studied. In *Corynebacterium* sp., *C. callunae*, *L. plantarum*, and *L. cellobiosus* most of the D-alanine replacing glycine occurs C-terminal in position 4, and in *C. insidiosum* and *S. aureus* glycine is found C-terminal in position 5. It is suggested that the modified peptidoglycan precursors are accumulated by being poor substrates for some of the enzymes involved in peptidoglycan synthesis. Two mechanisms leading to a more loosely cross-linked peptidoglycan and to morphological changes of the cells are considered. First, the accumulation of glycine-containing precursors may lead to a disrupture of the normal balance between peptidoglycan synthesis and controlled enzymatic hydrolysis during growth. Second, the modified glycine-containing precursors may be incorporated. Since these are poor substrates in the transpeptidation reaction, a high percentage of muropeptides remains uncross-linked. The second mechanism may be the more significant in most cases.

The inhibitory effect of glycine on bacterial growth has been known for a long time (5, 6, 12-14, 37, 41, 43, 44). Since the morphological effects of glycine are similar to those of penicillin, i.e., cell elongation and spheroplast formation, it has been assumed that the cell wall is the main site of glycine action. Strominger and Birge (38) studied the glycine effect in *Staphylococcus aureus* and demonstrated the accumulation of uridine diphosphate-*N*-acetyl muramic acid (UDP-MurNAc) and other cell wall precursors in which L-alanine was replaced by glycine. A more detailed study of the inhibition of the L-alanine-adding enzyme by glycine was carried out by Hishinuma et al. (15). Both authors propose that growth inhibition is at least partly due to inhibition of the "L-Ala-adding-enzyme". Neither of the two authors have investigated whether glycine-containing precursors are incorporated into the peptidoglycan.

Preliminary experiments of Stern (Diplom Thesis; Technical University München, 1971) have shown that the peptidoglycan of several lactobacilli grown under high glycine concentrations (1 to 10%) contains glycine, whereas the

amount of alanine was reduced. Some of the glycine residues obviously replaced L-alanine residues, as shown by a decrease of the amount of L-alanine and the occurrence of the peptides Gly-D-Glu and muramyl-glycine (Mur-Gly), in addition to L-Ala-D-Glu and Mur-L-Ala in the partial acid hydrolysates of the cell walls. The amount of glycine was significantly higher, however, than the decrease of L-alanine. This indicates that glycine residues are incorporated in some additional positions of the peptidoglycan. The experiments described in this paper have demonstrated that glycine replaces not only L-alanine but also D-alanine and that it is the latter replacement that causes growth inhibition and morphological aberrations.

MATERIALS AND METHODS

Organisms. The organisms used in this study are listed in Table 1. *Corynebacterium* sp. was isolated in our laboratory from an airborne contamination. *Staphylococcus aureus* strain Copenhagen was obtained from J. M. Ghuysen, Liege, Belgium. *Bacillus subtilis* W23 was provided by A. R. Archibald, Newcastle upon Tyne, England; and *Lactobacillus plantarum* NIZO 3610 was provided by the Nederland Instituut voor Zuivelonderzoek, Ede, The Netherlands.

Growth conditions. Lactobacilli were grown in MRS-medium (20); all the other strains were grown in yeast extract glucose broth (23) containing 0.5% sodium chloride. Concentrated glycine solutions were autoclaved separately and added to the medium in necessary amounts under sterile conditions. The organisms were grown at 30 C (*Corynebacterium* sp., *C. callunae*, *C. insidiosum*, *C. mediolanum*, and *L. plantarum*) or at 37 C (*B. subtilis*, *L. cellobiosus*, and *S. aureus*) either aerobically on a shaker or under conditions favorable to microaerophilic growth (only lactobacilli). The uniformity of the cultures was checked by phase-contrast microscopy and by dilution on agar plates. Growth was followed by measuring the optical density at 578 nm.

Determination of the glycine sensitivity. A series of flasks containing 30 ml of medium and increasing concentration of glycine was inoculated with 0.1 ml of an overnight grown culture. The optical density was measured at 578 nm after 24 and 48 h of growth. The percentage of inhibition was calculated in comparison to the growth of a noninhibited sample.

Electron microscopy. Samples were prefixed in 0.1% OsO_4 and then fixed in 1% OsO_4 by the method of Ryter et al. (33). After dehydration in ethanol and propylene oxide, they were embedded in Durcupan ACM (Fluka, Buchs, Switzerland) and sectioned on a ultratom III microtome (LKB, Stockholm, Sweden). The sections were examined in a Zeiss electron microscope (EM 9A).

Preparation of cell walls. Cell walls were prepared in the usual way (35). Before disruption the cells were suspended in 50 to 100 ml of distilled water and boiled for 10 min. This procedure was employed to inactivate the autolytic enzymes and to extract, at the same time, the nucleotide-activated precursors. The cells were disintegrated with glass beads in a cell mill (Bühler, Tübingen, BRD). The crude cell walls were treated with trypsin to digest proteins. Cell walls containing teichoic acid were suspended in 0.1 M boric acid-NaOH buffer (pH 9.0, 20 C) for 1 h to remove ester-linked alanine residues. The cell walls were thoroughly washed and freeze-dried.

Analytical procedure. For determination of amino acids and amino sugars, the cell walls were completely hydrolyzed in 4 N HCl (0.1 ml/mg cell wall) at 100 C in sealed tubes for 16 h. To get complete hydrolysis of δ-(aminosuccinyl)-ornithine, the cell walls of *L. cellobiosus* were hydrolyzed in 6 N HCl at 125 C for 16

TABLE 1. *Sensitivity of the various bacteria toward glycine*

Species	80% inhibition of growth by X% glycine	Morphology	Peptidoglycan type
L. plantarum NIZO 3610	3	Elongation and deformations	meso-Dpm-direct
B. subtilis W 23	0.4	Elongated cells	meso-Dpm-direct
Corynebacterium sp.	4	Elongation and spheroplast formation	meso-Dpm-direct
C. callunae ATCC 15991	7	Coccoid cells and spheroplast formation	meso-Dpm-direct
L. cellobiosus ATCC 11739	10	Elongated and swollen cells	L-Orn-D-Asp
S. aureus Copenhagen	5	Irregular size	L-Lys-$(Gly)_{5-6}$
C. mediolanum ATCC 14004	1.0	Coccoid cells and spheroplast formation	(L-Dab)-D-Glu-D-Dab
C. insidiosum ATCC 10253	0.55	Coccoid cells and spheroplast formation	

h. Under these conditions the amino sugars were degraded, and therefore the values for the amino sugars had to be calculated from 4 N HCl hydrolysates of cell walls. Dinitrophenylated cell walls and peptides were hydrolyzed in 6 N HCl at 100 C for 6 h. Quantitative amino acid and amino sugar determinations were carried out with an automatic amino acid analyzer (Beckman 120 C). Partial acid hydrolysis was performed in 4 N HCl at 100 C. Cell walls were usually hydrolyzed for 45 min; nucleotide-activated precursors and muropeptides(peptidoglycan fragments, 42) were hydrolyzed from lysozyme lysates for only 10 min. Isolation and identification of peptides in the partial acid hydrolysate of cell walls were carried out as described earlier (35, 36). The following solvent systems were employed for the separation of amino acids, amino sugars, and peptides by paper chromatography: (i) isopropanol-acetic acid-water (75:10:15); (ii) α-picoline-25% NH_4OH-water (70:2:28); (iii) n-butanol-propionic acid-water, (750:352:498). Ninhydrin was used as spray reagent.

N-terminal amino acids were determined by dinitrophenylation. Cell walls were dinitrophenylated by the method of Takebe (39); peptides, muropeptides, and precursors were dinitrophenylated by the method of Ghuysen et al. (10). Total amino groups were also measured after dinitrophenylation (11). Mono-dinitrophenyl (DNP) L diamino acids were prepared as follows. An 80-mg amount of diamino acid was dissolved in 20 ml of 2.5% of a sodium bicarbonate solution; 40 ml of ethanol and 0.073 ml of 1-fluoro-2,4-dinitrobenzene (FDNB) were added; and the solution was incubated at 50 C for 30 min. Ethanol was removed in a stream of warm air, and unreacted FDNB was extracted with ether. The aqueous phase was acidified with 2 ml of 4 N HCl. Di-DNP-diamino acids were extracted with ethyl acetate, and mono-DNP-diamino acids were extracted with a mixture of ethyl acetate and n-butanol (1:1, vol/vol). The DNP-amino acids were separated and identified by thin-layer chromatography on silica gel in the following solvent systems: (iv) benzene-acetic acid (8:2); (v) chloroform-methanol-acetic acid (95:5:1); (vi) chloroform-methanol-acetic acid-water (65:25:13:8). Quantitative determinations of these derivatives were carried out photometrically (30) and by comparison of the amino acid content of untreated and dinitrophenylated samples. C-terminal amino acids were determined after hydrazinolysis. Anhydrous hydrazine was prepared by the method of Fraenkel-Conrat and Tsung (8). Samples containing 0.3 to 0.4 μmol of each C-terminal amino acid were dried in tubes. A 0.3-ml amount of anhydrous hydrazine was added and the tube was immediately sealed and incubated at 80 C for 48 h. The excess reagent was removed in vacuo over concentrated H_2SO_4. The dried samples were dissolved in 0.3 ml of an aqueous norleucine solution (0.1 μmol/0.3 ml). Norleucine was employed as an internal standard. Redistilled benzaldehyde (0.05 ml) was added, and the tubes were thoroughly mixed for 10 s at 5-min intervals for 1 h. The emulsion was centrifuged at 12,000 $\times$ g for 5 min, and the clear supernatant fluid was treated with benzaldehyde in the same way. The supernatant fraction was then extracted twice with 0.3 ml of ether and finally dried in vacuo. The amount of C-terminal amino acid was determined by the amino acid analyzer or by photometric measurement of the DNP-derivative; the hydrazinolytic procedure was standardized by using cell walls of *Micrococcus luteus* (*lysodeikticus*), in which all of the glycine residues are C-terminal, and authentic peptides Ala-Gly, Gly-Ala, and Gly-Lys. It was established that the hydrazinolysis of cell walls is not as effective as that of soluble peptides. Yields of 65 to 68% of glycine were obtained in the case of cell walls of *M. luteus*. The peptides have given yields of 80 to 85% of the theoretical amount of C-terminal amino acid. These figures were used as correction factors. The quantitative determination of L- and D-alanine was performed by the method of Larson et al. (18), L-valine used as internal standard. Total *N*-acetylamino sugars were determined by the modified Morgan-Elson procedure of Reissig et al. (31).

Isolation of muropeptides. A 2-g amount of purified cell wall was suspended in 200 ml of 0.1 M ammonium acetate buffer (pH 6.2), and 200 mg of hen egg white lysozyme (Boehringer, Mannheim) was added. The suspension was incubated at 37 C for 20 min and then centrifuged at 35,000 $\times$ g for 45 min. The clear supernatant fluid (lysate) was concentrated under reduced pressure to a final volume of about 10 ml. Separation of the lysate was carried out by gel filtration on two columns (80 by 2.5 cm): Sephadex G-50 (fine) and Sephadex G-25 (fine), connected in series with a flow rate of 20 ml/h at 20 C. For each separation only 1.0 ml of the concentrated lysate was applied and eluted with distilled water. The low-molecular-weight fraction was further separated by preparative chromatography on Whatman paper 3 MM in the solvent system vii: n-butanol-acetic acid-water (62:25:25).

Isolation of nucleotide-activated peptidoglycan precursors. Nucleotide-activated precursors were either extracted by boiling water or by extraction with 25% (wt/vol) trichloroacetic acid at 4 C for 30 min (2). Trichloroacetic acid was extracted with ether, and the aqueous phase was neutralized, concentrated under reduced pressure at 20 C, and applied to a column (80 by 2.5 cm) of Sephadex G-25 by the procedure of Rosenthal and Sharon (32). The elution was carried out with distilled water in a cold room (4 C) at a flow rate of 20 ml/h. Fractions absorbing at 260 nm and containing bound *N*-acetylamino sugar (31) were pooled, concentrated, and separated by chromatography on Whatman no. 3 MM paper in solvent system viii: isobutyric acid-25% NH_4OH-water (250:5:145). The nucleotide-activated precursors were observed on chromatograms by ultraviolet (UV) absorption. The UV-absorbing spots were eluted and rechromatographed in solvent system ix: ethanol-1 M ammonium acetate (7:3).

RESULTS

Growth inhibition and morphological changes. The investigation was carried out with organisms which contain peptidoglycan of the four most common types (36). Fragments of the primary structure of these four types and the abbreviations employed in this paper are

given in Fig. 1. The meso-diaminopimelic (Dpm)-direct type is represented by four strains, and the [L-Dab]D-Glu-D-Dab (Dab, diaminobutyric acid) type is represented by two strains with different sensitivity toward glycine. The sensitivity toward glycine of the investigated strains, their morphological response toward the inhibition and their peptidoglycan type are summarized in Table 1.

The morphological effects were similar in all strains. There is an elongation accompanied by swelling of the cells (Fig. 2). The formation of spheroplasts is frequent in some and rare in other strains. In case of *S. aureus*, little change is seen. The cocci become more or less irregular in size, however.

Electron microscope investigations were carried out on ultrathin sections of normal and glycine-inhibited cells of *L. cellobiosus*. We observed that glycine inhibition leads to swollen cells with a very loose cell wall (Fig. 3 and 4). Additional layers are seen, and the outermost layer is disintegrating. It is obvious that the cell wall is heavily affected by glycine.

Effect of glycine on the primary structure of the peptidoglycan and of peptidoglycan precursors. To elucidate the mechanism of the glycine inhibition, the primary structure of the peptidoglycan of glycine inhibited cells and that of the peptidoglycan precursors accumulating during glycine inhibition were determined.

L. cellobiosus (L-Orn-D-Asp-type): primary

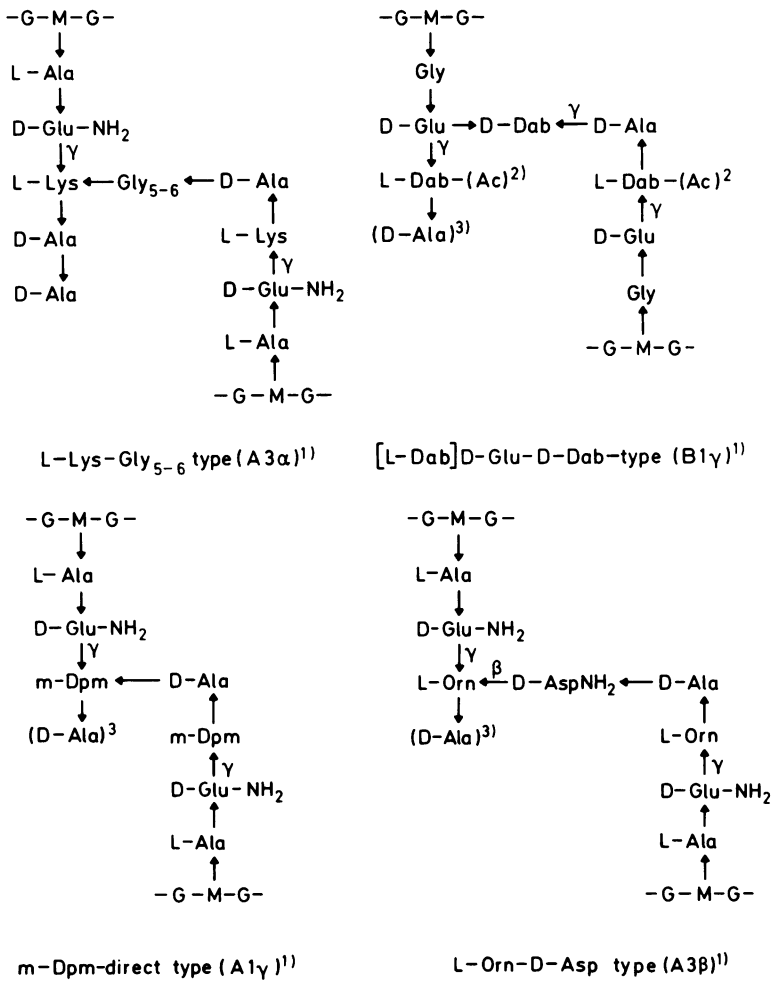

FIG. 1. *Fragments of the primary structure of the four different types of peptidoglycan. (1) Abbreviations according to Schleifer and Kandler (36). (2) Only present in C. insidiosum, not in C. mediolanum. (3) Absent in a certain percentage of the peptide subunits, when the diamino acid is C-terminal. G, N-acetylglucosamine; M, N-acetyl muramic acid; m-Dpm, meso-2,6-diaminopimelic acid.*

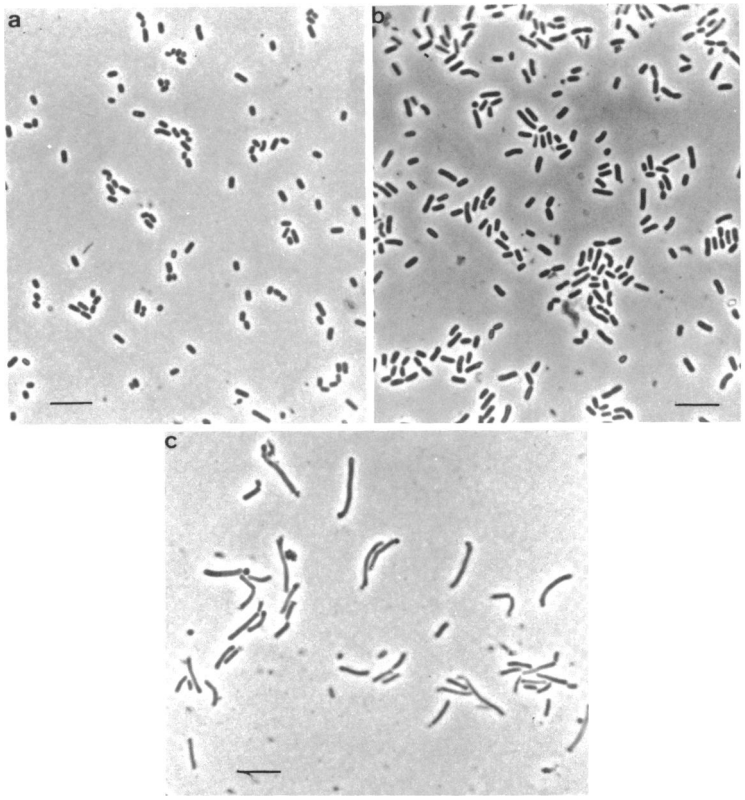

FIG. 2. *Phase-contrast microscope photographs of L. cellobiosus grown without (a) and with 4% (b) or 12% (c) glycine. Bar indicates 10 μm (from G. Stern diplom thesis, Technical University, München, 1970).*

structure of peptidoglycan. Cell walls of *L. cellobiosus* grown under either normal conditions or in the presence of 8% glycine were prepared. The results of the quantitative amino acid and amino sugar composition, the configuration of the amino acids, and the amounts of C- and N-terminal residues are given in Table 2. The main differences between the control and the inhibited sample are the following. (i) The peptidoglycan of the inhibited cells contains 0.71 mol of glycine per mol of glutamic acid, whereas the amount of alanine is decreased. The sum of alanine and glycine is equal to the amount of alanine in the control cells. (ii) The amount of aspartic acid is decreased by about 20% in the inhibited sample, and a corresponding increase of unsubstituted ω-amino groups of ornithine and lysine is found. (iii) Ornithine is the only diamino acid in the peptidoglycan of the control cells, whereas the inhibited sample contains small but significant amounts of lysine. The sum of ornithine and lysine is equal to the amount of ornithine in the control sample. (iv) The calculation of the extent of cross-linkage, based either on the amounts of C-terminal or N-terminal residues, shows a decrease of cross-linkage in the inhibited sample by about 30%.

The location of glycine within the peptidoglycan is indicated by the fact that not only is the amount of L-alanine decreased by about 50%, but also that of D-alanine by 30%. Therefore glycine is expected to replace half (0.5 mol) of the L-alanine residues in position 1 and ⅓ (0.21 mol) of D-alanine residues in position 4 of the peptide subunit. In fact, 0.18 mol of glycine was found to be C-terminal in the inhibited sample, whereas the amount of C-terminal alanine and diamino acids (Orn + Lys) did not change. This means that the amount of tetrapeptide subunits with C-terminal alanine is unchanged, but most of the peptide subunits containing glycine residues in position 4 remain uncross-linked. Only an insignificant amount of glycine may be involved in cross-linkages. Figure 5 shows the two-dimensional paper chromatograms of partial acid hydrolysates of control and inhibited samples. The replacement of L-alanine in position 1 of the peptide subunit is demonstrated by the occurrence of the peptides Mur-Gly and

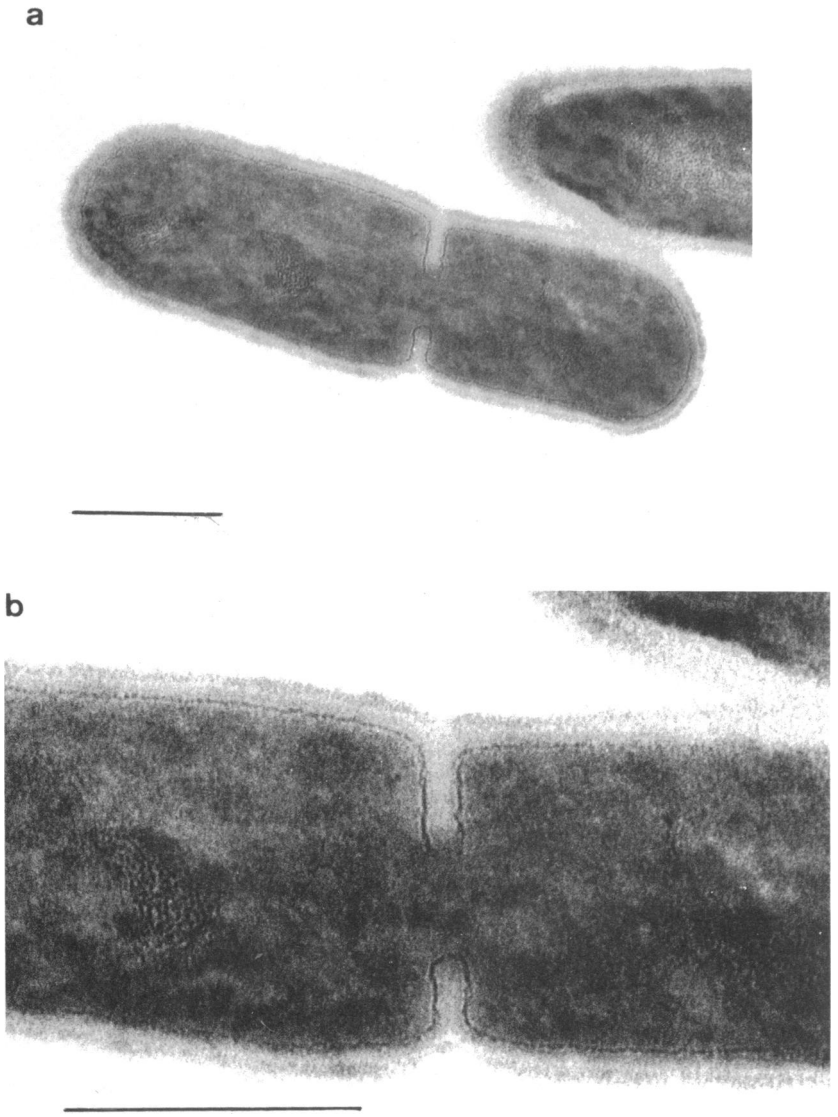

FIG. 3. (a) *Electron micrograph of an ultrathin section of L. cellobiosus grown in MRS medium. Bar indicates 0.5 μm.* (b) *Detail of Fig. 3a. Bar indicates 0.5 μm.*

Gly-D-Glu in the hydrolysate of inhibited cells. The expected peptide Orn-Gly was not found. Close to the origin a mixture of peptides was located (no. 16) which was difficult to separate. They contained all amino acids present in the peptidoglycan, and some of them also contained muramic acid. Unlike earlier studies of this peptidoglycan type (26, 27), δ-(aminosuccinyl)-ornithine was present only in traces, whereas the corresponding acyclic peptide N^{δ}-β-D-Asp-L-Orn appeared in fair amounts. To get direct evidence for the replacement of D-alanine by glycine, we investigated the muropeptides of a lysozyme lysate. In agreement with the much poorer cross-linkage of walls from cells grown in the presence of 1.1 M glycine, the lysis by lysozyme proceeds much faster and more completely than in the control (Fig. 6). The lysate of 2 g of cell walls of the inhibited sample was separated by gel filtration on combined columns of Sephadex G25 and G50. The fraction F (Fig. 7), containing the highest amount of *N*-acetylglucosamine derivatives of relative low molecular weight, was further separated by one-dimen-

sional paper chromatography. Although some ninhydrin-positive material remained close to the origin (Fig. 8), most material moved further and formed four poorly separated bands (F2–F5). They were eluted and rechromatographed, but a complete separation was not achieved.

The molar ratio of amino acids and the relative amounts of C- and N-terminal amino acids of the four fractions are given in Table 3. The slower-moving fraction (F2) contains obviously a mixture of dimers since only 0.5 mol of both of C- and N-terminal amino acids is present. The other poorly separated fractions contain mixtures of monomers which differ in their content of lysine. The slow-moving fraction F3 contains almost exclusively ornithine, whereas the dominant diamino acid in the fast-moving fraction F5 is lysine.

Partial acid hydrolysates were prepared from all fractions. A scheme of the two-dimensional paper chromatogram of the hydrolysate of F2 is shown in Fig. 9. It is very similar to that of total cell walls (Fig. 5). Since less material remained

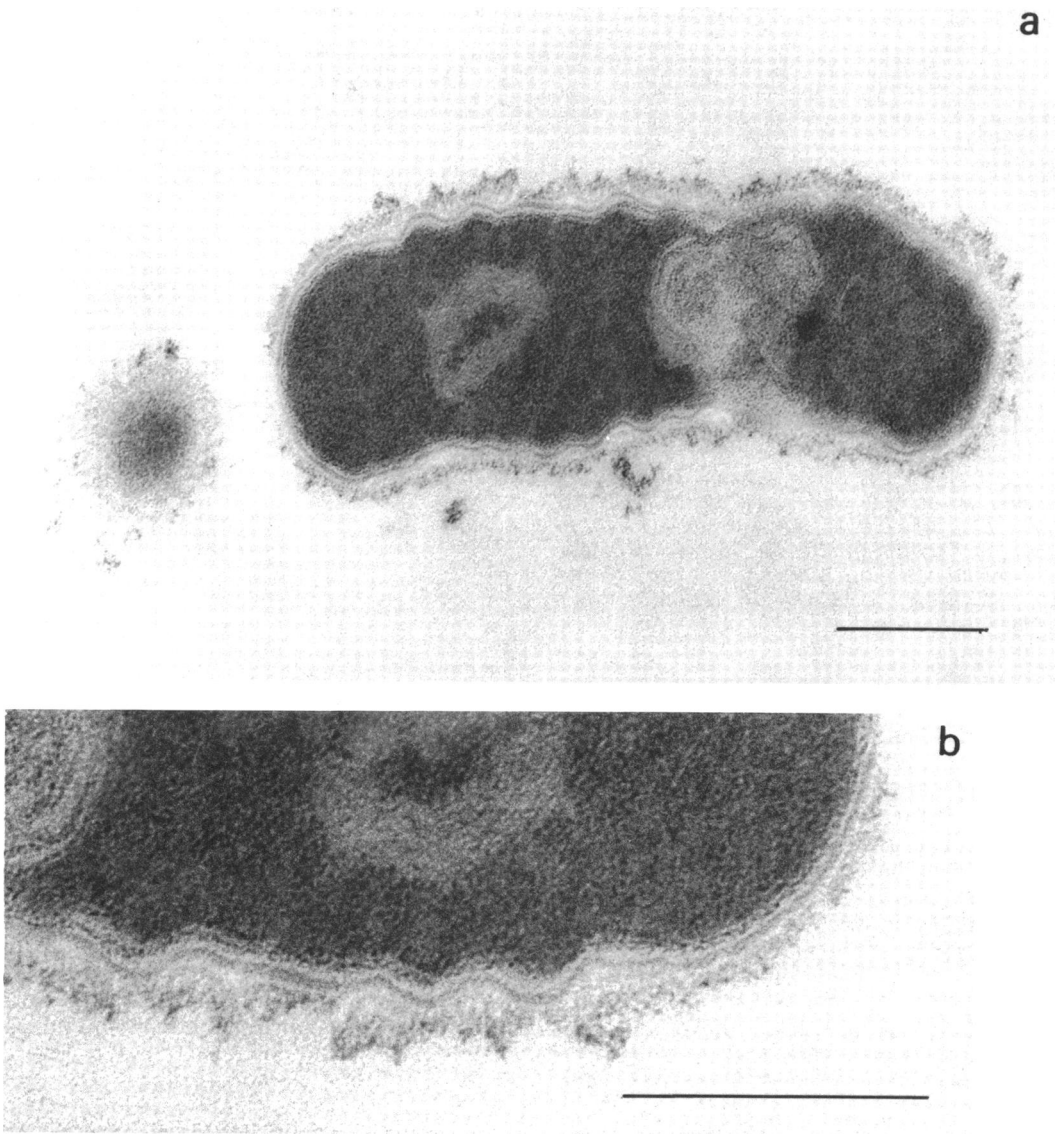

FIG. 4. *(a) Electron micrograph of an ultrathin section of L. cellobiosus grown in MRS medium containing 12% glycine. Bar indicates 0.5 μm. (b) Detail of Fig. 4a. Bar indicates 0.5 μm.*

TABLE 2. *Molar ratio of the amino sugars and amino acids of the peptidoglycan of L. cellobiosus*[a]

Growth conditions	Determination	Mur	$GlcNH_2$	Glu	Ala	Gly	Lys	Orn	Asp	L-Ala	D-Ala	Cross-linkage (%)
Normal	Molar ratio	1.03	1.13	1.0	1.61	—	—	0.96	0.89	0.99	0.61	
	C-terminal	—	—	—	0.06*	—	—	0.37	—	ND	ND	57
	N-terminal	—	—	—	—	—	—	0.07	0.37	ND	ND	
8% Glycine added	Molar ratio	1.18	0.99	1.0	0.91	0.71	0.14	0.81	0.67	0.50	0.41	
	C-terminal	—	—	—	0.06*	0.18	0.08	0.29	—	ND	ND	40
	N-terminal	—	—	—	—	—	0.04	0.23	0.31	—	—	

[a] The numbers of the N- and C-terminal amino acids are also based on the amount of D-glutamic acid of the peptidoglycan. Symbols: ND, not determined; *, configuration of the C-terminal alanine was not investigated.

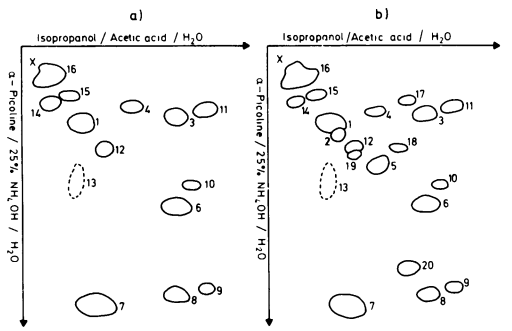

FIG. 5. *Scheme of a two-dimensional paper chromatogram of a partial acid hydrolysate of cell walls of L. cellobiosus. (a) Control sample; (b) inhibited sample. 1, Orn; 2, Lys; 3, Glu; 4, Asp; 5, Gly; 6, Ala; 7, $GlcNH_2$; 8, Mur; 9, Mur-L-Ala; 10, Mur-L-Ala-D-Glu; 11, L-Ala-D-Glu; 12, L-Orn-D-Ala; 13, δ-(aminosuccinyl)-ornithine; 14, N^5-β-D-Asp-L-Orn; 15, γ-D-Glu-L-Orn; 16, unidentified tri- and tetrapeptides; 17, Gly-D-Glu; 18, Mur-Gly-D-Glu; 19, L-Lys-D-Ala; 20, Mur-Gly.*

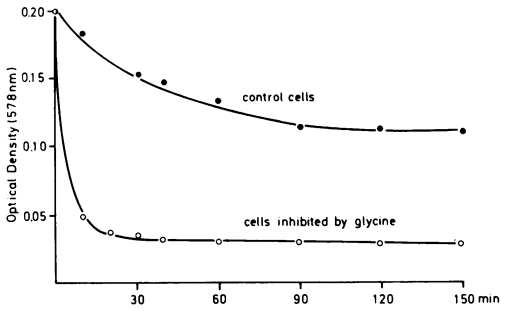

FIG. 6. *Lysis of cell walls of L. cellobiosus by lysozyme. Cells were suspended in 0.1 M ammonium acetate buffer (pH 6.2), and 200 μg lysozyme was added per ml suspension.*

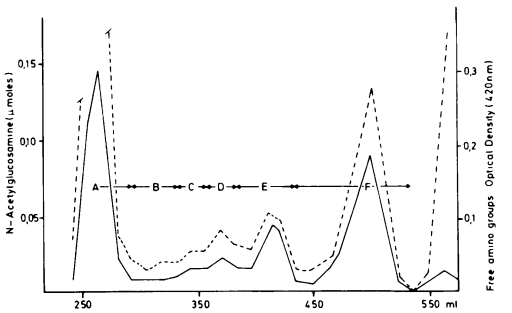

FIG. 7. *Elution profile of muropeptides from a lysozyme digest of cell walls of L. cellobiosus inhibited by glycine. Solid line, N-acetylglucosamine; broken line, free amino groups. For further details see Material and Methods.*

close to the origin of the chromatogram, it was possible to purify spot no. 21 by repeated one-dimensional paper chromatography. It contained Glu, Gly, Orn in a molar ratio of 1:1:1. In the hydrolysate of the dinitrophenylated peptides DNP-Glu, DNP-Gly, and δ-DNP-Orn were found in a molar ratio of approximately 1:1:2. This indicates the presence of a mixture of Gly-γ-D-Glu-L-Orn and γ-D-Glu-L-Orn-Gly. Unfortunately it was not possible to separate the two peptides. The most likely structures of the four fractions compatible with the data of Table 3 and the peptides found in the partial acid hydrolysates are shown in Fig. 10a. The dimers of fraction 2 consist predominantly of two tetrapeptides cross-linked by isoasparagine. About ⅓ of the peptide subunits, carrying a C-terminal amino acid, are existent as tripeptides with a C-terminal L-Orn instead of a C-terminal Gly. About 30% of the dimers contain a second mole of N-terminal isoasparagine. The position of alanine could not be determined exactly. Since L-Ala-D-Glu and L-Orn-D-Ala are found, the 0.2 mol of alanine residues per mol of glutamic acid must be located partly in position 1 and in position 4, as indicated in Fig. 10. In most dimers the cross-linkage occurs via gly-

cine, although in the total cell wall this is a relatively rare case. For unknown reason the glycine-containing dimers moved a little slower on the Sephadex column and were predominantly collected together with the bulk of monomers.

Fraction F3 is obviously a mixture of monomers, since 1 mol of both of C- and N-terminal amino acids per mol of glutamic acid is found. It may be composed of tetrapeptides, as depicted in Fig. 10b and c, since the tripeptides are predominantly found in the faster-moving fractions F4 and F5. In addition, C-terminal ornithine and N-terminal isoasparagine are present in equal amounts. However, the occurrence of tripeptides in fraction F3 cannot completely be ruled out. The partial acid hydrolysate showed qualitatively the same peptide pattern as that of fraction F2, which is compatible with the structures shown in Fig. 10b and c. Fraction F4 and F5 are mixtures of tri- and tetrapeptides shown in Fig. 10d, e, f, and g. The lysine-containing tripeptide is obviously the fastest-moving component since it dominates in F5. The chromatograms of the partial acid hydrolysates showed the same peptide pattern as that of F2, but the aspartic acid-containing peptides and L-Orn-D-Ala were missing. The analysis of the muropeptides of the lysozyme lysate is in agreement with the conclusion drawn from the data of the total cell wall. It proved the occurrence of glycine in position 1 and 4 of the peptide subunit and the presence of many monomers which contain no aspartic acid.

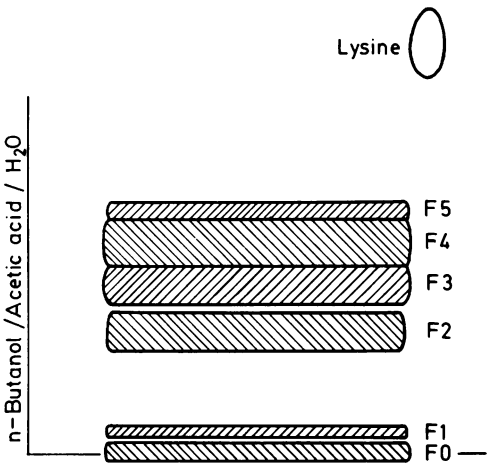

FIG. 8. *Scheme of the paper chromatographic separation of muropeptides of fraction F from the lysozyme lysate.*

Accumulation and structure of the precursors. The accumulation of precursors at the beginning was about the same for all glycine concentrations, but stopped early in the case of 12% glycine (Fig. 11). The maximal increase of the precursors in comparison with the control was only twofold. This is, by comparison with a 10-fold increase in *S. aureus* (14), a relatively poor accumulation. After separation by gel filtration and paper chromatography in two solvent systems, five UV-absorbing fractions were obtained. The results of their analyses are

TABLE 3. *Molar ratio of amino sugars and amino acids of 4 fractions of the lysozyme lysate of cell walls of L. cellobiosus grown in the presence of 8% glycine*[a]

Fraction	Determinations	Mur	$GlcNH_2$	Glu	Ala	Gly	Lys	Orn	Asp	Remarks
F 2[b]	Molar ratio	1.1	0.96	1.0	0.20	1.69	0.05	0.94	0.68	Mixture of
	N-terminal	—	—	—	—	—	0.05	0.33	0.14	dimers
	C-terminal	—	—	—	—	0.33	—	0.17	—	
F 3[b]	Molar ratio	1.1	0.95	1.0	0.40	1.15	0.08	0.86	0.39	Mixture of monomers
	N-terminal	—	—	—	—	—	0.04	0.5	0.39	(tetrapeptides)
	C-terminal	—	—	—	0.06	0.55	0.03	0.37	—	
F 4[b]	Molar ratio	1.04	0.97	1.0	0.76	0.60	0.21	0.76	—	Mixture of monomers
	N-terminal	—	—	—	—	—	0.21	0.71	—	(tri- and tetrapep-
	C-terminal	—	—	—	0.03	0.34	0.15	0.48	—	tides)
F 5[b]	Molar ratio	0.98	0.89	1.0	0.91	0.39	0.61	0.38	—	Mixtures of monomers
	N-terminal	—	—	—	—	—	0.56	0.25	—	(tri- and tetrapep-
	C-terminal	—	—	—	0.10	0.16	0.61	0.11	—	tides)

[a] The N- and C-terminal groups are also based on the amount of D-glutamic acid.

[b] Based on the content of glutamic acid, the following amounts were obtained: F 2, 6.6 μmol; F 3, 32.8 μmol; F 4, 28.5 μmol; F 5, 18 μmol.

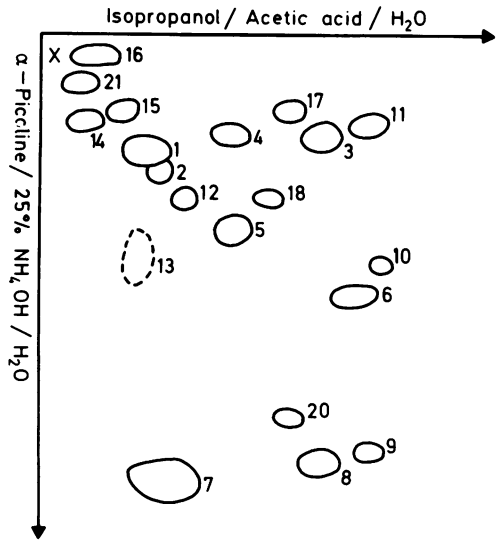

FIG. 9. *Scheme of a two dimensional paper chromatogram of a partial acid hydrolysate of F 2. For 1 to 20 see Fig. 5; 21, Gly-γ-D-Glu-L-Orn and γ-D-Glu-L-Orn-Gly.*

summarized in Table 4. The two UDP-activated amino sugars were present in large amounts. The incomplete precursors UDP-MurNAc-Gly and UDP-MurNAc-L-Ala were found in small amounts, whereas the UDP-MurNAc-tetrapeptides were the compounds accumulated in largest amount. The C-terminal amino acid of the tetrapeptides is glycine, whereas position 1 is taken either by glycine or L-alanine, and in position 3 L-ornithine is partly replaced by L-lysine. The partial acid hydrolysate of the fraction of tetrapeptides yielded L-Ala-D-Glu, Gly-D-Glu, L-Orn(L-Lys)-Gly, and γ-D-Glu-L-Orn(L-Lys), but no L-Orn-D-Ala, Gly-D-Ala, or D-Ala-Gly. This shows clearly that no pentapeptide precursor was present in this fraction. According to the known mechanism of biosynthesis of peptidoglycan, there has to be a pentapeptide precursor formed, otherwise no transpeptidation could take place. We assume that the concentration of this precursor was too low to be detected by our procedure.

The occurrence of a tetrapeptide precursor is quite unusual. So far UDP-MurNAc-tetrapeptides were only found by Anwar et al. (1) in *Aerobacter cloacae* and by Plapp and Kandler (25) in *L. cellobiosus*. The latter authors showed (28) that the normal pentapeptide precursor also occurs in *L. cellobiosus*. We tried to demonstrate the accumulation of the pentapeptide precursor by penicillin, but we found, besides small amounts of tripeptide, only the tetrapeptide precursor UDP-MurNAc-L-Ala-γ-D-Glu-L-Orn-D-Ala (Table 5). Even in non-inhibited cells only tetrapeptides were found. The UDP-MurNAc-tetrapeptides may have arisen from pentapeptides by the action of a DD-carboxypeptidase described by Izaki et al. (16, 17) in *E. coli* as carboxypeptidase I. This enzyme exhibits a high affinity to UDP-activated precursors but acts only poorly on peptidoglycan. Another hypothetical possibility for the synthe-

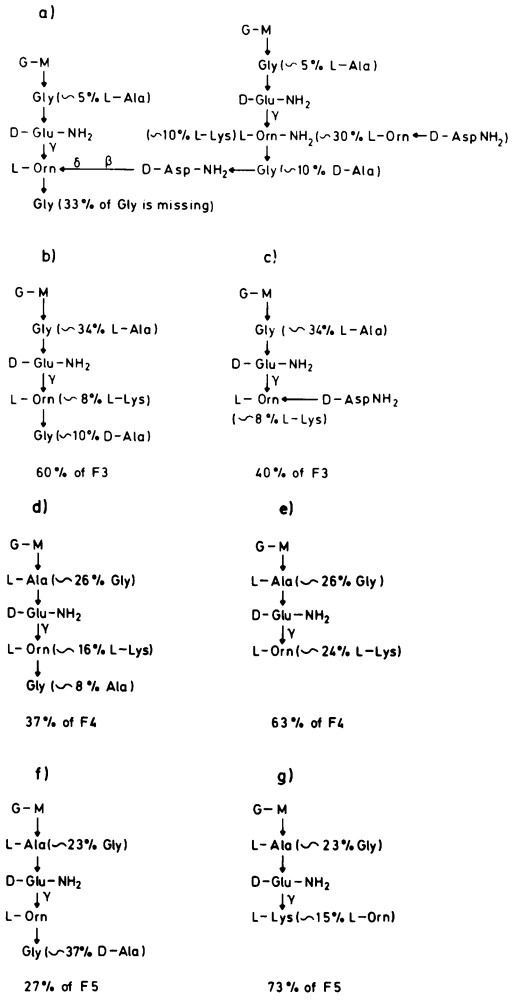

FIG. 10. *Suggested primary structures of the muropeptides (a to g) present in the various fractions. Although the amount of ammonia could not be exactly determined since the eluates from the paper chromatograms contained additional free ammonia, the amides of glutamic acid and aspartic acid are included in the formula based on the present knowledge of the primary structure of the peptidoglycan of L. cellobiosus (Fig. 1).*

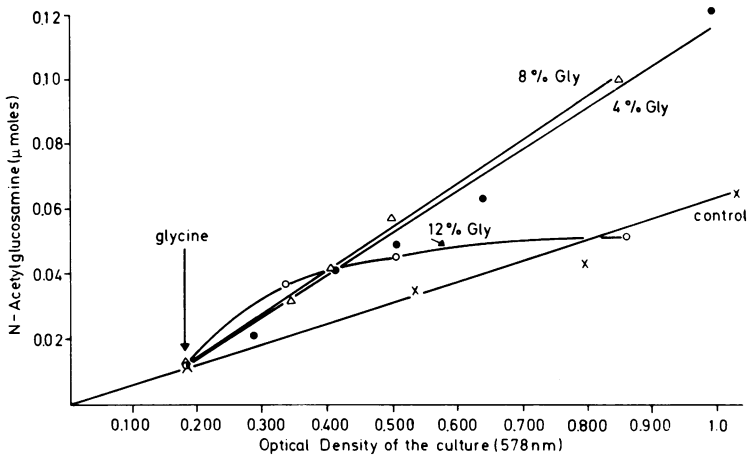

FIG. 11. *Accumulation of N-acetylamino sugars and their derivatives in cells of L. cellobiosus during growth with and without inhibiting concentrations of glycine.*

TABLE 4. *Amount and molar ratio of the amino acids of peptidoglycan precursors of L. cellobiosus grown in 10-liters of medium and inhibited by 4% glycine*[a]

Fraction	μmol	Mur	$GlcNH_2$	L-Ala[b]	Glu	Lys	Gly	Orn	C-terminal Gly	Precursors
I	8.15	—	+	—	—	—	—	—	—	UDP-GlcNAc
II	14.00	+	—	—	—	—	—	—	—	UDP-MurNAc
III	0.90	1.0	—	—	—	—	1.0	—	—	UDP-MurNAc-Gly
IV	0.20	1.0	—	1.0	—	—	—	—	—	UDP-MurNAc-L-Ala
V	26.00	1.0	—	0.5	1.0	0.15	1.5	0.85	1.0	UDP-MurNAc-Gly(L-Ala)-γ-D-Glu-L-Orn(L-Lys)-Gly

[a] When only one compound is present a cross instead of a number is given.
[b] D-Alanine was not present.

sis of tetrapeptide precursors is a direct synthesis from tripeptides by adding only one D-alanine or glycine residue instead of the dipeptide. However, such a reaction is not described so far. The inhibition of peptidoglycan synthesis in *L. cellobiosus* by glycine is most likely related to the replacement of D-alanine by glycine, leading to the accumulation of tetrapeptides with C-terminal glycine, which may be incorporated into the peptidoglycan either after or before substitution with aspartic acid. This would result in a low rate of cross-linkage and therefore in a less rigid cell wall.

Effect of glycine on the m-Dpm-direct type of peptidoglycan. Strains with very different sensitivity toward glycine (Table 1) were used to study the effect of glycine on peptidoglycan of the meso-Dpm-direct type. The results of the determination of the amino acid composition of cell walls from normally grown and inhibited cells are summarized in Table 6. The amount of glycine incorporated per mole of glutamic acid varies between 0.15 in the most sensitive organism (*B. subtilis*) and 0.73 in *L. plantarum*. The molar ratio of D-alanine is decreased in all strains, whereas that of L-alanine is significantly lower only in *L. plantarum*. Correspondingly, only in the partial acid hydrolysate of cell walls of *L. plantarum* significant amounts of the peptides Mur-Gly and Gly-D-Glu were found (Fig. 12), whereas meso-Dpm-Gly was found in the partial acid hydrolysate of all strains.

Although most of the glycine residues replacing D-alanine residues are C-terminal, some glycine residues are involved in cross-linkages. This is directly shown by the occurrence of the peptide Gly-meso-Dpm in the partial acid hydrolysate. It also follows from the fact that not all of the glycine residues which replace D-alanine residues are C-terminal.

Peptidoglycan precursors were isolated and analyzed from three strains (Table 7). The difference between *L. plantarum* and the other organisms is again evident. In *Corynebacterium*

TABLE 5. *Molar ratio of amino acids of peptidoglycan precursors of penicillin (0.5 U/ml) inhibited cells of L. cellobiosus*

Fraction	Mur	Ala	Glu	Orn	C-terminal Ala	Precursors
I	1.1	1.34	1.00	0.95	—	UDP-MurNAc-L-Ala-γ-D-Glu-L-Orn
II	1.2	1.96	1.00	0.96	1.0	UDP-MurNAc-L-Ala-γ-D-Glu-L-Orn-D-Ala

sp. and *B. subtilis* very little UDP-MurNAc, but 12 or 23 times more UDP-MurNAc-pentapeptide, was found. All the glycine residues were located in position 4 and 5. The structures of the precursors given in Fig. 13 are compatible with the data in Table 7, as well as with the peptide pattern found in the partial acid hydrolysates. Since neither D-Ala-D-Ala nor Gly-Gly could be detected (the position of both dipeptides on the chromatogram is well known), the terminal D-Ala-D-Ala is completely replaced by the mixed dipeptides Gly-D-Ala and D-Ala-Gly, both present in the partial acid hydrolysate. Only traces of Gly-D-Glu were seen. Therefore, trace amounts of a third species of precursor containing a glycine residue in position 1 may be present in the mixture of pentapeptides. Also in the total cell walls only traces of L-alanine are replaced by glycine. The "L-alanine-adding" enzyme seems in vivo not to be significantly inhibited by glycine, since no accumulation of UDP-MurNAc occurs and almost no L-alanine residues are replaced by glycine residues. The harmful action of glycine should, therefore, be the formation of modified UDP-MurNAc-pentapeptides containing glycine in position 4 or 5. They are incorporated into the peptidoglycan, but the peptide subunits containing glycine are less cross-linked as indicated by the predominant occurrence of glycine on the C-terminus. This is most likely also true for *C. callunae*. In contrast to the two organisms mentioned before, UDP-MurNAc and UDP-MurNAc-Gly are accumulated in *L. plantarum*. Also UDP-MurNAc-tripeptides are found. Most of them contain glycine residues, and only ¼ contain L-alanine residues. The accumulation of UDP-MurNAc indicates an inhibition of the L-alanine-adding enzyme as known for *S. aureus* (38). No UDP-MurNAc-pentapeptide could be found. Instead, UDP-MurNAc-tetrapeptides were accumulated as in *L. cellobiosus*. Most of them contain glycine residues in position 1 or 4, or both. The peptides found in the partial acid hydrolysate of the UDP-MurNAc-tetrapeptides were in agreement with the structure shown in Fig. 13b. Gly-D-Glu and Mur-Gly were much more pronounced than in the UDP-MurNAc-pentapeptides of *Corynebacterium* sp., whereas Gly-D-Ala and D-Ala-Gly were missing. Again, one has to assume that UDP-MurNAc-pentapeptides of normal structure also have to be formed, since the peptidoglycan of the inhibited sample shows 30% cross-linkage. The pool of such precursors is probably too small to be detected by our methods. The origin of the UDP-MurNAc-tetrapeptides may be the same as already discussed in the case of *L. cellobiosus*.

Effect of glycine on 2,4-diaminobutyric acid containing peptidoglycan of group B. In most peptidoglycans of group B the position 1 of the peptide subunits is occupied by a glycine residue (Fig. 1). Therefore, glycine should have no inhibitory effect if the inhibition of the L-alanine-adding enzyme is the main site of action. Nevertheless, organisms containing this type of peptidoglycan are very sensitive to glycine. Among 11 strains tested, the most resistant strain (*C. mediolanum*) was completely inhibited by 1.25% glycine, and the most sensitive strain (*C. insidiosum*) showed complete inhibition by 0.8% glycine. The effect of glycine on the primary structure of the peptidoglycan was studied in these two extreme cases. Table 8 shows the molar ratio of the amino acids and of the C- and N-terminal residues of control and inhibited samples. Although the peptidoglycans of the two strains belong to the same type, they differ in some details.

In *C. insidiosum*, the γ-amino group of the diaminobutyric acid of the peptide subunit, is acetylated, whereas it is free in *C. mediolanum* (7). Moreover, there are differences in the amount of D-alanine and, consequently, in the degree of cross-linkage. The effect of glycine is also different. In *C. insidiosum* the glycine content of the peptidoglycan in the inhibited sample is increased, whereas it is unchanged in *C. mediolanum*. Although no additional glycine is found in the peptidoglycan of the inhibited cells of *C. mediolanum*, the degree of cross-linkage is significantly lower.

The calculation of the degree of cross-linkage in the peptidoglycans of group B is not quite as simple as in case of group A, but also here we

TABLE 6. *Effect of glycine on molar ratio of amino acids and amino sugars and C-terminal and N-terminal residues of the peptidoglycan of four strains of the m-Dpm-direct type*[a]

Species and concn of glycine	Type	Molar ratios							C-terminal			N-terminal	Cross-linkage (%)	Inhibition of cross-linkage in % of control
		Mur	$GlcNH_2$	Glu	meso-Dpm	L-Ala	D-Ala	Gly	meso-Dpm[b]	Ala	Gly	meso-Dpm		
L. plantarum (2%)	a	0.90	1.37	1.0	0.97	1.0	0.59	—	0.46	0.03	—	0.49	51	42
	b	1.09	1.06	1.0	1.07	0.46	0.29	0.73	0.56	0.04	0.1	0.7	30	
B. subtilis (0.4%)	a	0.49	1.1	1.0	1.01	1.0	0.59	—	0.52	0.03	—	0.55	45	18
	b	0.85	1.04	1.0	1.04	0.96	0.44	0.15	0.50	0.03	0.1	0.63	37	
Corynebacterium sp. (4%)	a	0.97	1.08	1.0	0.96	1.0	0.81	—	0.15	0.10	—	0.25	75	37
	b	1.0	0.91	1.0	0.96	0.98	0.40	0.61	0.05	0.095	0.385	0.53	47	
C. callunae (7%)	a	1.1	0.8	1.0	0.9	1.0	0.79	—	0.16	0.2	—	0.36	64	23
	b	1.2	0.82	1.0	0.92	0.96	0.5	0.42	0.05	0.13	0.32	0.50	50	

[a] (a) Control; (b) inhibited by glycine.

[b] Since the *m*-Dpm is amidated, C-terminal *m*-Dpm does not occur as free amino acid after hydrazinolysis. The value was, therefore, calculated from the difference between N- and C-terminal amino acids.

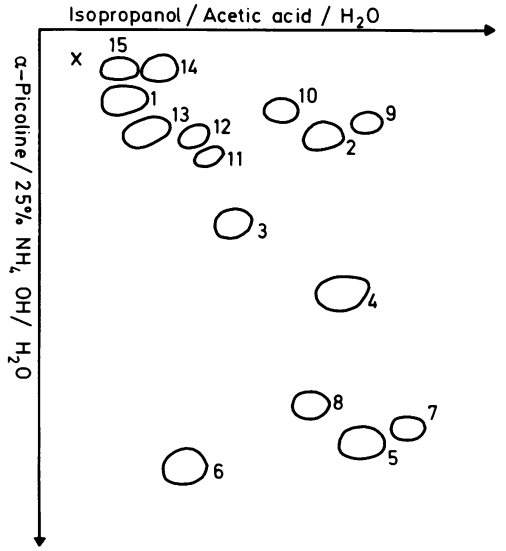

FIG. 12. *Scheme of a paper chromatogram of a partial acid hydrolysate of walls of glycine inhibited cells of L. plantarum. 1, meso-Dpm; 2, Glu; 3, Gly; 4, Ala; 5, Mur; 6, GlcNH$_2$; 7, Mur-L-Ala; 8, Mur-Gly; 9, L-Ala-D-Glu; 10, Gly-D-Glu; 11, meso-Dpm-D-Ala; 12, D-Ala-meso-Dpm; 13, meso-Dpm-Gly and Gly-meso-Dpm; 14, D-Glu-meso-Dpm-D-Ala; 15, L-Ala-D-Glu-meso-Dpm.*

can use the data given in Table 8 to calculate the cross-linkages either on the basis of the amount of the C-terminal or of the N-terminal residues.

Calculation based on the C-terminal residues. All the diaminobutyric acid bound to the α-carboxyl group of D-glutamic acid should be C-terminal (Fig. 1). Therefore the amount of C-terminal diaminobutyric acid exceeding 1.0 is due to the L-diaminobutyric acid located within the peptide subunit. In case of the control sample of *C. mediolanum*, the amount of 0.27 mol of C-terminal L-diaminobutyric acid agrees well with the lack of 0.27 mol of D-alanine. We can, therefore, assume that 73% of the peptide subunits of the peptidoglycan are tetrapeptides, whereas 27% are tripeptides. Pentapeptides are not present. This was shown by chromatographic analysis of the peptide pattern of a partial acid hydrolysate. Pentapeptides would give rise to D-Ala-D-Ala. No trace of this peptide could be detected. Since 0.03 mol of D-alanine per mol of glutamic acid are found to be C-terminal, about 3% of the tetrapeptide subunits are not cross-linked. The degree of cross-linkage is then 70%. This calculation is based on a molar ratio of 2.0 mol of diaminobutyric acid per mol of glutamic acid, whereas only 1.88 mol

TABLE 7. *Total amount and molar ratio of amino sugars, amino acids, and C-terminal residues of peptidoglycan precursors of three species containing peptidoglycan of the m-Dpm-direct type*

Organism	Factor	μmol	Mur	$GlcNH_2$	Ala	Glu	Dpm	Gly	C-terminal		Precursors
									Ala	Gly	
Corynebacterium sp.	I	1.5	—	+	—	—	—	—	—	—	UDP-GlcNAc
	II	Spur	+	—	—	—	—	—	—	—	UDP-MurNAc
	III	18.6	1.1	—	1.96	1.0	0.97	1.03	0.57	0.43	UDP-MurNAc-pentapeptides
B. subtilis	I	0.1	+	—	—	—	—	—	—	—	UDP-MurNAc
	II	2.3	1.1	—	1.71 (0.77) (D-Ala)	1.0	0.94	0.99	0.21	0.79	UDP-MurNAc-pentapeptides
L. plantarum	I	7.2	+	—	—	—	—	—	—	—	UDP-MurNAc
	II	0.6	1.0	—	—	—	—	1.0	—	—	UDP-MurNAc-Gly
	III	0.6	1.0	—	0.25	1.0	1.0	0.75	—	—	UDP-MurNAc-Gly-γ-D-Glu-meso-Dpm (L-Ala-γ-D-Glu-meso-Dpm)
	IV	8.4	1.1	—	0.48	1.0	1.0	1.41	0.2	0.8	UDP-MurNAc-tetrapeptides

was found. We assume, that most of the difference is due to the methodical error of the amino acid analysis. It may be, however, that some of the D-diaminobutyric acid residues are actually missing. In this case not all of the α-carboxyl groups of the D-glutamic acid residues are substituted. Consequently, the degree of cross-linkage would be somewhat lower, but then higher values for the amount of C-terminal residues should have been found. Taking into account a methodical error of the determination of C-terminal residues of about 10%, the agreement is reasonable.

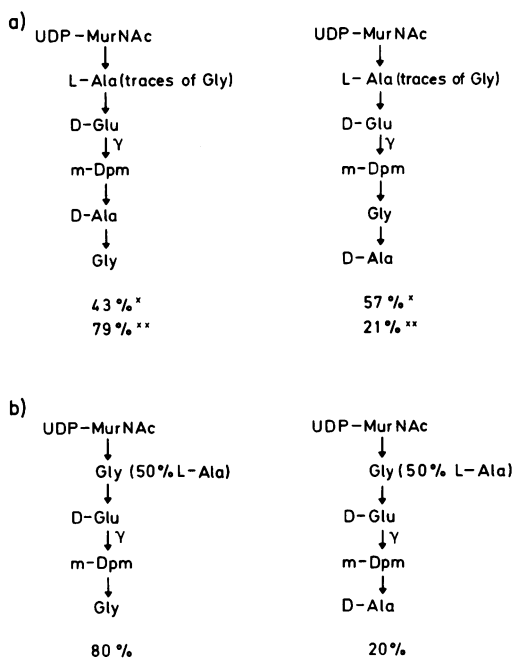

FIG. 13. *Proposed structure and proportion of the UDP-MurNAc-pentapeptide precursors (a) of Corynebacterium sp. and B. subtilis cells and of the UDP-MurNAc-tetrapeptide precursors (b) of L. plantarum cells inhibited by glycine. ×, Figures for Corynebacterium sp.; ××, figures for B. subtilis.*

Calculation based on the N-terminal residues. All the L-diaminobutyric acid residues of the interpeptide bridge are N-terminal since the peptidoglycan of *C. mediolanum* is not acetylated (7). The presence of 1.26 mol of N-terminal total diaminobutyric acid indicates, therefore, that 0.26 mol of D-diaminobutyric acid residues are N-terminal and not cross-linked. A degree of 74% of cross-linkage would be the maximal value. It becomes somewhat lower if we assume that less than 1.0 mol of D-diaminobutyric acid is present as mentioned above. Considering both ways of calculation, we may suppose a degree of cross-linkage of about 70% in the control sample of *C. mediolanum*. In case of *C. insidiosum* we have to consider the acetylation of the γ-amino group of the L-diaminobutyric acid residues of the peptide subunit. The number of the N-terminal diaminobutyric acid directly gives, therefore, the amount of uncross-linked interpeptide bridges.

Analogous calculations in case of the inhibited cells of *C. mediolanum* show that the portion of tripeptide subunits is highly increased, whereas the portion of uncross-linked tetrapeptides is unchanged. The increase in tripeptide subunits accounts completely for the decrease of cross-linking by about 40%.

The situation is different in *C. insidiosum*. Here the control sample contains about 10% of tripeptide subunits, while the degree of cross-linking is only 50% as shown by the N-terminal Dab. Pentapeptides are absent, since no D-Ala-D-Ala could be found in the partial acid hydrolysate. In the inhibited sample 0.4 mol of additional glycine was found. In contrast to *C. mediolanum*, no decrease, but an increase of D-alanine, is observed. The sum of D-alanine and additional glycine is 1.48. This means that

TABLE 8. *Molar ratio of amino acids, amino sugars, and C- and N-terminal residues in the peptidoglycan of normal and glycine-inhibited cells of C. mediolanum and C. insidiosum*

Organism	Type	Molar ratio						C-terminal			N-terminal	
		Mur	$GlcNH_2$	D-Ala	Glu	Dab	Gly	Dab	Ala	Gly	Dab	Cross-linkage (%)
C. mediolanum	Control	1.03	2.5	0.73	1.0	1.88	1.05	1.27	0.03	—	1.26	70
	+ 1% Glycine	0.90	1.8	0.46	1.0	2.04	1.04	1.55	0.03	—	1.59	42
C. insidiosum	Control	0.90	1.46	0.90	1.0	1.92	1.00	1.14	0.34	—	0.49	50
	+ 0.3% Glycine	0.98	0.94	1.07	1.0	2.02	1.41	1.00	0.29	0.39	0.70	30

no tripeptides, but about 50% tetra- and 50% pentapeptide subunits, are present. The absence of tripeptides is supported by the fact that only 1.0 mol of C-terminal diaminobutyric acid was found, as it is to be expected if only the D-diaminobutyric acid residues of the interpeptide bridge are C-terminal. The presence of pentapeptides is proven by the occurrence of the peptide D-Ala-Gly and small amounts of D-Ala-D-Ala in the partial acid hydrolysate. The peptide Gly-D-Ala was absent, however. Together with the fact that practically all additional glycine is C-terminal, we can assume that in the peptidoglycan of the inhibited cells about 50% of the peptide subunits are pentapeptides, carrying either D-Ala-Gly (40%) or D-Ala-D-Ala (10%), whereas the other 50% are tetrapeptides which are either cross-linked (30%) or C-terminal (20%).

Peptidoglycan precursors from glycine-inhibited cells of C. mediolanum. The peptidoglycan precursors were extracted by trichloroacetic acid from glycine-inhibited cells of *C. mediolanum*. After separation on a Sephadex column and twofold paper chromatography, five fractions containing nucleotide-activated precursors were found. Fraction 1 contained UDP-GlcNAc, whereas UDP-MurNAc was not detected (Table 9). Fractions II and III contained UDP-MurNAc-mono- and tripeptides, fractions IV and V contained UDP-MurNAc-pentapeptides. Fraction IV is a mixture of 90% UDP-MurNAc-Gly-γ-D-Glu-L-Dab-Gly-D-Ala and about 10% UDP-MurNAc-Gly-γ-D-Glu-L-Dab-D-Ala-Gly, since only 0.11 mol of glycine, but 0.89 mol of alanine, are C-terminal. The peptide pattern of the partial acid hydrolysate agreed with this proposed structure. Among others, the peptides Gly-D-Ala and D-Ala-Gly were found, but no D-Ala-D-Ala. In the hydrolysate of fraction V, however, mainly D-Ala-D-Ala and only little of D-Ala-Gly and Gly-D-Ala are present. This fraction, therefore, contains mainly the normal precursor UDP-MurNAc-Gly-γ-D-Glu-L-Dab-D-Ala-D-Ala and only some of the modified precursors of fraction IV. The dinitrophenylation of the pentapaptide fraction yielded 1.0 mol of ε-N-DNP-L-Dab. Therefore, the L-diaminobutyric acid is not acetylated, in contrast to the precursor of *C. insidiosum* investigated by Perkins (24). This is also in accordance with the finding that the peptidoglycan of *C. mediolanum* is not acetylated. The experiments show that glycine is incorporated mainly in position 4 of the precursors, although no additional glycine is found in the peptidoglycan.

Peptidoglycan precursors from glycine-inhibited cells of C. insidiosum. The peptidogly-

TABLE 9. *Molar ratio of amino acids, amino sugars, and C- and N-terminal residues in the peptidoglycan of normal and glycine-inhibited cells of C. mediolanum and C. insidiosum*

Organism	Fraction	μmol	Mur	$GlcNH_2$	Ala	Glu	Dab	Gly	C-terminal		Precursors
									Ala	Gly	
C. mediolanum	I	1.0	—	+	—	—	—	—	—	—	UDP-GlcNAc
	II	0.15	1.0	—	—	—	—	1.0	—	—	UDP-MurNAc-Gly
	III	0.25	1.0	—	—	1.0	1.0	1.0	—	—	UDP-MurNAc-Gly-γ-D-Glu-L-Dab
	IV	5.5	1.1	—	1.0	1.0	1.0	2.0	0.89	0.11	UDP-MurNAc-pentapeptides
	V	0.7	1.1	—	1.68	1.0	0.95	1.25	0[a]	0	UDP-MurNAc-pentapeptides
C. insidiosum	I	0.6	—	+	—	—	—	—	—	—	UDP-GlcNAc
	II	0.5	1.0	—	—	—	—	1.0	—	—	UDP-MurNAc-Gly
	III	0.25	1.0	—	—	1.0	—	1.0	—	—	UDP-MurNAc-Gly-D-Glu
	IV	0.9	1.0	—	—	1.0	1.0	1.0	—	—	UDP-MurNAc-Gly-γ-D-Glu-L-Dab
	V	0.35	1.0	—	2.0	1.0	1.0	1.0	1.0	—	UDP-MurNAc-Gly-γ-D-Glu-L-Dab-D-Ala-D-Ala
	VI	0.8	0.9	—	1.39	1.0	0.97	1.52	0.52	0.48	UDP-MurNAc-Gly-γ-D-Glu-L-Dab-D-Ala-Gly

[a] Not determined.

can precursors were extracted by hot water from the glycine-inhibited cells prior to the preparation of cell walls. The separation on a Sephadex column followed by twofold paper chromatography yielded six fractions of different chemical composition (Table 9). The first fraction contained UDP-GlcNAc, whereas UDP-MurNAc was not found. Fractions II to IV contained incomplete precursors; fraction V contained the normal UDP-MurNAc-pentapeptide. The fraction IV was a mixture of about 50% normal UDP-MurNAc-pentapeptide and 50% UDP-MurNAc-Gly-γ-D-Glu-L-Dab-D-Ala-Gly. In agreement with the finding of Perkins (24), this precursor was not susceptible to dinitrophenylation, indicating that the γ-amino group of L-diaminobutyric acid is substituted (probably acetylated). Since practically all of the glycine exceeding 1.0 mol/mol of glutamic acid is C-terminal, the additional glycine can occur only in position 5 of the precursor. This is in agreement with the peptide pattern of the partial acid hydrolysate. Besides the usual peptides, occuring in partial acid hydrolysates of walls of non-inhibited cells, only D-Ala-D-Ala and D-Ala-Gly were found, but no Gly-D-Ala. Unlike *C. mediolanum* where glycine enters predominantly position 4, in *C. insidiosum* only the D-alanine in position 5 is replaced by glycine. The modified precursor is not significantly accumulated and is obviously used up by the translocase and polymerase reactions since the peptidoglycan contains a relatively high amount of C-terminal glycine in position 5.

Effect of glycine on S. aureus. The organisms described previously accumulated precursors which contain glycine not only in position 1 but also in position 4 or 5 (or both). In *S. aureus* only precursors containing glycine in position 1 were reported (38). Therefore we reinvestigated this organism to be sure that it actually differs from the other organisms studied. A comparison of the amino acid composition and the content of N- and C-terminal residues of normal and inhibited cells (3% glycine) is given in Table 10. The control sample shows the typical molar ratio of amino acids and amino sugars. The high amount of glycine corresponds to an interpeptide bridge consisting of penta- or hexaglycine. About 20% of the peptide subunits are pentapeptides, indicated by the extent by which D-alanine exceeds a molar ratio of 1.0 and by the corresponding amount of C-terminal alanine. The 0.05 mol of C-terminal glycine probably resulted from the action of autolytic endopeptidases on interpeptide bridges before the cells were killed, and should not be used for the calculation of the extent of cross-linkage. The small amount of N-terminal L-alanine may also have arisen from the action of autolytic enzymes (40). It is also possible that small amounts of L-alanine are bound, instead of the pentaglycine, to the ϵ-amino group of lysine. As shown by Schleifer et al. (K. H. Schleifer, J. Gen. Microbiol. **57**:XIV, 1969; 34), such a replacement of pentaglycine by L-alanine occurs frequently in staphylococci. Since this modified interpeptide bridge is not cross-linked, some N-terminal L-alanine is found. As a consequence of this second possibility, the molar ratio of L-alanine should increase above 1.0, and the peptide N^6-L-Ala-L-Lys should occur in the partial acid hydrolysate. Neither of the two consequences could be found. Therefore, the second possibility seems most unlikely.

The amount of N-terminal glycine was determined only by the direct determination of DNP-glycine in the hydrolysate of dinitrophenylated cell walls. The determination by the difference between the glycine content of normal and dinitrophenylated cell walls was not suitable in case of *S. aureus* because of the large error of the difference due to the extremely high total amount of glycine. Some of the 0.21 mol of DNP-glycine may have arisen from the action of autolytic enzymes on interpeptide bridges analogous to the C-terminal glycine, but the main portion results probably from uncross-linked interpeptide bridges. Considering the amount of C- and N-terminal residues, the degree of cross-linkage is about 80%. The amino acid composition of the peptidoglycan of the inhibited sample is only slightly different. L-Alanine is decreased by about 20%; D-alanine is decreased by about 10%. Glycine is slightly increased. The increase of C- and N-terminal glycine is much more pronounced. Some C-terminal L-lysine is also present which was not found in the control sample. From the amounts of C- and N-terminal residues, a degree of cross-linkage of about 50% is calculated. This means a decrease of 38%, compared to the 80% cross-linkage in the control.

The analysis of the peptide pattern of the partial acid hydrolysate of the cell walls of normal and inhibited cells (Fig. 14) showed that the following peptides appear in the inhibited sample in addition to the peptides of the control: Gly-D-Glu (small amounts), L-Lys-Gly, N^6-Gly-L-Lys-Gly, and Gly-D-Ala. This demonstrates that glycine replaces L-alanine at position 1 and D-alanine at position 4 of the peptide subunit.

If glycine residues occur also in position 5, the peptide D-Ala-Gly would arise during hydrol-

ysis. This peptide arises, however, also from the normally cross-linked peptide subunit. Its presence in the partial acid hydrolysate is, therefore, no evidence for peptide subunits with D-Ala-Gly at the C-terminal end. We can calculate the distribution of the various types of peptide subunits in the inhibited cells from the data in Table 10 if we assume that the same amount of C-terminal glycine has arisen from autolytic processes as in the control sample and that no glycine residues at position 4 are involved in cross-linkage. Then we find that 50% tetrapeptide subunits with D-alanine or Gly residues in position 4 are involved in cross-linkage, whereas 6% tripeptide, 13% pentapeptide subunits with Gly-D-Ala or D-Ala-D-Ala, and 30% with D-Ala-Gly are uncross-linked.

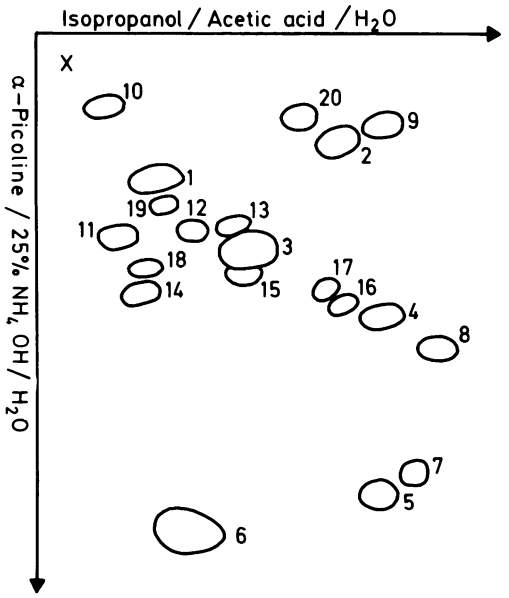

FIG. 14. *Scheme of a paper chromatogram of a partial acid hydrolysate of walls of inhibited cells of S. aureus. 1, Lys; 2, Glu; 3, Gly; 4, Ala; 5, Mur; 6, $GlcNH_2$; 7, Mur-L-Ala; 8, D-Ala-D-Ala; 9, L-Ala-D-Glu; 10, γ-D-Glu-L-Lys; 11, N^6-Gly-L-Lys; 12, L-Lys-D-Ala; 13, Gly-Gly-Gly; 14, N^6-Gly-L-Lys-D-Ala; 15, Gly-Gly (in previous papers [36, 37] a lower R_f value than that of Gly in α-picoline was observed; in recently obtained batches of α-picoline it moves faster than Gly); 16, D-Ala-Gly; 17, Gly-D-Ala; 18, N^6-Gly-L-Lys-Gly; 19, L-Lys-Gly; 20, Gly-D-Glu; no. 17 to 20 were not detected in normally grown cells.*

Peptidoglycan precursors from glycine-inhibited cells of S. aureus. During logarithmic growth, 3.75% glycine was added. The cells were harvested after 3 h of further incubation (inhibition of the growth rate about 25%), and the precursors were extracted and separated. Three fractions of precursors were found. Fraction 1 contained UDP-MurNAc, fraction 2 contained a mixture of about 20% UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys and 80% MurNAc-Gly-γ-D-Glu-L-Lys (the sequence was proved by partial acid hydrolysis), and fraction 3 was a mixture of UDP-MurNAc-pentapeptides (Table 11). From the analysis of the peptide pattern of the partial acid hydrolysate (Fig. 15) and the data of Table 11, the structure shown in Fig. 16 can be deduced.

The precursor UDP-MurNAc-Gly-γ-D-Glu-L-Lys-D-Ala-D-Ala reported by Strominger and Birge (38) could not be found. The chromatogram of the hydrolysate showed no trace of D-Ala-D-Ala which should arise on partial hydrolysis of such a precursor. This does not mean, however, that no normal UDP-MurNAc-pentapeptide was present at all. Its concentration is most likely under the limit of detection by our method. It could not be determined whether glycine at position 1 is preferentially combined with one of the two precursors of Fig. 16 or if it is statistically distributed among the two types of precursors. Statistically about every third precursor should carry L-Ala instead of glycine.

The high percentage of glycine in position 1 of the precursor is in contrast to the relative low percentage of glycine in the peptide subunits of the peptidoglycan. It shows that this kind of precursor is much more discriminated by the

TABLE 10. *Molar ratio of amino acids, amino sugars, and C- and N-terminal residues in the peptidoglycan of normal and glycine-inhibited cells of S. aureus*

Type	Determination	Mur	$GlcNH_2$	Ala	Glu	Lys	Gly	L-Ala	D-Ala	Cross-linkage (%)
Control	Molar ratio	1.01	2.10	2.2	1.0	1.0	5.2	1.02	1.18	
	C-terminal	—	—	0.19	—	—	0.05	—	0.19	80
	N-terminal	—	—	0.05	—	—	0.21	0.05	—	
3% Glycine	Molar ratio	1.32	2.26	1.87	1.0	1.0	5.39	0.83	1.04	
	C-terminal	—	—	0.13	—	0.06	0.36	—	0.13	50
	N-terminal	—	—	0.06	—	0.08	0.4	0.06	—	

TABLE 11. *Molar ratio of amino acids, amino sugars, and C- and N-terminal residues in the peptidoglycan of normal and glycine-inhibited cells of S. aureus*

Fraction	μmol	Mur	$GlcNH_2$	Ala	Glu	Lys	Gly	L-Ala	D-Ala	C-terminal		Precursors
										Gly	Ala	
I	2.9	+	—	—	—	—	—	—	—	—	—	UDP-MurNAc
II	0.8	1.1	—	0.2	1.0	0.85	0.92	0.22	—	—	—	UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys (Gly-γ-D-Glu-L-Lys)
III	2.4	1.1	—	1.1	1.0	0.9	1.74	0.34	0.76	0.65	0.35	UDP-MurNAc-pentapeptides

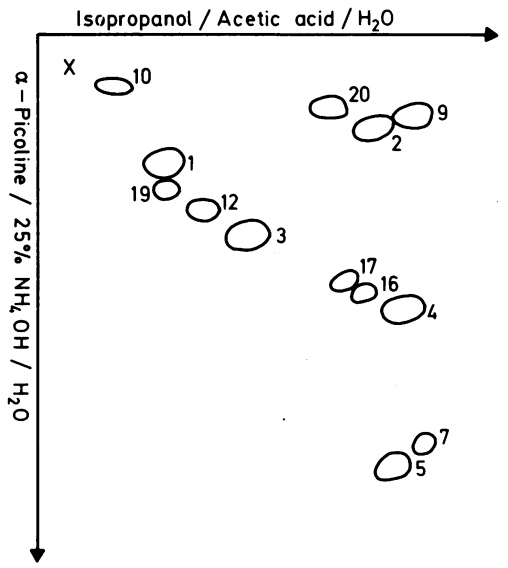

FIG. 15. *Scheme of a paper chromatogram of a partial acid hydrolysate of the UDP-MurNAc-pentapeptide fraction of S. aureus. For explanation of numbers see Fig. 14.*

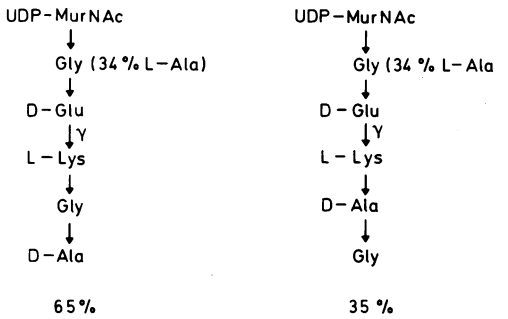

FIG. 16. *Proposed structure and proportion of the UDP-MurNAc-pentapeptide precursors of S. aureus cells inhibited with glycine.*

final steps of peptidoglycan synthesis than is the precursor containing glycine in position 5.

Reversal of transpeptidase reaction by glycine. As found by Izaki et al. (16), the transpeptidase reaction can be reversed by D-amino acids and by glycine in particulate enzyme fractions of *E. coli*. If this occurs also in vivo, glycine should appear in position 5 of the peptide subunits when cells are incubated with glycine. To test this possibility we had to apply an organism which does not contain a DD-carboxypeptidase, otherwise the glycine incorporated by the reverted transpeptidation would be split off. Therefore, we used *L. acidophilus* (63

Gasser) that contains no DD-carboxypeptidase, as shown by Coyette and Ghuysen (3, 4).

At first the sensitivity against glycine was tested during the growth period. The lowest concentration causing significant inhibition was 0.3%, whereas at 2% no growth occurred. To test the uptake of glycine in the peptidoglycan by reversal of transpeptidation, cells from the log and early stationary phase were harvested, suspended in 0.1 M phosphate buffer (pH 6.3), and incubated at 37 C for 12 h with and without 7.5% glycine. About 5% of the control cells were lysed; the other cells showed no morphological changes. Most of the glycine-treated cells were optically less dense; about 20% were unchanged. The cells were centrifuged and disintegrated for cell wall preparation. The molar ratios of the amino acids of the cell walls were identical to the control. No glycine was incorporated into the peptidoglycan.

This negative result may not be significant since the experiment was not carried out under growth conditions. However, the addition of glycine during growth would not allow one to distinguish between the incorporation of glycine during synthesis on one hand and that resulting from reversal of transpeptidation on the other hand. Similarly the explanation of the experiments of Izaki et al. (16) mentioned above is not unequivocally correct, since Pollock et al. (29) have shown that the N-terminus of peptide subunit or interpeptide bridge is a good acceptor for the transpeptidase reaction so is a free D-amino acid or glycine. Therefore, the replacement of D-alanine at position 5 of the peptide subunit by glycine does not require an intermediary cross-linkage.

DISCUSSION

The investigations have shown that glycine interferes with several steps of peptidoglycan synthesis. The contribution of the diverse sites of glycine action to the final inhibition varies in different organisms, thus nearly each organism tested shows another spectrum of changes caused by glycine. Table 12 presents a survey of the observed changes in the peptidoglycan and the precursors, respectively, and the presumed inhibitions of particular steps of biosynthesis. The only features of the glycine inhibition common to all species is a decrease (20 to 40%) in the extent of cross-linking of the peptidoglycan and the synthesis of modified UDP-activated precursors in which alanine residues are partly replaced by glycine residues. Which one of the three alanine residues becomes replaced and by what mechanism the poorer cross-linkage is caused varies. The replacement of L-alanine residues (38) and the inhibition of the L-alanine-adding enzyme (15) is only one of several possibilities. As a matter of fact, glycine may act as an analogue either of L-alanine or of D-alanine. One can divide the eight organisms investigated into three major groups according to the extent to which glycine acts as a L-alanine analogue. (i) The replacement of L-alanine residues by glycine does not occur at all in organisms which contain peptidoglycan of group B, since this peptidoglycan does not contain L-alanine but glycine residues in position 1 of the peptide subunit (Fig. 1) (*C. insidiosum*, *C. mediolanum*). (ii) In *Corynebacterium* sp., *C. callunae*, and *B. subtilis*, only trace amounts of L-alanine residues are replaced by glycine. UDP-MurNAc is not accumulated as would be expected if the L-alanine-adding enzyme were inhibited significantly. Replacement of L-alanine is insignificant or at most of minor importance for growth inhibition. (iii) In *L. plantarum*, *L. cellobiosus*, and *S. aureus*, L-alanine residues are replaced by glycine to a significant extent and UDP-MurNAc is accumulated. But even in these organisms the decrease of cross-linkage is probably caused by the replacement of D-alanine residues, although the limitation of synthesis of precursors by the inhibition of the L-alanine-adding enzyme may contribute significantly to the growth inhibition by glycine.

The two organisms of the first group show different sites of glycine action, although the inhibition of growth by affecting peptidoglycan synthesis can be caused only by the replacement of D-alanine residues in both species. The peptidoglycan of the inhibited cells of *C. insidiosum* contains additional glycine residues located in position 5 of the peptide subunit, whereas no additional glycine is found in case of *C. mediolanum*. The accumulated UDP-MurNAc-pentapeptides of *C. insidiosum* contain additional glycine as in the peptidoglycan exclusively in position 5, whereas most of the glycine is localized in position 4 in *C. mediolanum* (Table 12). Based on such data, we can suggest which steps of the biosynthesis of the peptidoglycan are affected in vivo during the exposure of cells to glycine. The effect of glycine on the first steps of the incorporation of D-alanine, i.e., the formation of D-Ala-D-Ala and its incorporation into the precursor, can not be distinguished. Since no Gly-D-Ala was found in the accumulated precursors of *C. insidiosum* this peptide is either not formed at all or it is completely discriminated by the D-Ala-D-Ala-adding enzyme. On the other hand, D-Ala-Gly is very readily formed and incorporated. In *C. mediolanum* Gly-D-Ala is most easily formed

TABLE 12 *Survey on the proposed effects of glycine on the biosynthesis and structure of peptidoglycan*

Species	Peptidoglycan type	Inhibition of cross-linkage (%)	Incorporation (mol/mol of Glu) of Gly into position X of						Most accumulated precursor UDP-X	Presumed limitations of peptidoglycan synthesis in vivo							
			Peptidoglycan			UDP-MurNAc-penta-(tetra)-peptide				Inhibition of L-Ala adding enzyme	Formation and incorporation of		Discrimination of precursors containing Gly in position			Inhibition of transpeptidation	Degradation of precursors by carboxypeptidase
			1	4	5	1	4	5			D-Ala-Gly	Gly-D-Ala	1	4	5		
C. insidiosum	[L-Dab]D-Glu-D-Dab	40	0.0 (1.0)	0.0	0.39	0.0 (1.0)	0.0	0.5	MurNAc-tri- and pentapeptides	—[a]	No	++	—	—	Slight	++	No
C. mediolanum	[L-Dab]D-Glu-D-Dab	~30	0.0 (1.0)	0.0	0.0	0.0 (1.0)	0.9	0.1	MurNAc-pentapeptides	—	++	+	—	Yes (complete)	?	++	Yes
Corynebacterium sp.	*m*-Dpm-direct	37	Trace	0.61	0.0	Trace	0.57	0.43	MurNAc-pentapeptides	No	++	++	No	No	?	++	No
C. callunae	*m*-Dpm-direct	23	Trace	0.42	0.0	0	0	0	0	No	0[b]	0	0	0	0	++	0
B. subtilis	*m*-Dpm-direct	18	0.04	0.14	0.0	0.06	0.2	0.8	MurNAc-pentapeptides	No	+	++	No	Slight	?	++	No
L. plantarum	*m*-Dpm-direct	42	0.54	0.19	0.0	0.60	0.8	No pentapeptide	MurNAc and MurNAc-tetrapeptides	Yes	?[c]	++?[c]	Slight	Yes	?	Indirect	Yes
L. cellobiosus	L-Orn-D-Asp	30	0.50	0.21	0.0	0.53	1.0	No pentapeptide	MurNAc and MurNAc-tetrapeptides	Yes	?[c]	++?[c]	No	Yes	?	Indirect	Yes
S. aureus	L-Lys-Gly_{5-6}	38	0.17	0.1	0.31	0.66	0.65	0.35	MurNAc and MurNAc-pentapeptides	Yes	++	++	Yes	Yes	Slight	++	No

[a] —Not relevant.

[b] 0, Not investigated.

[c] Since only UDP-MurNAc-tetrapeptides were found, the incorporation of Gly-D-Ala and D-Ala-Gly is only presumed. The incorporation of Gly-D-Ala is most likely since Gly was found in position 4.

and incorporated, whereas D-Ala-Gly is strongly, but not completely, discriminated.

Studies on the substrate specificity of the enzyme involved in these reactions were carried out by Neuhaus (21) and Neuhaus and Struve (22) in *S. faecalis*. They found that the D-Ala-D-Ala synthetase catalyzed only the formation of D-Ala-Gly and not of Gly-D-Ala, but that neither D-Ala-Gly nor Gly-D-Ala were utilized in vitro by the D-Ala-D-Ala-adding enzyme. Therefore, the formation of UDP-MurNAc-pentapeptides containing glycine in position 4 or 5 would be very unlikely, and an accumulation of UDP-MurNAc-tripeptide should be expected. The different results of our in vivo experiments indicate that the substrate specificity of the two enzymes is different from that of *S. faecalis* in both organisms studied.

The occurrence of Gly in position 4 of the peptide subunit in all tested bacteria but one and the fact that the distribution of D-Ala and Gly within positions 4 and 5 is variable lead us to suggest that there are different specificities in the enzymes involved in D-Ala incorporation among the various bacteria.

The next group of reactions which can be judged only as a whole includes the translocation of the precursor to the lipid carrier, its completion by adding *N*-acetylglucosamine and the interpeptide bridge, and its polymerisation to peptidoglycan. The pentapeptide precursors containing C-terminal glycine residues are only slightly discriminated against the normal ones in case of *C. insidiosum*, since the amount of glycine in position 5 within the peptidoglycan is only slightly lower than that in the isolated mixture of UDP-MurNAc-pentapeptides. There may be even no discrimination at all if some of the incorporated modified pentapeptides have undergone transpeptidation through which the C-terminal glycine is lost. The majority of the modified pentapeptides are not cross-linked, however, thus leading to a lower degree of cross-linkage in the peptidoglycan.

In case of *C. mediolanum*, none of the modified precursors containing a glycine residue in position 4 is found in the peptidoglycan. The main difference between the peptidoglycan of the control and the inhibited sample is the increased amount of tripeptide subunits in the latter. There are two possibilities for the formation of an increased amount of tripeptide subunits. The first possibility is that the accumulated modified precursors are broken down to UDP-MurNAc-tripeptides by carboxypeptidases which exhibit a high affinity to UDP-activated precursors similar to the ones found in *E. coli* (17). These tripeptides may then become incorporated into the peptidoglycan. An incorporation of UDP-MurNAc-tripeptides was observed in a cell-free preparation of *E. coli* (16), however, at a much lower rate (10 to 15%) compared to the incorporation of the corresponding pentapeptides. Under the conditions of glycine inhibition, when the concentration of the normal UDP-MurNAc-pentapeptide is low and modified pentapeptide precursors are accumulated, the incorporation of tripeptides may be favored, however. Since tripeptide subunits can not undergo transpeptidation, the degree of cross-linkage is limited by the extent of incorporation of incomplete precursors. The second possibility is that the glycine-containing pentapeptide precursors are incorporated, but then completely discriminated during transpeptidation and subsequently degraded to tripeptide subunits by carboxypeptidases. This seems unlikely, however, since it was shown (19) that, at least in the case of *Streptomyces albus*, carboxypeptidase and transpeptidase exhibit the same substrate specifity. Therefore, the modified precursors should be discriminated by both processes. Little can be said about the fate of the pentapeptide precursor which contains C-terminal glycine. It may be discriminated during the peptidoglycan synthesis or it may be incorporated and then undergo transpeptidation, losing its C-terminal glycine residue.

The comparison of the effect of glycine on the two strains shows that the most severe limitation for the cross-linkage is in case of *C. insidiosum* for inhibition of transpeptidation by pentapeptide subunits containing C-terminal glycine, whereas in case of *C. mediolanum* the incorporation of UDP-MurNAc-tripeptides most likely derived from precursors containing glycine at position 4 or 5. The inhibition of transpeptidation in the latter case is only indirect.

The organisms of the second group are characterized by the incorporation of glycine mainly in position 4 of the peptide subunit in the peptidoglycan, whereas no significant amount of L-alanine at position 1 is replaced by glycine. Here the UDP-MurNAc-pentapeptides which contain glycine in position 4 are not, or are not strongly, discriminated against the normal ones from incorporation into the peptidoglycan. They are, however, at least partly excluded from transpeptidation since most of the glycine in the peptidoglycan is C-terminal. A minor portion is involved in cross-linkage as indicated by the occurrence of the peptide Gly-*m*-Dpm in the partial acid hydrolysates. UDP-MurNAc-

pentapeptides containing glycine in position 5 may be incorporated, but then the glycine is lost during transpeptidation or by the action of carboxypeptidase.

The two organisms of group 2 investigated in more detail react very similar. The only distinction concerns the different proportion of incorporation of Gly-D-Ala (predominant in *B. subtilis*) and D-Ala-Gly (predominant in *Corynebacterium* sp.) As shown by Hishinuma et al. (15), the L-alanine-adding enzyme of *B. subtilis* is inhibited in vitro by glycine. They assumed that this may be at least partly the reason for the growth inhibition by glycine. Our in vivo experiments, however, indicate only a weak competition between L-alanine and glycine which does not contribute significantly to the growth inhibition. The latter is brought about by the stronger effect of the D-alanine replacement by Gly. The "no" in the line "inhibition of L-alanine adding enzyme" of Table 12 is, therefore, not in contradiction to the finding of Hishinuma et al. (15). It means, however, that this reaction step is not decisive in growth inhibition in vivo.

In all three organisms of group 3, an inhibition of the L-alanine-adding enzyme is also indicated in vivo, since UDP-MurNAc is found in large amounts and precursors containing glycine residues in position 1 are formed. The extent to which they are incorporated into the peptidoglycan differs among the three species. In *S. aureus* the amount of glycine in position 1 of the peptide subunit of the peptidoglycan is much lower than it is in the accumulated mixture of precursors, whereas it is about equal in case of the two lactobacilli. This indicates a discrimination against these precursors during the incorporation into peptidoglycan in *S. aureus*, whereas there is no such discrimination in the lactobacilli.

The competition between glycine and D-alanine leads in *S. aureus* to the accumulation of UDP-MurNAc-pentapeptides which contain either Gly-D-Ala or D-Ala-Gly at the C-terminus of the peptide subunit. The normal precursor containing D-Ala-D-Ala is probably present in undetectable amounts only. The UDP-MurNAc-pentapeptides which contain glycine in position 4 must be more strongly discriminated against than the normal ones in incorporation, since in the peptidoglycan more glycine is found in position 5 than in position 4.

The large amount of C-terminal glycine, compared to the small amount of C-terminal D-alanine in the peptidoglycan of the inhibited sample, shows that the peptide subunits with C-terminal glycine are poorly used for transpeptidation. We consider this the main reason for the decrease of the degree of cross-linkage. The origin of 6% tripeptide subunits, which also contribute to the lower degree of cross-linkage in the inhibited sample, is unknown. They may be directly incorporated from UDP-MurNAc-tripeptides found in the cell extract in small amounts or they may have arisen by the action of carboxypeptidases on uncross-linked pentapeptide subunits of the peptidoglycan. The latter seems very unlikely, since *S. aureus* most likely contains none of these enzymes. The uncross-linked pentapeptides are not degraded in the control sample and are also present in the inhibited peptidoglycan.

In the two lactobacilli no UDP-MurNAc-pentapeptides, but only UDP-MurNAc-tetrapeptides, were found. They contain mainly C-terminal glycine and may have arisen from UDP-MurNAc-pentapeptides with C-terminal Gly-D-Ala by the action of a DD-carboxypeptidase as described by Izaki and Strominger (17) in *E. coli*. *L. cellobiosus* contains UDP-MurNAc-L-Ala-γ-D-Glu-L-Orn-D-Ala in normally grown cells or in cells inhibited by penicillin. It is possible that the incorporation of such incomplete precursors is involved in the regulation of the extent of cross-linking not only in inhibited but also in uninhibited cells. Under normal conditions the C-terminal D-alanine residues of the tetrapeptide subunits are split off in the peptidoglycan by carboxypeptidase II (an LD-carboxypeptidase) (17). Therefore a high percentage of tripeptide subunits is found in the peptidoglycan of normally grown cells. The tetrapeptide subunits with C-terminal glycine may be a poorer substrate for carboxypeptidase II. Consequently, a higher percentage of such subunits remains in the peptidoglycan of glycine-inhibited cells. Some of the proposed UDP-MurNAc-pentapeptides containing C-terminal Gly-D-Ala must have been incorporated and must have undergone transpeptidation since a minor portion of glycine is involved in cross-linkages in both organisms.

Unlike *S. aureus*, the decrease of the degree of cross-linkage in *L. plantarum* and *L. cellobiosus* may be caused by the breakdown of modified UDP-MurNAc-pentapeptides to the corresponding tetrapeptides which are incorporated but are not suitable for transpeptidation. The inhibition of cross-linking is therefore of indirect nature. In case of *L. cellobiosus*, the specificity of the L-ornithine-adding enzyme seems to be affected by glycine since a significant amount of L-lysine is incorporated into the

glycine-inhibited sample. Also the incorporation of the interpeptide bridge (D-Asp) is hindered. These two effects were not seen in other organisms. They may not be related to the competition of glycine with L- or D-alanine, thus indicating a third type of glycine action.

Careful enzymatic studies will be necessary to prove the various conclusions drawn from the distribution of glycine in peptidoglycan and in UDP-activated precursors formed in glycine-inhibited cells. However, the principles of the action of glycine on peptidoglycan became fairly clear. There seems to be no particular enzymatic reaction common in all bacteria, which is most sensitive to glycine and solely responsible for growth inhibition. Although we have interpreted the effects of glycine as resulting mainly from the incorporation of subunits containing glycine in positions 4 and 5 with consequent inhibition of cross-linkage, the more loosely cross-linked cell wall could also be the consequence of a disruption of the normal balance between peptidoglycan synthesis and controlled enzymatic hydrolysis due to a decreased incorporation of precursors. The latter mechanism would most likely lead to an increase of C-terminal D-alanine. Since in most cases an increase of C-terminal glycine was found, we consider this mechanism less important. The closest analogy to the action of glycine has been found in investigations of growth inhibition by D-amino acids. They also replace D-alanine in position 4 or 5 of the peptidoglycan precursor. The modified precursors become discriminated against normal ones during the following reactions, thus leading to a decrease of the degree of cross-linkage of the peptidoglycan (Trippen et al., manuscript in preparation). A similar diversity of differentially sensitive reactions may be the reason for the apparently contradictory data on the effect of penicillin, whose action on cell morphology, i.e., spheroplast or L-phase induction, has often been compared with that of glycine (5, 6, 9).

ACKNOWLEDGMENTS

This work was supported by a grant of the Deutsche Forschungsgemeinschaft.

We are indebted to F. Schötz and H. Bathelt for their advice and cooperation in preparing the electron micrographs, and E. Hagner for her excellent technical assistance.

LITERATURE CITED

1. Anwar, R. A., C. Roy, and R. W. Watson. 1963. Isolation and structure of uridine nucleotide-peptides from *Aerobacter cloacae* NRC 492. Can. J. Biochem. **41**:1065–1072.
2. Chatterjee, A. N., and H. R. Perkins. 1966. Compounds formed between nucleotides related to the biosynthesis of bacterial cell wall and vancomycin. Biochem. Biophys. Res. Commun. **24**:489–494.
3. Coyette, J., and J.-M. Ghuysen. 1970. Structure of the walls of *Lactobacillus acidophilus* strain 63 AM Gasser. Biochemistry **9**:2935–2943.
4. Coyette, J., and J.-M. Ghuysen. 1970. Wall autolysin of *Lactobacillus acidophilus* strain 63 AM Gasser. Biochemistry **9**:2952–2955.
5. Dienes, L., J. H. Weinberger, and S. Madoff. 1950. The transformation of typhoid bacilli into L-forms under various conditions. J. Bacteriol. **59**:755–764.
6. Dienes, L., and P. C. Zamecnik. 1952. Transformation of bacteria into L-forms by amino acids. J. Bacteriol. **64**:770–771.
7. Fiedler, F., and O. Kandler. 1973. Die Aminosäuresequenz von 2,4-Diaminobuttersäure enthaltenden Mureinen bei verschiedenen coryneformen Bakterien und *Agromyces ramosus*. Arch. Mikrobiol. **89**:51–66.
8. Fraenkel-Conrat, H., and C. H. Tsung. 1967. Hydrazinolysis, p. 151–155. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology vol. 11, Academic Press Inc., New York.
9. Fung, P. H., and A. J. Winter. 1968. Effects of penicillin and glycine on cell wall glycopeptides of the two varieties of *Vibrio fetus*. J. Bacteriol. **96**:1889–1894.
10. Ghuysen, J.-M., E. Bricas, M. Lache, and M. Leyh-Bouille. 1968. Structure of the cell walls of *Micrococcus lysodeikticus*. III. Isolation of a new peptide dimer, N^{α}-(L-alanyl-γ-(α-D-glutamyl - glycine)) - L - lysyl - D-alanyl-N^{α}-(L-alanyl-γ-(α-D-glutamyl-glycine)-L-Lysyl-D-alanine. Biochemistry **7**:1450–1460.
11. Ghuysen, J.-M., and J. L. Strominger. 1963. Structure of the cell wall of *Staphylococcus aureus*, strain Copenhagen. I. Preparation of fragments by enzymatic hydrolysis. Biochemistry **2**:1110–1119.
12. Gordon, J., and M. Gordon. 1943. Involution forms of the genus *Vibrio* produced by glycine. J. Pathol. Bacteriol. **55**:63–68.
13. Gordon, J., R. A. Hall, and L. H. Stickland. 1949. A comparison of the degree of lysis by glycine of normal and glycine-resistant organisms. J. Pathol. Bacteriol. **61**:581–585.
14. Hishinuma, F., K. Izaki, and H. Takahashi. 1969. Effects of glycine and D-amino acids on growth of various microorganisms. Agr. Biol. Chem. **33**:1577–1586.
15. Hishinuma, F., K. Izaki, and H. Takahashi. 1971. Inhibition of L-alanine adding enzyme by glycine. Agr. Biol. Chem. **35**:2050–2058.
16. Izaki, K., M. Matsuhashi, and J. L. Strominger. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reaction in strains of *Escherichia coli*. J. Biol. Chem. **243**:3180–3192.
17. Izaki, K., and J. L. Strominger. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIV. Purification and properties of two carboxypeptidases from *Escherichia coli*. J. Biol. Chem. **243**:3193–3201.
18. Larson, D. M., D. C. Setsinger, and P. E. Waibel. 1971. Procedure for determination of D-amino acids. Anal. Biochem. **39**:395–401.
19. Leyh-Bouille, M., J.-M. Ghuysen, R. Bonaly, M. Nieto, H. R. Perkins, K. H. Schleifer, and O. Kandler. 1970. Substrate requirements of the *Streptomyces albus* G DD carboxypeptidase. Biochemistry **9**:2691–2970.
20. Man, J. C. de, M. Rogosa, and M. E. Sharpe. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23**:130–135.
21. Neuhaus, F. C. 1962. The enzymatic synthesis of D-alanyl-D-alanine I. Purification and properties of D-alanyl-D-alanine synthetase. J. Biol. Chem. **237**:778–786.
22. Neuhaus, F. C., and W. G. Struve. 1965. Enzymatic synthesis of analogs of the cell wall precursor. I. Kinetics and specificity of uridine diphospho-N-acetylmuramyl-L-alanyl-D-glutamyl-L-lysyl: D-alanyl-D-

alanine ligase (adenosine diphosphate) from *Streptococcus faecalis* R. Biochemistry **4**:120-131.
23. Niebler, E., K. H. Schleifer, and O. Kandler. 1969. The amino acid sequence of L-glutamic acid containing mureins of *Micrococcus luteus* and *M. freudenreichii*. Biochem. Biophys. Res. Commun. **34**:560-568.
24. Perkins, H. R. 1968. Cell wall mucopeptide of *Corynebacterium insidiosum* and *Corynebacterium sepedonicum*. Biochem. J. **110**:47-48.
25. Plapp, R., and O. Kandler. 1967. Vergleichende Untersuchungen über die Anreicherung UDP-aktivierter Mureinvorstufen durch Antibiotika bei verschiedenen Bakterien, p. 79-87. *In* Internationales Symposium: Wirkungsmechanismen von Fungiziden und Antibiotika. Akademie-Verlag, Berlin.
26. Plapp, R. and O. Kandler. 1967. Die Aminosäuresequenz des Asparaginsäure enthaltenden Mureins von *Lactobacillus coryneformis* und *Lactobacillus cellobiosus*. Z. Naturforsch. **22b**:1062-1067.
27. Plapp, R., and O. Kandler. 1967. Identification of L-ornithine and δ-aminosuccinyl ornithine in cell wall hydrolysates of *Lactobacillus cellobiosus*. Nature (London) **213**:803-804.
28. Plapp, R., and O. Kandler. 1967. Isolation of an ornithine-containing cell wall precursor of *Lactobacillus cellobiosus*. Biochem. Biophys. Res. Commun. **28**:141-145.
29. Pollock, J. J., J. M. Ghuysen, R. Linder, M. R. J. Salton, H. R. Perkins, M. Nieto, M. Leyh-Bouille, J. M. Frere, and K. Johnson. 1972. Transpeptidase activity of Streptomyces D-alanyl-D-carboxypeptidases. Proc. Nat. Acad. Sci. U.S.A. **69**:662-666.
30. Rao, K. R., and H. A. Sober. 1954. Preparation and properties of 2,4-dinitrophenyl-L-amino acids. J. Amer. Chem. Soc. **76**:1328-1331.
31. Reissig, J. L., J. L. Strominger, and L. F. Leloir. 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem. **217**:959-966.
32. Rosenthal, S., and N. Sharon. 1964. The use of Sephadex G-25 for the isolation of nucleotide sugar derivatives from *Micrococcus lysodeikticus*. Biochim. Biophys. Acta **83**:378-380.
33. Ryter, A., E. Kellenberger, A. Birch-Andersen, and O. Maaløe. 1958. Etude au microscope électronique de plasmas contenant de l'acide désoxyribonucleique. I. Les nucléoides des bacteries en croissance active. Z. Naturforsch. **13b**:597-605.
34. Schleifer, K. H., L. Huss, and O. Kandler. 1969. Die Beeinflussung der Aminosäuresequenz des serinhaltigen Mureins von *Staphylococcus epidermidis* Stamm 24 durch die Nährbodenzusammensetzung. Arch. Mikrobiol. **68**:387-404.
35. Schleifer, K. H., and O. Kandler. 1967. Zur chemischen Zusammensetzung der Zellwand der Streptokokken. I. Die Aminosäuresequenz des Mureins von *Str. thermophilus* und *Str. faecalis*. Arch. Mikrobiol. **57**:335-364.
36. Schleifer, K. H., and O. Kandler. 1972. The peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36**:407-477.
37. Snell, E. E., and B. M. Guirard. 1943. Some interrelationship of pyridoxine, alanine and glycine in their effect on certain lactic acid bacteria. Proc. Nat. Acad. Sci. U.S.A. **29**:66.
38. Strominger, J. L., and C. H. Birge. 1965. Nucleotide accumulation in *Staphylococcus aureus* by glycine. J. Bacteriol. **89**:1124-1127.
39. Takebe, I. 1965. Extent of cross linkage in the murein sacculus of *Escherichia coli* B cell wall. Biochim. Biophys. Acta **101**:124-126.
40. Tipper, D. J. 1969. Mechanism of autolysis of isolated cell walls of *Staphylococcus aureus*. J. Bacteriol. **97**:837-847.
41. Vela, G. R., and R. S. Rosenthal. 1972. Effect of peptone on *Azotobacter* morphology. J. Bacteriol. **111**:260-266.
42. Weidel, W., and H. Pelzer. 1964. Bag-shaped macromolecules. A new outlook on bacterial cell walls. Advan. Enzymol. **26**:193-232.
43. Welsch, M. 1958. Formation de protoplasts d'*Escherichia coli* sous l'influence de la glycine et d'autres acides amines. Schweiz. Z. Allg. Pathol. Bacteriol. **21**:741-768.
44. Welsch, M., and P. Osterrieth. 1958. A comparative study of the transformation of gram-negative rods into "protoplasts" under the influence of penicillin and glycine. Antonie van Leeuwenhoek J. Microbiol. Serol. **24**:257-272.