Franklin, T. J. (1967), *Biochem. J. 105,* 371.
Franklin, T. J., and Higginson, B. (1970), *Biochem. J. 116,* 287.
Hirata, H., Fukui, S., and Ishikawa, S. (1969), *J. Biochem. 65,* 843.
Hopfer, U., Lehninger, A. L., and Thompson, T. E. (1968), *Proc. Nat. Acad. Sci. U. S. 59,* 484.
Jacob, F., Siminovitch, L., and Wollman, E. (1952), *Ann. Inst. Pasteur, Paris 83,* 295.
Keith, A. D., and Snipes, W. (1974), *Science 183,* 666.
Kunugita, K., and Matsuhashi, M. (1970), *J. Bacteriol. 104,* 1017.
Luria, S. E. (1970), *Science 168,* 1166.
Luria, S. E. (1973), *in* Bacterial Membranes and Walls, Leive, L., Ed., New York, N.Y., Marcel Dekker, p 293.
Luria, S. E., Adams, J. N., and Ting, R. C. (1960), *Virology 12,* 348.
Lusk, J. E., and Nelson, D. L. (1972), *J. Bacteriol. 112,* 148.
Lusk, J. E., Williams, R. J. P., and Kennedy, E. P. (1968), *J. Biol. Chem. 243,* 2618.
Nagel de Zwaig, R., and Luria, S. E. (1967), *J. Bacteriol. 94,* 1112.
Nomura, M. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28,* 315.
Nomura, M., and Maeda, A. (1965), *Zentralbl. Bakteriol., Parasitenk., Infektionskr. Hyg., Abt. 1: Orig. 196,* 216.
Phillips, S. K., and Cramer, W. A. (1973), *Biochemistry 12,* 1170.
Plate, C. A. (1973), *Antimicrob. Agents Chemother. 4,* 16.
Plate, C. A., and Luria, S. E. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 2030.
Plate, C. A., Suit, J. L., Jetten, A. M., and Luria, S. E. (1974), *J. Biol. Chem.* (in press).
Rosen, B. P. (1973), *Biochem. Biophys. Res. Commun. 53,* 1289.
Senior, B. W., and Holland, I. B. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 959.
Silver, S., and Clark, D. (1971), *J. Biol. Chem. 246,* 569.
Sinensky, M. (1974), *Proc. Nat. Acad. Sci. U. S., 71,* 522.
Träuble, H., and Eibl, H. (1974), *Proc. Nat. Acad. Sci. U. S. 71,* 214.
Wendt, L. (1970), *J. Bacteriol. 104,* 1236.
Yguerabide, J. (1972), *Methods Enzymol. 26,* 498.
Yguerabide, J., Epstein, H. F., and Stryer, L. (1970), *J. Mol. Biol. 51,* 573.

Penicillin-Induced Secretion of a Soluble, Uncross-Linked Peptidoglycan by *Micrococcus luteus* Cells†

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ABSTRACT: Incubation of *Micrococcus luteus* cells in the presence of penicillin G leads to accumulation in the culture medium of a linear uncross-linked peptidoglycan. The amount of secreted peptidoglycan was dependent on the concentration of penicillin and paralleled the rate of cell wall synthesis, and the secretion was not accompanied by any cell lysis. Analysis of the peptidoglycan revealed that over 84% of its weight could be accounted for by *N*-acetylglucosamine, *N*-acetylmuramic acid, Ala, Glu, Gly, and Lys in the molar ratios of 2:2:3:1:1:1. The product was digested by lysozyme to yield mainly the disaccharide GlcNAc-β-(1→4)-MurNAc and the disaccharide-hexapeptide GlcNAc-β-(1→4)-MurNAc-L-Ala-D-*i*-Glu(Gly)-L-Lys-D-Ala-D-Ala. The molecular weight of the secreted peptidoglycan, estimated by ultracentrifugation, was found to be 38,000, corresponding to a chain length of approximately 50 disaccharide units, half of which are substituted by the hexapeptide on their *N*-acetylmuramic acid residues. A similar value for the molecular weight of the peptidoglycan was obtained from periodate oxidation and β-elimination studies. Muramic acid was found at the reducing end of the peptidoglycan. In addition to the uncross-linked peptidoglycan, penicillin also promoted the secretion of a hexapeptide in equimolar amounts to that of unsubstituted muramic acid residues in the secreted peptidoglycan. It is concluded that the secretion of the peptidoglycan is due to the inhibition by penicillin of the attachment of newly synthesized peptidoglycan strands by transpeptidation to the preexisting cell wall. The secretion of the free hexapeptide is assumed to be the result of the action of a penicillin-insensitive amidase which acts on the newly synthesized peptidoglycan strands.

Studies on the biosynthesis of bacterial cell walls have indicated that two types of enzymatic reactions are involved in the attachment of newly synthesized peptidoglycan to the preexisting one in the wall: (1) penicillin-insensitive transglycosylation reactions which build up linear peptidoglycan strands by a series of transfers from the two nucleotide precursors, UDP-GlcNAc and UDP-MurNAc-pentapeptide, and a lipid carrier (Strominger, 1970) and (2) penicillin-sensitive transpeptidation reactions which bind the newly synthesized linear peptidoglycan chains to suitable acceptor sites on the preexisting peptidoglycan of the cell wall (Mirelman *et al.,* 1972, 1974a; Fiedler and Glaser, 1973).

If in fact both types of reactions operate in the biosynthesis and assembly of the bacterial cell wall peptidoglycan, it could be expected that in the presence of penicillin, bacterial cells will continue to synthesize linear peptidoglycan

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chains. However, since penicillin would inhibit the attachment of the newly synthesized chains by transpeptidation to the preexisting cell wall, part of these chains should accumulate either in the cells or in the medium. A search was therefore undertaken for such linear peptidoglycan chains using *Micrococcus luteus* cells pulsed for short periods of time with radioactive L-lysine and then chased with unlabeled L-lysine in the presence of penicillin. We have indeed found that cells incubated in the presence of penicillin secrete into the medium a soluble linear peptidoglycan with a composition and structure very similar to that present in the walls of *M. luteus*. In addition to the peptidoglycan, penicillin also caused the secretion of a free hexapeptide, the structure of which is

L-Ala-D-*i*-Glu-L-Lys-D-Ala-D-Ala
|
Gly

In this paper we report on the conditions required for the formation of these materials and describe some of their chemical and physical properties.

Experimental Section

Materials. Penicillin G and 6-aminopenicillanic acid were gifts from the Beecham Research Laboratories, Betchworth, England, and vancomycin was a gift from Ely Lilly Co. UDP-MurNAc-L-Ala-D-*i*-Glu-L-[^{14}C]Lys-D-Ala-D-Ala was prepared according to the literature (Chatterjee and Park, 1964). Uniformly labeled L-[^{14}C]lysine, L-[^{14}C]alanine, and D-[^{14}C]glucose, all more than 99% pure, were obtained from the Radiochemical Centre, Amersham, England, and $NaBT_4$ was from New England Nuclear, Boston, Mass. *Streptomyces* amidase was a gift from Dr. J. M. Ghuysen. All other compounds were commercial products of the highest purity available.

Incorporation of Radioactive Precursors. Early logarithmic phase cells of *M. luteus* NCTC 2665, grown as described before (Mirelman *et al.*, 1974b), were harvested by filtration on a Millipore filter (pore size 0.45 μ) and washed with phosphate buffer (0.08 M, pH 6.8). Cells (2×10^8/ml) were resuspended in 20 ml of a minimal medium suitable for wall synthesis but inadequate for growth. The minimal medium contained glycine (1 mM), L-glutamic acid (1 mM), and D-glucose (0.0285 M) together with salts and cofactors as described (Chatterjee and Park, 1964).

Pulse-chase experiments were carried out using the early logarithmic phase cells of *M. luteus*. During the pulse period (10 min) the cells (2×10^8/ml) were suspended with shaking in 20 ml of the minimal medium to which L-alanine (0.5 mM) and L-[^{14}C]lysine (24 nmol/ml, 10 mCi/mmol) were added. In some of the experiments penicillin G (10 μg/ml) was also added. At the end of the pulse, the cells were rapidly harvested by filtration on a Millipore filter (pore size 0.45 μ) and extensively washed on the filter for 1 min with prewarmed (32°) unlabeled minimal medium (20 ml). The radioactive cells (4×10^9) were then resuspended in 20 ml of the unlabeled minimal medium, the suspension was divided into two equal parts, and penicillin G (10 μg/ml) was added to one of these. The samples were reincubated under the above conditions and aliquots (2 ml of cells) were taken at 10-min intervals and filtered on Millipore filters and the cells extensively washed with water (10 ml). The cells were then suspended by shaking in 5 ml of water. An aliquot was taken for the determination of total radioactivity and the bulk of the cells used for the preparation of walls, as described before (Mirelman *et al.*, 1974b).

For the study of the incorporation of radioactive L-lysine, unlabeled L-alanine (0.5 mM) was added to the minimal medium together with penicillin G (10 μg/ml) and after incubation of the cells for 3 min with shaking at 32°, uniformly labeled L-[^{14}C]lysine (sp act. 318 mCi/mmol) was added to a final concentration of 450 pmol/ml. In some experiments unlabeled L-lysine was added together with the L-[^{14}C]lysine so that the final concentration of the amino acid was 98 nmol/ml (sp act. 1.6 mCi/mmol). For studies involving the incorporation of radioactive L-alanine, unlabeled L-lysine (1 mM) was added to the minimal medium together with penicillin G (10 μg/ml) and, after incubation for 3 min with shaking at 32°, uniformly labeled L-[^{14}C]alanine (sp act. 159 mCi/mmol) was added to a final concentration of 1.2 nmol/ml. For the study of the incorporation of radioactive glucose, unlabeled D-glucose in the minimal medium was replaced with uniformly labeled D-^{14}C]glucose (sp act. 311 mCi/mmol) at a final concentration of 2.25 nmol/ml, and penicillin G (10 μg/ml) was added. In all cases the cells were incubated with shaking at 32° for different periods of time (usually for 30 min) and the optical density of the culture was monitored in a Klett Summerson spectrophotometer using filter No. 66. After the incubation, the cells were rapidly harvested by filtration on a Millipore filter (pore size 0.45 μ). The cells were washed with 20 ml of phosphate buffer (pH 6.8, 0.08 M) and the culture filtrate and washings were collected.

Isolation of Secreted Peptidoglycan. RADIOACTIVELY LABELED. The radioactive filtrate, obtained after harvesting the cells incubated in one of the labeled minimal media, was routinely analyzed by paper chromatography (solvent II, 20 hr) using 0.5-ml aliquots. The bulk of the filtrate was concentrated to 3 ml and fractionated by gel filtration on a Sephadex G-100 (fine) column (2.3 × 57 cm) using water as eluent. Tubes containing 2.5 ml were collected and aliquots (100 μl) taken for determination of radioactivity.

UNLABELED PEPTIDOGLYCAN. Early logarithmic cells (4 l., 2×10^8 cells/ml) were harvested by centrifugation (6000*g*, 10 min), washed twice at room temperature with 2 l. of phosphate buffer (0.08 M, pH 6.8), and then resuspended in 1 l. of unlabeled minimal medium containing penicillin G (10 μg/ml). After incubation with shaking at 32° for 30 min, the cells were collected by centrifugation (6000*g*, 10 min) and the supernatant was further clarified by passing through a Millipore filter. The volume of the supernatant was reduced to 100 ml by evaporation *in vacuo* at 25°, and the concentrated solution was extensively dialyzed in the cold against 4 × 10 l. of water using Visking No. 18 dialysis bags which exclude compounds of a molecular weight below 8000. The nondialyzable material was evaporated to a final volume of 10 ml. It was applied to a Sephadex G-100 column (2.3 × 57 cm) and eluted with water. The effluent from the column was monitored spectrophotometrically at 220 nm, and fractions (2.5 ml) were collected.

Analytical Methods. Reducing groups (Park and Johnson, 1949), total neutral sugars (Dubois *et al.*, 1956), and organic and total phosphate (Bartlett, 1959) were determined by procedures described in the literature. Nitrogen was determined by the micro-Kjeldahl technique. For the assay of neutral sugars, samples were hydrolyzed in 1 M H_2SO_4 for 6 hr at 100° and the hydrolysate was neutralized with $BaCO_3$. After evaporation, the dried residue was dissolved in water and aliquots were subjected to descending paper chromatography in solvent I. Reducing compounds were detected on paper chromatograms by the silver nitrate

reagent (Sharon and Jeanloz, 1960). Amino acids and amino sugars were detected with ninhydrin. For amino acid analysis, hydrolysis was carried out in sealed tubes with 6 M HCl at 110° for 18 hr, and analyses of the hydrolysates were done on the Beckman 120 C amino acid analyzer using the integration constants described (Mirelman and Sharon, 1967). Radioactivity in the hydrolysate was monitored by passing the effluent from the amino acid analyzer column directly through a flow scintillation spectrometer (Packard Tricarb 3200).

The extent of cross-linkage at the ϵ-NH_2 group of the L-[^{14}C]lysine incorporated into the peptidoglycan was determined by the nitrous acid deamination procedure (Mirelman *et al.*, 1974b), and cross-linkage at the α-NH_2 moiety of the L-alanine residue was determined after partial acid hydrolysis according to Schleifer and Kandler (1972) as modified by us (Mirelman and Bracha, 1974).

The ratio of radioactive D-alanine to L-alanine in acid hydrolysates was determined with the aid of D-amino acid oxidase (Ghuysen *et al.*, 1966). Assays for glycerol, glucose, and pyruvic acid in hydrolysates of the peptidoglycan were done by enzymatic techniques according to established procedures (Wieland, 1963). Uronic acid was analyzed by the carbazole reaction using D-glucono-γ-lactone as standard and pentoses were assayed by the orcinol reaction using D-ribose as standard (Dische, 1962). Reduction with $NaBT_4$ (244 mCi/mmol) was done by the procedure of McLean *et al.* (1973). The product was purified by gel filtration, hydrolyzed, and analyzed by chromatography in solvent I or on the amino acid analyzer to which the flow scintillation counter was attached. The disaccharide-pentapeptide GP-2 (Mirelman and Sharon, 1967) and *N*-acetyl-D-glucosamine were used as standards in the experiments with $NaBT_4$.

Periodate oxidation and β-elimination studies were done as described (Ward and Perkins, 1973). Digestion with *Streptomyces* amidase and lysozyme were done as described (Mirelman and Sharon, 1967).

Radioactivity was routinely determined by counting aqueous samples in an ethoxyethanol containing fluid (Bray, 1960) with an efficiency of 72%. Samples on paper or on filter were counted in a toluene containing fluid (counting efficiency 74%).

Paper Chromatography and Paper Electrophoresis. Descending paper chromatograms were run on Whatman No. 1, with solvent I, *n*-butyl alcohol–acetic acid–water (4:1:5, v/v, upper phase); II, isobutyric acid–ammonia (1 M) (5:3); III, ethyl acetate–pyridine–water (5:2:7, v/v, upper phase). Paper electrophoresis was carried out on Whatman No. 3MM paper in pH 3.5 buffer (pyridine–acetic acid–water) at 50 V/cm for 90 min.

Radioactive compounds on paper chromatograms were detected by exposure of the chromatogram to an X-ray film for 24–72 hr. Compounds were eluted from chromatograms as earlier described (Eshdat and Mirelman, 1972).

Determination of Molecular Weight. The experiments were performed with a Spinco Model E analytical ultracentrifuge equipped with an RTIC unit and Schlieren phase plate optics. Sedimentation velocity measurements of the soluble peptidoglycan (1% solution in 0.9% NaCl) were performed at 20° and at 59780 rpm. Apparent sedimentation coefficients were calculated by the least-squares method and corrected to standard conditions ($s^0_{20,w}$) (Schachman, 1957). The intrinsic diffusion coefficient ($d^0_{20,w}$) was determined with a synthetic boundary cell according to Ehrenberg (1957). The value for the partial specific volume of the secreted peptidoglycan was taken as $\bar{\nu}$ = 0.69 (Mirelman and Sharon, 1967).

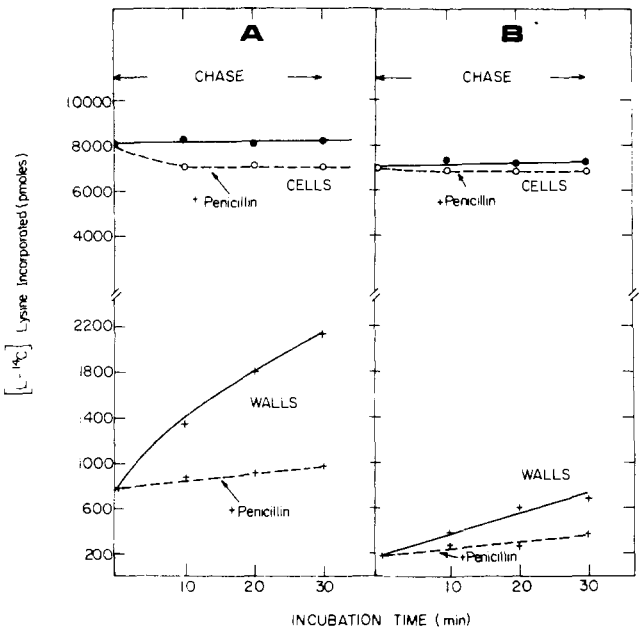

FIGURE 1: Effect of penicillin G on the incorporation of lysine into intact cells and cell walls of *M. luteus* in pulse-chase experiments. (A) Cells (2×10^8 cells/ml) were pulsed with L-[^{14}C]lysine (10 mCi/mmol, 24 nmol/ml) as described in text, after which the cells were washed. The radioactive cells (4×10^9) were resuspended in 20 ml of the unlabeled minimal medium, the suspension was divided into two equal parts, and penicillin G (10 μg/ml) was added to one of these parts. The pulsed cells were then reincubated (t = 0) and samples (4×10^8 cells) were taken at 10-min intervals and analyzed as described in the text. (B) As in A, except that the pulse was done in the presence of penicillin G (10 μg/ml). Results are expressed in pmol/2 $\times 10^8$ cells (○), or cell walls from the same number of organisms (+).

Results

When cells were pulsed with L-[^{14}C]lysine for 10 min in the absence of penicillin and then reincubated ("chased") for 30 min in an unlabeled minimal medium in the absence of penicillin, no change in the radioactivity of the intact cells was observed (Figure 1A). However, after such a chase, there was a threefold increase in the radioactivity found in the cell walls. A different result was obtained when the chase was done in the presence of penicillin (10 μg/ml); under these conditions the total radioactivity of the cells decreased by 15% while the net increase in the radioactivity of the cell walls during the chase was only 30%. Furthermore, the amount of radioactivity found in the cells after a chase done in the presence of penicillin (Figure 1A) was very close to that found at the end of a pulse of L-[^{14}C]lysine done in the presence of penicillin G (10 μg/ml) (Figure 1B).

Secretion of Soluble Peptidoglycan. EFFECT OF PENICILLIN CONCENTRATION. The minimal medium containing L-[^{14}C]lysine, in which the *M. luteus* cells were incubated for 30 min in the presence of different concentrations of penicillin G, was analyzed by paper chromatography in solvent II. At penicillin concentrations of 10 μg/ml, the amount of material which remained at the origin of chromatograms, as calculated from its L-[^{14}C]lysine content, was approximately 40 pmol/ml of culture medium. This amount was close to the difference found between the amount of radioactive lysine that was incorporated into the cell walls in the absence of penicillin (66.8 pmol/ml, Table

TABLE I: Effect of Antibiotics on the Secretion of the Soluble Peptidoglycan into the Culture Medium.[a]

Antibiotic Added	μg/ml	L-[^{14}C]Lysine[a] Intact Cells	L-[^{14}C]Lysine[a] Cell Walls	Radioact. per ml[b] Filtered Culture Medium	Radioact. per ml[b] Chromatographically Immobile Peptidoglycan
None		262	66.8	101	0.50
Penicillin G	0.1	258	69.0	88	2.68
Penicillin G	1	232	62.1	135	8.95
Penicillin G	10	136	21.4	230	38.20
Penicillin G	100	122	13.6	243	35.40
6-Aminopenicillanic acid	100	215	38.3	154	11.03
Vancomycin	100	270	14.9	33	0.05

[a] Cells (5 ml, 2 × 10^8/ml) were incubated for 30 min in a minimal medium containing L-[^{14}C]lysine (450 pmol/ml, sp act. 318 mCi/mmol). Cells and cell walls were obtained as described under Methods. Results are expressed in picomoles of L-lysine in 2 × 10^8 cells or of cell walls obtained from the same number of cells. [b] An aliquot of the culture fluid (0.5 ml) after lyophilization was chromatographed in solvent II for 20 hr. Material remaining at the origin of the chromatograms was counted. Results are expressed in picomoles of L-lysine in culture medium (1 ml) or in the chromatographically immobile peptidoglycan.

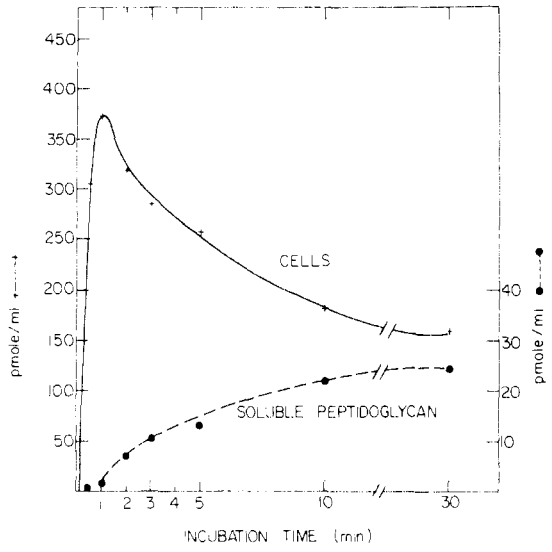

FIGURE 2: Effect of time on the incorporation of L-[^{14}C]lysine into intact cells in the presence of penicillin G (10 μg/ml) and on the secretion of soluble peptidoglycan into the medium. Isolation of the peptidoglycan is described in the text: (O–O) secreted peptidoglycan (picomoles/milliliter culture medium); (+---+) amount of L-[^{14}C]lysine in intact cells (pmol/2 × 10^8 cells).

I) and the amount that was incorporated in the presence of 10 μg/ml of penicillin G (21.4 pmol/ml). At penicillin concentrations below 10 μg/ml, the amount of material which remained at the origin of chromatograms was considerably smaller (Table I). Paper chromatographic analysis of the radioactive culture medium revealed that in addition to the material remaining at the origin, three other radioactive spots were present. One of these migrated as authentic L-[^{14}C]lysine and another as the UDP-MurNAc-pentapeptide (R_F 0.17, solvent II). The third compound migrated at a rate similar to the free hexapeptide (R_{Ala} 0.30 in solvent I and R_F 0.70 in solvent II). (For further identification of this compound see below.)

In addition to penicillin G, 6-aminopenicillanic acid was also found to cause the secretion of soluble peptidoglycan which did not migrate on paper chromatography (Table I). However, no such peptidoglycan was found in the medium of cells incubated in the presence of vancomycin at a concentration as high as 100 μg/ml, although this antibiotic caused a very marked inhibition of incorporation of L-[^{14}C]lysine into the cell wall (Table I).

EFFECT OF TIME AND CONCENTRATION OF PRECURSORS. Incubation of the cells with penicillin G (10 μg/ml) in the minimal medium labeled with radioactive lysine, for different periods of time, caused the secretion of the chromatographically immobile material as early as after 1 min of incubation. The amount of this material increased with time (Figure 2). Measurement of the amount of label in the whole cells revealed a very rapid incorporation during the first minute of incubation, followed by a slow decrease in the radioactivity of the cells. Maximal secretion of soluble peptidoglycan into the medium in the presence of penicillin G (10 μg/ml) by 2 × 10^8 cells/ml in 30 min was observed at approximately 90 nmol/ml of L-lysine (Figure 3).

Isolation of Labeled Peptidoglycan. Fractionation of culture fi' rates (20 ml) from 4 × 10^9 cells on columns of Sephadex G-100 (2.3 × 57 cm), separated between the high molecular weight, nondialyzable peptidoglycan and the low molecular weight compounds present in the medium. Four columns were run, two for the medium labeled with lysine at different concentrations (Figure 4B,C) and one each for L-alanine (Figure 4A) and D-glucose (Figure 4D). The two radioactive peaks which were obtained from each of the columns were pooled and lyophilized. Analysis of peak I from the [^{14}C]alanine-labeled medium (Figure 4A) by paper chromatography in solvents I and II revealed that the radioactivity remained exclusively at the origin of chromatograms (40 pmol/ml of culture medium). Analysis of peak II from the same column on paper electrophoresis (pH 3.5) and paper chromatography (solvents I and II) revealed the presence of three radioactive compounds (**1**, **2**, and **3**). Compounds **1** and **2** were identified as alanine and UDP-MurNAc-pentapeptide (7 pmol/ml of culture medium) by comparison of their rates of migration in the above two solvent systems with those of authentic compounds. Compound **3** migrated on paper chromatography (R_F 0.70, solvent II) and paper electrophoresis (M_{Ala} 3.0, pH 3.5) at the same rate as the radioactive hexapeptide

L-Ala-D-*i*-Glu-L-Lys-D-Ala-D-Ala
|
Gly

isolated from amidase digests of the [^{14}C]alanine-labeled soluble peptidoglycan (Figure 4A, peak I). Moreover, the migration of compound 3 in these systems was very close to that of the pentapeptide

L-Ala-D-*i*-Glu-L-Lys-D-Ala
|
Gly

isolated by digestion of GP-2 with *Streptomyces* amidase (R_F 0.68 in solvent II) (Mirelman and Sharon, 1967).

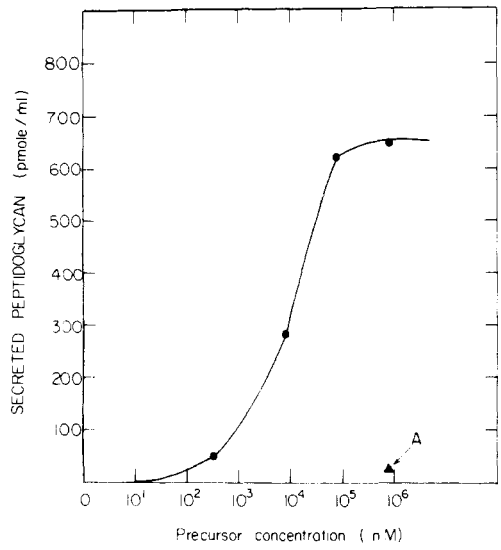

FIGURE 3: Effect of concentration of L-[^{14}C]lysine on the secretion of the soluble, chromatographically immobile peptidoglycan by *M. luteus* (2×10^8 cells/ml) incubated for 30 min in the presence of penicillin G (10 μg/ml). Results expressed in picomoles of peptidoglycan per milliliter of culture medium. (A) Amount of chromatographically immobile product obtained when cells were incubated in the absence of penicillin G.

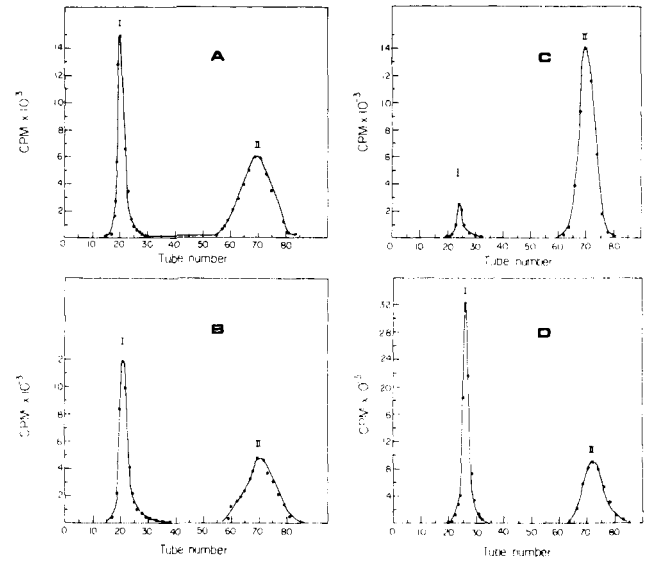

FIGURE 4: Gel filtration of the radioactively labeled culture filtrate obtained after incubation of *M. luteus* cells (20 ml, 2×10^8 cells/ml) in the minimal medium to which penicillin G (10 μg/ml) and different radioactive precursors were added. For experimental details, see text. Fractionation was on a Sephadex G-100 column (2.3 × 57 cm) using water as eluent. Fractions containing 2.5 ml were collected and aliquots (100 μl) taken and counted for radioactivity. The radioactive precursors were: (A) L-[^{14}C]alanine (159 mCi/mmol, 1.2 nmol/ml); (B) L-[^{14}C]lysine (318 mCi/mmol, 0.45 nmol/ml); (C) L-[^{14}C]lysine (1.6 mCi/mmol, 98 nmol/ml); and (D) D-[^{14}C]glucose (311 mCi/mmol, 2.25 nmol/ml).

Acid hydrolysates of the [^{14}C]alanine-labeled hexapeptide isolated from the culture medium showed that it contained two D-[^{14}C]alanine residues for every L-[^{14}C]alanine. Furthermore, partial acid hydrolysis (4 M HCl, 90 min, 100°) of the hexapeptide, followed by fractionation on the amino acid analyzer, afforded the same pattern of radioactive peptide fragments as that obtained from partial acid hydrolysates of cell walls labeled with [^{14}C]alanine in the presence of 10 μg/ml of penicillin G (Mirelman and Bracha, 1974). In particular, the dipeptide D-[^{14}C]Ala-D-[^{14}C]Ala was found in both hydrolysates.

Upon paper chromatographic analysis in solvents I and II of peak II from the [^{14}C]lysine-labeled media (Figure 4B,C) the same radioactive spots were obtained as from the [^{14}C]alanine-labeled medium. In the [^{14}C]glucose-labeled medium (Figure 4D), however, no radioactive spot that corresponded to the hexapeptide was detected in peak II.

In one of the experiments (Figure 4C), the specific activity of the labeled soluble peptidoglycan, as well as that of the free hexapeptide, was measured and found to be 1.47 mCi/mmol, a value very close to the specific activity of the L-[^{14}C]lysine added to the medium (1.6 mCi/mmol).

Analysis of acid hydrolysates (6 N HCl, 20 hr, 110°) of the soluble peptidoglycan obtained from cells incubated with D-[^{14}C]glucose in the presence of penicillin G (10 μg/ml) revealed that radioactivity resided mainly in glucosamine (48%) and muramic acid (41%). The rest of the radioactivity (11%) was found in an unidentified compound which migrated at a rapid rate on paper chromatograms (R_{Glc} 2.5, solvent I) and appeared as a nonionic compound on the amino acid analyzer (eluted after 25 min as compared to 65 min for muramic acid and 110 min for glucosamine).

Analysis of the labeled peptidoglycan done after acid hydrolysis under milder conditions (3 N HCl, 2 hr, 100°), either on paper chromatography (solvent I) or on the amino acid analyzer, afforded a smaller proportion (7%) of the unidentified compound, indicating that it may be a degradation product of the labeled amino sugars. Indeed, paper chromatograms (solvent I) of acid hydrolysates (6 N, HCl, 2 hr, 110°) of the authentic disaccharide GlcNAc-MurNAc afforded, in addition to glucosamine and muramic acid, a reducing compound which migrated at the same rate as the above compound.

Periodate oxidation of the sugar-labeled peptidoglycan followed by reduction with $NaBT_4$ afforded a radioactive compound which migrated as glycerol (R_F 0.70 in solvent III). The radioactivity found in this spot represented 0.4% of the total radioactivity in the labeled polymer and is presumably derived from oxidation of a GlcNAc moiety at the nonreducing end of the peptidoglycan.

The uncross-linked, linear structure of the secreted peptidoglycan was established by deamination with HNO_2 of the L-[^{14}C]lysine-labeled polymer (Figure 4B) followed by analysis of its acid hydrolysate (Mirelman *et al.*, 1974b). More than 99% of the L-[^{14}C]lysine residues were deaminated by this procedure and converted into α-aminocaproic acid. Partial acid hydrolysates (Schleifer and Kandler, 1972; Mirelman and Bracha, 1974) of the [^{14}C]alanine-labeled peptidoglycan (Figure 4A) failed to reveal the presence of any radioactive D-Ala-L-Ala, suggesting the absence of cross-linkages involving this dipeptide. Of the total amount of radioactive alanine present in the soluble peptidoglycan, 34.8% was of the L configuration and 65.2% of the D configuration. Treatment of the [^{14}C]alanine-labeled peptidoglycan under conditions leading to β elimination (4 M ammonia, 37°, 6 hr) followed by paper chromatography (solvent II) revealed that approximately 4.5% of total radioactivity was released in the form of a fast moving low molecular weight compound (R_F 0.72, solvent II) which migrated at a rate almost identical with that of the free hexapeptide (R_F 0.71) obtained by *Streptomyces* amidase digestion of the same polymer.

Digestion of the Labeled Peptidoglycan with Lytic En-

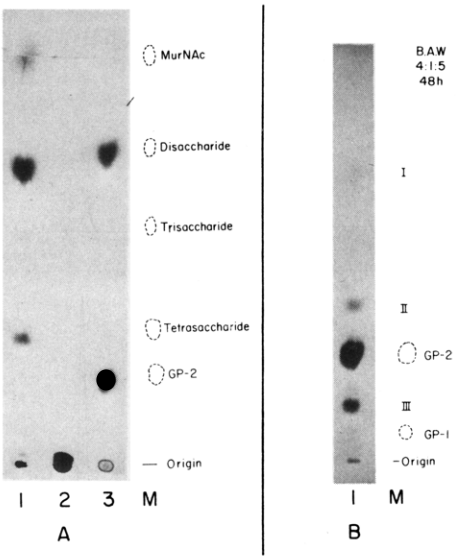

FIGURE 5: Paper chromatography autoradiogram of enzymatic digests of labeled linear peptidoglycan. (A) Peptidoglycan labeled in its amino sugar constituents (Figure 4D, peak I): (1) *Streptomyces* amidase digests of the soluble peptidoglycan; radioactive material remaining at the origin of the chromatograms accounted for 12.5% of total radioactivity; (2) intact soluble peptidoglycan; (3) lysozyme digests of soluble peptidoglycan. Radioactive material remaining at the origin accounted for 11% of total radioactivity. Markers: the disaccharide is GlcNAc-β(1→4)-MurNAc and tetrasaccharide is its corresponding dimer. GP-2 is the disaccharide–pentapeptide and GP-1 is the disaccharide–pentapeptide dimer (Mirelman and Sharon, 1967). The trisaccharide is the GlcNAc-MurNAc-GlcNAc (Chipman *et al.*, 1968). (B) Lysozyme digests of the alanine-labeled peptidoglycan (Figure 4A, peak I). Spots I, II, and III have not yet been fully identified and may represent transglycosylation products (Chipman *et al.*, 1968). Material remaining at the origin accounts for only 1.5% of the total radioactivity.

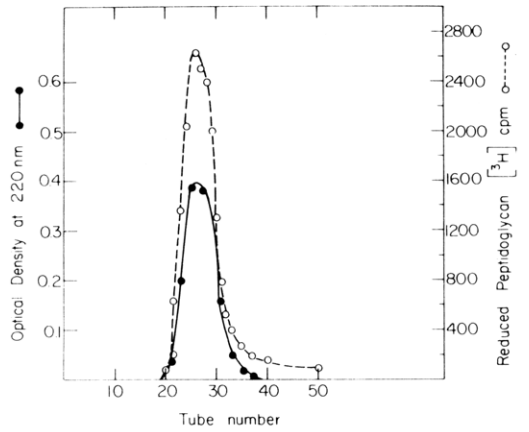

FIGURE 6: Gel filtration of the soluble peptidoglycan (3.4 mg) on a Sephadex G-100 column (2.3 × 57 cm) before and after reduction with $NaBT_4$. Elution of the soluble peptidoglycan was followed by measuring the optical density at 220 nm and that of the reduced peptidoglycan (2.4 mg) by measurement of radioactivity in 100-μl aliquots.

zymes. Lysozyme digests of the polymer obtained from D-[^{14}C]glucose as the radioactive precursor (Figure 4D, peak I) afforded a variety of radioactive compounds (Figure 5A). The free disaccharide (44%) and tetrasaccharide (3.5%) together accounted for approximately 50% of total radioactivity, and the disaccharide–hexapeptide for 45%. This is consistent with the analysis of the polymer (see Table II) which indicated a ratio of two disaccharide units per hexapeptide. *Streptomyces* amidase digests of the polymer labeled in the glycan moiety afforded a variety of oligosaccharides (Figure 5A); however, the amidase preparation is known to contain some glycosidase activity (J. M. Ghuysen, personal communication). The most abundant radioactive compound obtained by lysozyme digestion of the alanine-labeled polymer (Figure 4A) was the disaccharide–hexapeptide (85% of total radioactivity) which migrated very similarly to the disaccharide–pentapeptide GP-2 (Figure 5B) in solvent I (Mirelman and Sharon, 1967). The minor compounds (Figure 5B, I, II, and III) may be transglycosylation products of the lysozyme-catalyzed reaction (Chipman *et al.*, 1968).

Preparation and Properties of Unlabeled Soluble Peptidoglycan. The nondialyzable, high molecular weight material which was secreted into the medium by cells (2×10^{11}/l.) incubated in the presence of penicillin G (10 μg/ml) was separated by gel filtration on Sephadex G-100 (2.3 × 57 cm). Fractions of 2.5 ml were collected and the column was monitored by measuring the ultraviolet absorption at 220 nm. A number of overlapping peaks were eluted. The fast migrating peak (tubes 21–32) was pooled and after lyophilization it gave 7.8 mg of dried material. Peak II (tubes 33–55) which yielded 10.5 mg of material and peak III, which contained remnants of low molecular weight constituents of the culture medium together with material absorbing at 260 nm, were discarded. The material obtained from the first peak was rechromatographed on the same Sephadex column and emerged in tubes 21–32 as a single symmetrical peak with a recovery by weight of over 90% (Figure 6). This material was used for further analysis. It did not migrate on paper chromatography (solvent I) and gave a positive ninhydrin reaction.

The Schlieren pattern in the analytical ultracentrifuge showed a single symmetrical peak with an $s^0_{20,w}$ of 1.54 (Figure 7). Diffusion measurements gave a value of $d^0_{20,w}$ 3.45^1 and the calculated molecular weight was 38,000. The ultraviolet spectrum of this material (at 1 mg/ml) showed no absorption above 240 nm. The amino acid and amino sugar composition of the purified material is given in Table II. The sum of components by weight and the recovery of

TABLE II: Chemical Composition of the Purified Soluble Peptidoglycan.[a,b]

Component	nmol/mg	Molar Ratio
Glucosamine	1190	1.97
Muramic acid	1110	1.84
Glutamic acid	*603*	*1.00*
Glycine	573	0.95
Alanine	1650	2.73
Lysine	553	0.91
Neutral sugar[c]	180	0.29
Phosphate	<5	
Glucose	<10	
Glycerol	<10	
Nitrogen	8.53%	
Total recovery	84.1%	

[a] Analysis was carried out on dry unlabeled peptidoglycan secreted after incubation of cells in the presence of penicillin in a minimal medium. For details see text. [b] Samples were acid hydrolyzed and analyzed on the amino acid analyzer as earlier described (Mirelman and Sharon, 1967). [c] Neutral sugar determination was by the phenol–sulfuric acid procedure using glucose as standard (Dubois *et al.*, 1956).

nitrogen are over 84%, indicating that only a small amount of nonpeptidoglycan matter is present in the preparation.

Analysis by paper chromatography of an acid hydrolysate (6 N HCl, 20 hr, 110°) of the polymer revealed in addition to muramic acid and glucosamine, a reducing compound which migrated rapidly on paper (R_{Glc} 2.5, solvent 1), similar to the compound obtained earlier from acid hydrolysates of the labeled polymer. The polymer contained only traces of phosphorus (<0.16 μg of P/mg of polymer). Only minute amounts (less than 10 nmol/mg of polymer) of glycerol, pyruvic acid, or glucose were detected by enzymatic analysis. Glucose was not detected on paper chromatograms of acid hydrolysates (1 M H_2SO_4, 100°, 6 hr) of the polymer. Determination of neutral sugars by the phenol–sulfuric acid procedure (Dubois *et al.*, 1956), using D-glucose as standard, gave a value of 180 nmol/mg of polymer. No uronic acids or pentoses were detected in the polymer.

Reduction of the unlabeled polymer (2.4 mg) with $NaBT_4$ (2 mg, 13 mCi), followed by acid decomposition of the unreacted $NaBT_4$ and gel filtration on the Sephadex G-100 column to remove traces of tritium-labeled compounds not bound to the peptidoglycan, afforded a high molecular weight product (containing 1.8×10^6 dpm) the elution pattern of which was the same as that of the unmodified polymer (Figure 6). A large amount of radioactivity (5.5×10^6 dpm) was eluted in tubes 65–80 (not shown in figure).

Paper chromatographic analysis in solvent I of acid hydrolysates (3N HCl, 2 hr, 100°) of the ^{3}H-labeled polymer revealed the presence of two radioactive compounds with R_F 0.05 and R_F 0.17 as compared to [^{3}H]glucosaminitol (R_F 0.16) and [^{3}H]muramicitol (R_F 0.18). Analysis of the same acid hydrolysates on the amino acid analyzer revealed the presence of [^{3}H]muramicitol (eluted after 35 min) and a basic compound which is eluted after 120 min as compared to 110 min for [^{3}H]glucosaminitol. The nature of this reduced compound has not yet been established.

Discussion

Our present studies indicate that the difference found between the amount of radioactive precursors incorporated into intact cells of *M. luteus* in the presence and absence of penicillin is mainly due to a leakage or secretion of radioactive material into the culture medium. The amount of L-[^{14}C]lysine which was incorporated into the cell wall during a pulse-chase experiment in the absence of penicillin G was approximately twice the amount which was incorporated when the pulse was in the absence and the chase was in the presence of the antibiotic (Figure 1A). Studies aimed at the detection of labeled cell wall components released from cells incubated in the presence of penicillin revealed that the antibiotic induces the secretion into the medium of a soluble, high molecular weight material which did not migrate on paper chromatograms in three different solvents. Such a radioactive polymer was formed in the presence of penicillin when the incubation was carried out in the presence of either radioactive L-lysine, L-alanine, or D-glucose (Figure 4). Radioactive lysine and alanine were present unchanged in the soluble polymer whereas radioactive glucose was converted into glucosamine and muramic acid (over 90%). The secreted polymer was completely digested by lysozyme and by *Streptomyces* amidase (Figure 5). The main products identified in the lysozyme digest (over 85%) were the free disaccharide GlcNAc-β-(1→4)-MurNAc and the disaccharide–hexapeptide which on paper chromatography migrated at an almost identical rate as the disaccharide–pentapeptide GP-2 (Mirelman and Sharon, 1967).

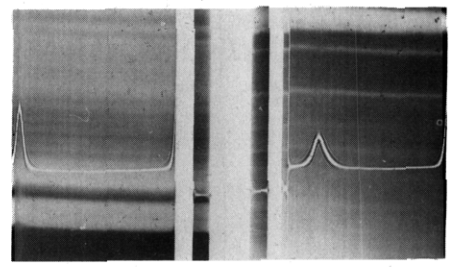

FIGURE 7: Schlieren patterns of the soluble peptidoglycan (1% in 0.9% NaCl). The photographs were taken approximately 16 (left) and 88 min after attaining a maximal speed of 59,780 rpm in the analytical ultracentrifuge.

From the amount of free di- and tetrasaccharide found in the lysozyme digests, it was concluded that approximately 50% of the muramic acid residues in the polymer were not substituted by a peptide chain. Digestion of the peptidoglycan with *Streptomyces* amidase which is contaminated by *N*-acetylmuramidase afforded a mixture of oligosaccharides (mainly di- and tetrasaccharide) as well as a free hexapeptide which, according to all indications, had the structure

L-Ala-D-*i*-Glu-L-Lys-D-Ala-D-Ala
|
Gly

The soluble peptidoglycan contained two D-alanine residues for each L-alanine residue. All the ϵ-amino groups of the lysine present in the soluble peptidoglycan were deaminated by the nitrous acid technique (Mirelman *et al.*, 1974a,b). Furthermore, no evidence for the presence of a D-Ala-L-Ala linkage was detected in partial acid hydrolysates of the polymer (Mirelman and Bracha, 1974). These findings clearly demonstrate that the soluble peptidoglycan is uncross-linked. Periodate oxidation of the peptidoglycan labeled in its glycan moiety, followed by reduction with $NaBT_4$, gave rise to radioactive glycerol suggesting that *N*-acetylglucosamine occupies the nonreducing end of the glycan chain. From the amount of the radioactive glycerol formed (0.4% of the total radioactivity of the glycan chain) it was concluded that the polymer has a chain length of approximately 50 disaccharide units.

To obtain more information on the nature of the secreted material, unlabeled peptidoglycan was prepared on a large scale at a yield of approximately 8 mg of polymer per liter (2×10^{11} cells) of minimal medium. Analysis of this material indicated that typical peptidoglycan components account for 85% of its weight (Table II). The polymer displayed a single band in the analytical ultracentrifuge (Figure 7) with a molecular weight of 38,000 indicating a glycan chain of approximately 50 disaccharide units, half of them having a hexapeptide substituted on the carboxyl of muramic acid (Figure 8). The soluble peptidoglycan did not contain any phosphate, glucose, or aminomanuronic acid—compounds known to be components of a heteropolysaccharide covalently bound to the peptidoglycan of *M. luteus* cell walls (Campbell *et al.*, 1969; Page and Anderson, 1972). The absence of phosphate is in line with the finding that no radioactivity was present in the polymer secreted from cells grown in a ^{32}P-labeled minimal medium (D. Mirelman, unpublished results).

Studies with tritiated sodium borohydride showed that muramic acid is present at the reducing end of the polymer,

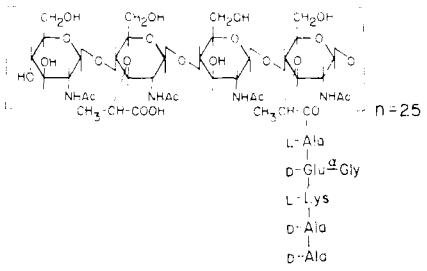

FIGURE 8: Proposed structure of the soluble uncross-linked peptidoglycan of *M. luteus*.

since [^{3}H]muramicitol was identified in acid hydrolysates of the ^{3}H-labeled peptidoglycan (65% of total radioactivity). Another, as yet unidentified, radioactive compound with basic properties was also present in the hydrolysate so that the possibility that not all glycan chains terminate in *N*-acetylmuramic acid cannot be excluded. Further evidence that muramic acid–hexapeptide is at the reducing end of the soluble peptidoglycan was obtained from β-elimination studies of the [^{14}C]alanine-labeled polymer that led to the release of a labeled peptide moiety. The amount of peptide released by β elimination (4.5%) is also in agreement with the molecular size of the polymer as determined by ultracentrifugation and periodate oxidation studies.

The secretion of the soluble peptidoglycan by *M. luteus* cells was dependent on the concentration of penicillin and of cell wall precursors in the medium. Maximal secretion (680 pmol/ml of medium, see Figure 3) was obtained at antibiotic concentrations over 10 μg/ml, and when the concentration of the four cell wall amino acids in the medium was at least 90 μM (Figure 3). No polymer, however, was detected in the medium in the absence of the antibiotic. The secretion is very fast: small amounts of peptidoglycan could be detected in the culture medium as early as after 1 min of incubation in the presence of penicillin (Figure 2). The possibility that the secretion could be the result of some autolysis of cells occurring during the incubation in the minimal medium in the presence of penicillin was not overlooked. No reduction in the turbidity of the cell cultures was observed during the short incubation periods (up to 30 min) used. Moreover, whenever measured, the specific radioactivities of the secreted ^{14}C-labeled polymer as well as of the free hexapeptide were almost the same as that of the L-[^{14}C]lysine precursor in the medium.

Penicillin G was found to promote not only the secretion of the linear peptidoglycan, but of two other compounds as well. These were identified as the free hexapeptide, which had the same chromatographic and electrophoretic properties as the hexapeptide released from [^{14}C]alanine-labeled soluble peptidoglycan by the *Streptomyces* amidase and small amounts of UDP-MurNAc-pentapeptide.

The secretion of the hexapeptide is apparently the result of a selective digestion of the peptidoglycan by a penicillin-insensitive *N*-acetylmuranyl-L-alanine amidase, which specifically utilizes the newly synthesized, linearly growing peptidoglycan strand as its substrate. It is possible that the hydrolytic action of this enzyme unmasks the N-terminal L-alanine residues of the hexapeptide, which are then utilized in the transpeptidation reaction leading to the formation of the D-Ala-L-Ala peptide cross-linkage found in this organism (Mirelman and Bracha, 1974). Studies are now underway to determine the fate of these cleaved, free hexapeptides in the cell and whether they may participate in an *in vitro* penicillin-sensitive transpeptidation reaction using the system developed by us for the study of cell wall biosynthesis (Mirelman *et al.*, 1972).

M. luteus cells appear to be very low in, or devoid of, lytic enzymes, and it is unlikely that endogenous glycosidases cleaved the peptidoglycan prior to its secretion. Thus, the chain length determined in the soluble polymer may represent the average original size of a glycan chain in the walls of this organism.

An interesting correlation was observed between the amount of soluble peptidoglycan secreted at any given penicillin concentration and the amount of labeled precursor that was incorporated into the cell walls of organisms grown in the presence of the same concentration of antibiotic. The amount of radioactivity found in the soluble peptidoglycan together with that incorporated into the cell wall was approximately equal to the amount of label which was incorporated into the cell walls of organisms grown in the absence of penicillin (Table I). These results strongly suggest that the soluble peptidoglycan is secreted into the medium because penicillin inhibits its attachment by transpeptidation to the preexisting cell wall. These findings lend further support to our previously proposed model, according to which cell wall elongation and growth are the result of a concerted action by both transglycosylation and transpeptidation, the latter serving for the covalent binding of newly synthesized linear peptidoglycan strands to free amino group acceptors on the preexisting cell wall (Mirelman *et al.*, 1972, 1974a).

Two types of peptide cross-linkages are present in *M. luteus* cell walls, the ϵ-Lys→D-Ala type and the D-Ala→L-Ala one (Schleifer and Kandler, 1972). We have recently found that the transpeptidation reactions leading to the formation of these two cross-linkages markedly differ in their sensitivity toward penicillin. Thus, whereas the formation of the ϵ-Lys→D-Ala cross-linkage is inhibited by 50% at 0.06 μg/ml, a concentration which also inhibits by 50% the growth of *M. luteus* cells, the D-Ala→L-Ala cross-linkage is inhibited by 50% at a much higher concentration of penicillin (~5 μg/ml) (Mirelman and Bracha, 1974). Our present results, which show that the amount of soluble peptidoglycan secreted at low concentrations of penicillin (0.5 μg/ml) was much smaller than at higher concentration of the antibiotic (Table I), may indicate that the two transpeptidation reactions which are responsible for the covalent binding of the linear strands to their respective acceptors on the preexisting cell wall may function independently.

While this report was in preparation, a paper appeared (Keglevic *et al.*, 1974) describing the excretion of a peptidoglycan complex from a mutant of *Brevibacterium divaricatum* grown in the presence of penicillin G. The nature of the excreted peptidoglycan complex bears some resemblance to the soluble peptidoglycan secreted by the *M. luteus* cells, though it apparently also contains appreciable amounts of phosphate, neutral sugars, and cell wall autolysis products.

The mechanism by which the soluble linear peptidoglycan chains are secreted from the cells is not yet understood. It is quite possible, however, that the penicillin promoted secretion of soluble peptidoglycan complexes is of more general occurrence in bacteria. Indeed, preliminary data show that it occurs to some extent also in *Staphylococcus aureus* H (D. Mirelman, unpublished results) though not in *Escherichia coli* W-7 (U. Schwarz, unpublished results).

The reasons for the secretion of the linear peptidoglycan

from the cells could be similar in the various cases and the antigenic as well as the clinical implications involved in the potential penicillin-induced secretion of macromolecules by penicillin-susceptible bacteria should be borne in mind.

Acknowledgments

We wish to thank Mr. N. Kaldor and his collaborators for growing the *M. luteus* cells.

References

Bartlett, G. R. (1959), *J. Biol. Chem. 234,* 466.
Bray, A. G. (1960), *Anal. Biochem. 1,* 279.
Campbell, J. N., Leyh-Bouille, M., and Ghuysen, J. M. (1969), *Biochemistry 8,* 193.
Chatterjee, A. N., and Park, J. T. (1964), *Proc. Nat. Acad. Sci. U.S. 51,* 9.
Chipman, D. M., Pollock, J. J., and Sharon, N. (1968), *J. Biol. Chem. 243,* 487.
Dische, Z. (1962), *Methods Carbohyd. Chem. 1,* 484.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28,* 350.
Ehrenberg, A. (1957), *Acta Chem. Scand. 11,* 1257.
Eshdat, Y., and Mirelman, D. (1972), *J. Chromatogr. 65,* 458.
Fiedler, F., and Glaser, L. (1973), *Biochim. Biophys. Acta 300,* 467.
Ghuysen, J. M., Tipper, D. J., and Strominger, J. L. (1966), *Methods Enzymol. 8,* 685.
Keglevic, D., Ladesic, B., Hadzija, O., Tomasic, J., Valinger, Z., Pokorny, M., and Naumski, R. (1974), *Eur. J. Biochem. 42,* 389.
McLean, C., Werner, D. A., and Aminoff, D. (1973), *Anal. Biochem. 55,* 72.
Mirelman, D., Bracha, R. (1974), *Antimicrob. Agent Chemother. 5,* 663.
Mirelman, D., Bracha, R., and Sharon, N. (1972), *Proc. Nat. Acad. Sci. U.S. 69,* 3355.
Mirelman, D., Bracha, R., and Sharon, N. (1974a), *Ann. N. Y. Acad. Sci. 235,* 326.
Mirelman, D., Bracha, R., and Sharon, N. (1974b), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 39,* 105.
Mirelman, D., and Sharon, N. (1967), *J. Biol. Chem. 242,* 3414.
Page, R. L., and Anderson, J. S. (1972), *J. Biol. Chem. 247,* 2471.
Park, J. T., and Johnson, M. J. (1949), *J. Biol. Chem. 181,* 149.
Schachman, H. K. (1957), *Methods Enzymol. 4,* 32.
Schleifer, K. H., and Kandler, O. (1972), *Bacteriol. Rev. 36,* 407.
Sharon, N., and Jeanloz, R. W. (1960), *J. Biol. Chem. 235,* 1.
Strominger, J. L. (1970), *Harvey Lect. (1968–1969) 64,* 179.
Ward, J. B., and Perkins, H. R. (1973), *Biochem. J. 135,* 721.
Wieland, O. (1963), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N.Y., Academic Press, pp 211–214.

Acyl Transfer Reactions of Retina†

J. G. Swartz* and J. E. Mitchell

ABSTRACT: Whole homogenate and subcellular fractions of the bovine retina incorporated labeled palmitate, oleate, and linoleate into phosphatidylcholine and phosphatidylethanolamine with and without added lysophosphatide acceptors. ATP and CoA were required, with the optimal concentration of CoA varying with the fatty acid supplied. Net phospholipid synthesis was noted with fatty acids and the lysophosphatides. Supplying multiple acyl donors as opposed to a single donor gave results indicating varying degrees of substrate preference and positional specificity of the transfer mediator(s) for the two lysophosphatides studied. Rod outer segments did not incorporate the fatty acids under the conditions of uptake by other retinal fractions but apparently an exchange of fatty acids occurred between lysolecithin molecules; this exchange was enhanced by addition of the 105,000*g* supernatant.

The demonstration of phospholipase activity in a number of tissues from various species (Waite, 1973; Franson *et al.*, 1971; Cooper and Webster, 1970; Blaschko *et al.*, 1967; de Haas *et al.*, 1971; Ottolenghi, 1964; Gatt, 1968) continues to raise questions concerning the removal of the monoacyl phosphoglycerides formed in these tissues. The high degree of specificity exhibited by the phospholipases and transacylases from different subcellular fractions (Robertson and Lands, 1964; Waite and Sisson, 1971) points to intricate cellular and subcellular mechanisms for the control of the concentration of fatty acids, lysophosphatides, and phospholipids with definite fatty acyl substituents (Wood and Harlow, 1969; Montfoort *et al.*, 1971).

Alterations in the fatty acid composition of structural phospholipids have been correlated with changes in the permeability of cell membranes (Walker and Kummerow, 1964) and Waite *et al.* (1969) demonstrated changes in membrane structure to accompany hydrolysis of mitochon-

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