BIOSYNTHESIS OF WALL-LINKED TEICHURONIC ACID BY A WALL-PLUS-MEMBRANE PREPARATION FROM *MICROCOCCUS LUTEUS*

Effect of antibiotics

A. WESTON and H. R. PERKINS

Department of Microbiology, Life Sciences Building, University of Liverpool, Liverpool L69 3BX, England

Received 15 February 1977

1. Introduction

Cell-walls of *Micrococcus luteus* contain two major polymers, peptidoglycan and a polysaccharide called teichuronic acid. The latter was first extracted from *M. luteus* cell walls with trichloroacetic acid and contained D-glucose and *N*-acetylmannosaminuronic acid in approximately equimolar amounts [1]. It was shown [2] to consist of repeating units of D-*N*-acetyl-mannosamin-pyranuronosyl-β(1,6)-D-glucose-linked α [1,4]. The in vitro biosynthesis of the polysaccharide by a particulate membrane preparation required the addition of the nucleotide precursors, UDP–glucose, UDP–*N*-acetyl-D-mannosaminuronic acid (UDP–ManNAcUA) and UDP–*N*-acetylglucosamine [3]. Synthesis of new polysaccharide is now demonstrated in a wall-plus-membrane preparation of *M. luteus*. As with the synthesis of peptidoglycan in this type of preparation [4–6], newly synthesized polysaccharide was either linked to pre-existing wall or found in the soluble fraction. Total polysaccharide synthesis was inhibited by bacitracin and tunicamycin but was not affected by benzylpenicillin. It was concluded that UDP–*N*-acetylglucosamine was incorporated by means of a lipid carrier into a link-piece between the polysaccharide and peptidoglycan. The inhibitory actions of tunicamycin and bacitracin suggest that the lipid carrier might be a polyisoprenyl phosphate.

2. Materials and methods

Micrococcus luteus NCTC 2665 was grown, harvested and the wall-plus-membrane preparations isolated as described previously [4–6]. UDP–*N*-Acetylhexosaminuronic acid (UDP–HexNAcUA) containing a mixture of UDP–ManNAcUA and UDP–GlcNAcUA, was prepared by penicillin inhibition of logarithmically growing cells of *M. luteus* [2,7]. UDP-D-[^{14}C]Glucose (200 mCi/mmol), UDP–D-[6-^{3}H]glucose (48 mCi/mmol) and UDP–N-acetyl[^{14}C]glucosamine (265 mCi/mmol) came from the Radiochemical Centre, Amersham. Tunicamycin [8] was given by Dr R. B. Sykes. UDP–MurNAc-Pentapeptide and all other substrates were prepared as before [6,9]. Reaction mixtures contained in a final volume of 0.2 ml: 50 mM Tris/HCl, pH 8.0, 20 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 100 mM NH_4Cl, 15 mM ATP, 2.5 mM glycine and wall-plus-membrane preparation (0.1 ml, protein concentration 3–8 mg/ml). Labelled and unlabelled substrates were added as indicated: UDP–HexNAcUA (0.075 mM), UDP–*N*-acetylglucosamine (0.5 mM), UDP–MurNAc-pentapeptide (0.125 mM) UDP–D-[^{14}C]glucose (0.05 mM, 12 mCi/mmol) or UDP–D-[^{3}H]glucose (0.05 mM, 22 mCi/mmol). The reaction mixtures were incubated at 28°C. Enzyme activity was halted by the addition of 0.2 ml sodium dodecyl sulphate (10% w/v) (SDS) and soluble polysaccharide and polysaccharide linked to pre-existing wall were isolated as in the purification of peptidoglycan [4–6]. The soluble polysaccharide remained at the origin of chromatograms. Paper chromatography was in solvent A, isobutyric acid: 0.5 M ammonia (5:3 v/v). All determinations of radioactivity and analytical methods were as previously described [6,9].

3. Results

3.1. *UDP–HexNAcUA and UDP–[^{14}C]glucose incorporation into newly synthesized polysaccharide by wall-plus-membrane preparation*

Assay mixtures were incubated for 2 h and soluble and insoluble polysaccharides were isolated. Incorporation of [^{14}C]glucose from UDP–[^{14}C]glucose into both soluble and insoluble polysaccharide was dependent on the addition of UDP–HexNAcUA. Biosynthesis of polysaccharide was not dependent on concomitant peptidoglycan synthesis but the addition of UDP–*N*-acetylglucosamine stimulated synthesis of both fractions. The best system for overall synthesis contained UDP–[^{14}C]glucose, UDP–HexNAcUA and UDP–*N*-acetylglucosamine. Addition of UDP–MurNAc-pentapeptide, whether or not UDP–*N*-acetylglucosamine was present, increased the amount of polysaccharide linked to existing cell-wall by 11% but decreased the soluble portion by 26% (table 1). Page and Anderson [3] described a soluble component called S100, which stimulated polysaccharide synthesis by a particulate enzyme preparation and they also observed a lag period of 15–20 min before synthesis commenced. With our wall-plus-membrane preparation S100 had no stimulatory effect on either type of synthesis and there was no lag-period. No radioactivity was found on chromatograms in the region of the lipid-intermediate (R_F 0.8–0.9), implying that UDP–[^{14}C]glucose was not linked to a lipid intermediate during synthesis.

Assay mixtures were set up containing all the nucleotide precursors. The effects of benzylpenicillin, bacitracin and tunicamycin on the synthesis of soluble and insoluble polysaccharide were determined. Benzylpenicillin (100 μg/ml) decreased the amount of [^{14}C]glucose incorporated into polysaccharide which was linked to existing wall. After 30 min the decrease was 6% rising to 33% after 120 min. This reduction was counterbalanced by an increase in the amount of soluble polysaccharide and overall polysaccharide synthesis was almost unchanged (fig.1). Bacitracin (100 μg/ml) inhibited the synthesis of both soluble and insoluble polysaccharide. Incorporation of [^{14}C]glucose into insoluble polysaccharide was decreased by 34% after 30 min and by 53% after 120 min, whereas soluble polysaccharide synthesis was decreased by 44% after 30 min and by 53% after 120 min (fig.1). Tunicamycin (200 μg/ml) also caused the inhibition of soluble and insoluble polysaccharide synthesis (fig.2). After 120 min, the amount linked to existing wall was decreased by 40% and the amount in the soluble fraction by 80%. Since polysaccharide synthesis was stimulated by UDP–*N*-acetylglucosamine, experiments were carried out to determine whether there were differences in the amount of UDP–*N*-acetyl[^{14}C]glucosamine incorporated into product when either or both UDP–glucose and UDP–

Table 1
Determination of requirements for polysaccharide synthesis from UDP–[^{14}C]glucose

Addition of substrates	[^{14}C]Glucose incorporated into insoluble cell-walls (pmol/mg protein/h)	[^{14}C]Glucose incorporated into soluble polysaccharide (pmol/mg protein/h)
UDP–*N*-Acetylglucosamine	67	62
UDP–*N*-Acetylglucosamine UDP–MurNAc-pentapeptide UDP–HexNAcUA	643	1412
UDP–*N*-Acetylglucosamine UDP–HexNAcUA	584	1919
UDP–HexNAcUA	335	817
UDP–HexNAcUA UDP–MurNAc-Pentapeptide	406	698

Wall-plus-membrane preparations were incubated for 2 h as described in Materials and methods.

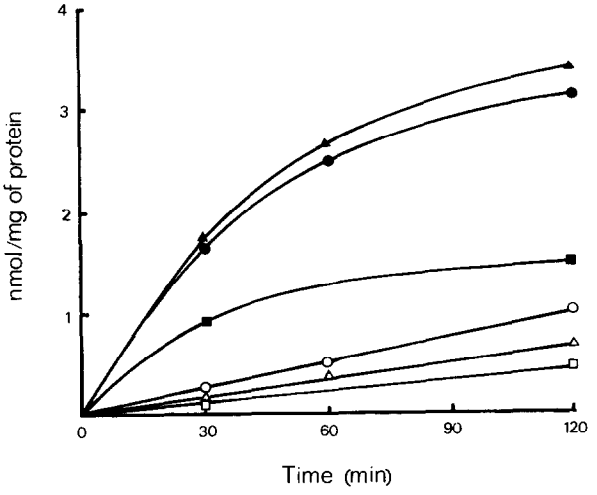

Fig.1. Effects of antibiotics UDP-[^{14}C]glucose incorporation into soluble and insoluble polysaccharide. Incubation mixtures contained the nucleotide precursors for both polysaccharide and peptidoglycan synthesis. Key: [^{14}C]Glucose incorporated into insoluble polysaccharide: Control (○). In the presence of benzylpenicillin (100 μg/ml) (△). Bacitracin (100 μg/ml) (□). SDS-Soluble polysaccharide: Control (●), in the presence of benzylpenicillin (▲), bacitracin (■).

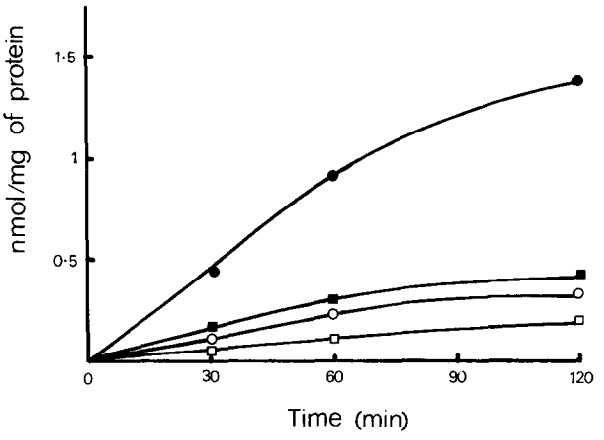

Fig.2. Effect of tunicamycin on UDP–[^{3}H]glucose incorporation into newly synthesized polysaccharide. Incubation mixtures contained nucleotide precursors for both polysaccharide and peptidoglycan synthesis. The graph shows the amount of [^{3}H]glucose incorporated into insoluble polysaccharide (open symbols) and total polysaccharide (closed symbols). Control, no addition (○,●). In the presence of tunicamycin (200 μg/ml) (□,■).

HexNAcUA were omitted from assay mixtures. None of the assay mixtures contained UDP–MurNAc-pentapeptide. In both fractions tested the incorporation of UDP–*N*-acetyl[^{14}C]glucosamine was almost entirely dependent upon the presence of UDP–HexNAcUA. The further addition of UDP–glucose produced a slight enhancement, but alone it had no effect (table 2).

4. Discussion

The synthesis of teichuronic acid by a wall-plus-membrane preparation was dependent on the addition

Table 2
UDP–*N*-Acetyl[^{14}C]glucosamine incorporation into the polysaccharide product

Fraction	Substrates added	*N*-Acetyl[^{14}C]glucosamine incorporation in product (pmol)
Residue (cell-walls)	UDP-HexNAcUA + UDP–glucose	215
	UDP–HexNAcUA	188
	UDP–Glucose	6
	None	6
Combined SDS-supernatant	UDP–HexNAcUA + UDP–glucose	258
	UDP–HexNAcUA	225
	UDP–Glucose	10
	None	11

Wall-plus-membrane preparations were incubated for 2 h as described in Materials and methods, the final concentration of UDP–*N*-acetyl[^{14}C]glucosamine being 0.05 mM (11.6 mCi/mmol). Samples were then extracted with 1% SDS in TMM buffer, first at 20°C, then at 100°C for 15 min.

of the nucleotides, UDP–glucose, UDP–HexNAcUA and UDP–*N*-acetylglucosamine, suggesting that *N*-acetylglucosamine might form a link-piece between the newly synthesized polysaccharide and the peptidoglycan. If so, the amount of polysaccharide synthesized in the absence of UDP–*N*-acetylglucosamine would be a measure of existing sites in the preparation which were primed for attachment of the polysaccharide. Most of the newly synthesized polysaccharide attached to insoluble wall was linked to 'old' sites, since concomitant peptidoglycan synthesis produced only a small effect. Addition of UDP–MurNAc-pentapeptide somewhat decreased the synthesis of soluble polysaccharide. This may have been because re-direction of UDP–*N*-acetylglucosamine towards peptidoglycan synthesis limited its availability for the initiation of polysaccharide synthesis. [^{14}C]Glucose from UDP–[^{14}C]glucose was never linked to a lipid carrier during polysaccharide synthesis, but UDP–*N*-acetylglucosamine may have been linked to a lipid-intermediate during the formation of initiation sites and link-pieces. Inhibition by tunicamycin would suggest that UDP–*N*-acetylglucosamine is linked directly to a lipid-intermediate. Although there are contradictory reports about the site of action of the antibiotic in *M. luteus* [8], it has been shown that tunicamycin prevents UDP–*N*-acetylglucosamine linking directly to the lipid intermediate in particulate enzyme preparations of *Bacillus subtilis* [10]. Tunicamycin also inhibited the incorporation of UDP–*N*-acetylglucosamine into an intermediate in the biosynthesis of the teichoic acid of *Staphylococcus aureus* [11,12]. If the polyprenyl phosphate intermediate were the same in both types of polymer synthesis, there would be competition between the UDP–MurNAc-pentapeptide for peptidoglycan synthesis and the UDP–*N*-acetylglucosamine for polysaccharide synthesis. Inhibition by bacitracin would suggest that if a lipid intermediate is involved in polysaccharide synthesis then it must first be dephosphorylated before UDP–*N*-acetylglucosamine can be linked. Penicillin reduced the amount of polysaccharide linked to pre-existing wall but the reduction was counterbalanced by an increase in soluble material similar to the effects of penicillin on peptidoglycan synthesis [5,6]. Since penicillin caused newly-formed cross-linked peptidoglycan to become soluble, any polysaccharide joined to it would also become soluble. Penicillin also allowed the expression of autolytic enzymes which could split some of the glycan bonds in the existing wall [6], so that some sites in the existing wall primed for attachment of polysaccharide would be lost to the soluble fraction together with the newly synthesized polysaccharide. In the absence of peptidoglycan synthesis the incorporation of UDP–*N*-acetyl[^{14}C]glucosamine was increased by the addition of UDP–HexNAcUA alone or with UDP–glucose, but not by the latter alone. This suggests that the availability of polysaccharide precursors may control the formation of the linkage-region. The results are supported by the recent preliminary report [13] suggesting that *N*-acetylglucosamine and *N*-acetylmannosaminuronic acid are present in a lipid intermediate of teichuronic acid synthesis.

Acknowledgements

We acknowledge the receipt from the Medical Research Council of a Programme Grant and also a Studentship to A. W.

References

[1] Perkins, H. R. (1963) Biochem. J. 86, 475–483.
[2] Hase, S. and Matsushima, Y. (1972) J. Biochem. 72, 1117–1128.
[3] Page, R. L. and Anderson, J. S. (1972) J. Biol. Chem. 247, 2471–2479.
[4] Mirelman, D., Bracha, R. and Sharon, N. (1972) Proc. Natl. Acad. Sci. USA 69, 3355–3359.
[5] Mirelman, D., Bracha, R. and Sharon, N. (1974) Ann. NY Acad. Sci. 235, 326–347.
[6] Weston, A., Ward, J. B. and Perkins, H. R. (1977) J. Gen. Microbiol. in press.
[7] Anderson, J. S., Page, R. L. and Salo, W. L. (1972) J. Biol. Chem. 247, 2480–2485.
[8] Tamura, G., Sasaki, T., Matsuhashi, M., Takatsuki, A. and Yamasaki, M. (1976) Agr. Biol. Chem. 40, 447–449.
[9] Ward, J. B. (1974) Biochem. J. 141, 227–241.
[10] Bettinger, G. E. and Young, F. E. (1975) Biochem. Biophys. Res. Commun. 67, 16–21.
[11] Bracha, R. and Glaser, L. (1976) Biochem. Biophys. Res. Commun. 72, 1091–1098.
[12] Hancock, I., Wiseman, G. and Baddiley, J. (1976) FEBS Lett. 69, 75–80.
[13] Levy, G. N., Ratnayake, J. H. and Anderson, J. S. (1976) Fed. Proc. 35, 1701.