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The Control of Synthesis of Bacterial Cell Walls

INTERACTION IN THE SYNTHESIS OF NUCLEOTIDE PRECURSORS

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Phosphoenolpyruvate–UDP-*N*-acetylglucosamine enolpyruvyltransferase, UDP-*N*-acetylglucosamine pyrophosphorylase and CDP-glycerol pyrophosphorylase activities were demonstrated in soluble extracts from *Bacillus licheniformis* A.T.C.C. 9945. The effect of various nucleotides, sugar nucleotides and sugar phosphates on the nucleotide pyrophosphorylases was investigated. UDP-*N*-acetylglucosamine pyrophosphorylase was inhibited by UDP-MurAc-pentapeptide (UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine) and CDP-glycerol. CDP-glycerol pyrophosphorylase was inhibited by UDP-MurAc-pentapeptide and stimulated by UDP-*N*-acetylglucosamine. Interaction between a precursor of one cell-wall polymer and an enzyme involved in the synthesis of a precursor of a second polymer has therefore been demonstrated. The possible role of such interaction in the control of bacterial cell-wall synthesis is discussed. Of the other compounds investigated mono- and di-nucleotides were shown to be inhibitory, indicating that nucleotide pyrophosphorylase activities may be influenced by the energy charge of the cell.

Inter-relation between the biosynthesis of peptidoglycan and teichoic acid in particulate enzyme preparations from *Staphylococcus lactis I* 3 (Watkinson *et al.*, 1971), *Bacillus subtilis* N.C.T.C. 3610 and *Bacillus licheniformis* A.T.C.C. 9945 (Anderson *et al.*, 1972) has been demonstrated. The precursor nucleotides for one polymer were shown to inhibit the synthesis of the other polymer, the inhibition being due, at least in part, to competition for the common polyisoprenyl phosphate acceptor. It was proposed that the concentration of the various nucleotide precursors might determine the distribution of the polyisoprenyl phosphate between the various polymer-synthesizing enzyme systems. If this is indeed the case then the concentration of the precursors must be carefully regulated and possibly the precursors for one polymer might be involved in control of the synthesis of the precursors for the other.

UDP-*N*-acetylglucosamine pyrophosphorylase and phosphoenolpyruvate – UDP-*N*-acetylglucosamine enolpyruvyltransferase (phosphoenolpyruvate–UDP-GlcNAc enolpyruvyltransferase) are the first specific enzymes concerned in the biosynthesis of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine (UDP-MurAc-pentapeptide), the precursors of peptidoglycan (Scheme 1). CDP-glycerol pyrophosphorylase, on the other hand, is the first specific enzyme for the synthesis of CDP-glycerol, a precursor of poly(glycerol phosphate), poly(glycerol phosphate glucose) and poly(glycerol phosphate galactose), the wall teichoic acids of *B. licheniformis* (Burger & Glaser, 1964, 1966). These three enzymes seemed likely targets for control and were selected for investigation in this study. Phosphoenolpyruvate–UDP-GlcNAc enolpyruvyltransferase is subject to feedback inhibition by UDP-*N*-acetylmuramic acid and UDP-MurAc peptides (Anwar *et al.*, 1971; Venkateswaran *et al.*, 1973; Wickus & Strominger, 1973).

Experimental

Materials

[1-^{14}C]Acetic anhydride and [1-^{14}C]glycerol were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. D-[U-^{14}C]Glyceric acid 3-phosphate was obtained from Calbiochem Ltd., London W1H 1AS, U.K. Other biochemicals were purchased from Sigma Chemicals, St. Louis, Mo., U.S.A. The solvents and chemicals used were of analytical grade and were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

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Radioactivity on chromatograms was determined quantitatively by counting appropriate areas of the paper in a scintillation counter (Beckman LS-150) in a liquid scintillant of the following composition: toluene, 2 litres; 2,5-diphenyloxazole, 8g; 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 0.2g. Radioactive samples in aqueous solution were counted in the liquid scintillant to which 1 litre of Triton X-100 (Lennig Chemicals Ltd.) had been added (Patterson & Green, 1965).

Other methods

Paper chromatography. This was carried out on Whatman no. 1 paper with the descending solvent system ethanol–1M-ammonium acetate, pH3.6 (5:2, v/v) (Paladini & Leloir, 1951). Products were detected by the alkaline $AgNO_3$ reagent for sugars and polyols (Trevelyan *et al.*, 1950) and molybdate–$HClO_4$ for phosphates (Hanes & Isherwood, 1949).

Culture conditions. The organism was *Bacillus licheniformis* A.T.C.C. 9945, grown as described by Hancock & Baddiley (1972).

Preparation of crude enzymes. Phosphoenolpyruvate–UDP-GlcNAc enolpyruvyltransferase was prepared by the method of Venkateswaran & Wu (1972). Acetone-dried cells (200mg) were extracted with 2ml of 0.05M-Tris–HCl buffer, pH7.4, containing 4mM-dithiothreitol, at 0°C for 15min. After centrifugation at 15000*g* for 10min, the supernatant (protein content 10.8mg/ml) was used as the crude enzyme preparation.

A crude preparation containing both UDP-GlcNAc and CDP-glycerol pyrophosphorylases was prepared as follows. Washed cells (10g wet wt.) were suspended in 50ml of 0.05M-sodium phosphate buffer, pH7.5, containing lysozyme (50mg), ribonuclease (2mg) and deoxyribonuclease (2mg). The cell suspension was incubated at 37°C until lysis was complete (30min) and was then centrifuged at 25000*g* for 20min. Protein was precipitated from the supernatant by the addition of finely ground $(NH_4)_2SO_4$ to give a 90% saturated solution of the salt, followed by centrifugation at 25000*g* for 30min. The precipitated protein was then dissolved in water and the solution was dialysed overnight against 5mM-Tris–HCl buffer, pH8.0. The dialysed solution containing about 25mg of protein/ml was used as the crude enzyme preparation for both pyrophosphorylases.

Preparation of substrates. CDP-glycerol was synthesized from the morpholidate of CMP (Sigma) by the general method of Roseman *et al.* (1961). D-[1,3-^{14}C]Glycerol 1-phosphate was prepared by the method described by Baddiley *et al.* (1968) and *N*-[*acetyl*-^{14}C]acetylglucosamine 1-phosphate by the method of Distler *et al.* (1958). UDP-MurAc-pentapeptide was prepared by the method described by Anderson *et al.* (1972). [U-^{14}C]Phosphoenolpyruvate was prepared enzymically from D-[U-^{14}C]glyceric acid 3-phosphate by the procedure of Gunetileke & Anwar (1968) as modified by Venkateswaran & Wu (1972) and had a specific radioactivity of 1.03×10^6c.p.m./μmol.

Measurement of enzymic activities. The activity of all three enzymes was measured as the amount of radioactively labelled nucleotide synthesized under the conditions below, which are not necessarily those for maximum enzyme activity. Phosphoenolpyruvate–UDP-GlcNAc enolpyruvyltransferase was determined as described by Venkateswaran & Wu (1972). Incubation mixtures contained NaF (1μmol), UDP-GlcNAc (0.5μmol), [U-^{14}C]phosphoenolpyruvate (0.013μmol) and crude enzyme (50μl), in a final volume of 0.1ml.

Incubation mixtures for the determination of UDP-GlcNAc pyrophosphorylase contained UTP (0.5μmol), $MgCl_2$ (0.5μmol), Tris–HCl buffer, pH 8.0 (1.0μmol), *N*-acetylglucosamine 1-phosphate (0.2μmol), *N*-[*acetyl*-^{14}C]acetylglucosamine 1-phosphate (0.01μmol, 5.27×10^4c.p.m.) and crude enzyme (50μl), in a final volume of 0.12ml. Reaction mixtures were incubated at 37°C for 30min, boiled for 2min, and then applied as bands to Whatman no. 1 paper and subjected to chromatography in ethanol–1M-ammonium acetate, pH3.6 (5:2, v/v), for 18h. Radioactive areas on chromatograms corresponding to UDP-GlcNAc were cut out and counted. Approximately 0.15μmol of UDP-GlcNAc was synthesized under these conditions.

Incubation mixtures for the determination of CDP-glycerol pyrophosphorylase contained CTP (0.4μmol), sodium DL-glycerol 1-phosphate (3.2μmol), D-[1,3-^{14}C]glycerol 1-phosphate (0.00075μmol, 3.36×10^7c.p.m./μmol), $MgCl_2$ (0.2μmol), Tris–HCl buffer, pH8.0 (30μmol), and 40μl of enzyme solution, in a total volume of 100μl. Reaction mixtures were incubated at 37°C for 15min and the reaction was terminated by placing the mixtures in a boiling-water bath for 2min. The mixtures were chromatographed as described above and areas corresponding to CDP-glycerol counted. The amount of CDP-glycerol synthesized was approximately 34nmol/min per mg of protein.

The standard deviation for the nucleotide pyrophosphorylase determinations is $\pm1.66\%$.

Results

Phosphoenolpyruvate–UDP-N-acetylglucosamine enolpyruvyltransferase

Under standard conditions about 2nmol of UDP-GlcNAc enolpyruvate was synthesized by the enzyme preparations. CDP-glycerol had no inhibitory effect on phosphoenolpyruvate–UDP-GlcNAc enolpyruvyltransferase activity at a concentration (5mM)

Table 1. *Effect of CDP-glycerol and UDP-MurAc-pentapeptide on phosphoenolpyruvate–UDP-N-acetylglucosamine enolpyruvyltransferase from B. licheniformis*

Enzyme was preincubated with inhibitor at the concentration shown for 10min at 37°C. After the addition of UDP-GlcNAc and [U-^{14}C]phosphoenolpyruvate as described in the Experimental section, mixtures were incubated for a further 30min and then UDP-GlcNAc-enolpyruvate was assayed.

Effector	UDP-GlcNAc-enolpyruvate formed (c.p.m.)
None	2028
CDP-glycerol (5mM)	2093
UDP-MurAc-pentapeptide (1mM)	610

Table 2. *Effect of various nucleotides and sugar nucleotides on UDP-N-acetylglucosamine pyrophosphorylase activity*

Incubations were carried out as described in the text with effectors added to give a final concentration of 4.2mM. Enzyme activity was measured as UDP-*N*-[*acetyl*-^{14}C]acetylglucosamine synthesized. With CDP-glucose, CDP-choline, UDP-glucose, TDP-glucose, ATP, CMP, CDP, CTP, UMP, UDP, P_i, DL-glycerol 1-phosphate, glucose 1-phosphate, fructose 1,6-diphosphate and phosphoenolpyruvate the relative enzyme activity was 99–104.

Effector	Relative enzyme activity
None	100
UDP-GlcNAc	94.8
PP_i	91.4
UDP-MurAc-pentapeptide	79.4
CDP-glycerol	88.4
AMP	88.2
ADP	89.8

equivalent to that of the substrate, UDP-GlcNAc (Table 1). By contrast, UDP-MurAc-pentapeptide was a potent inhibitor at a much lower concentration (1mM). This marked inhibition by the pentapeptide derivative is similar to that observed in *E. coli* and *B. cereus* by Venkateswaran *et al.* (1973).

UDP-N-acetylglucosamine pyrophosphorylase

Since CDP-glycerol did not inhibit phosphoenolpyruvate–UDP-GlcNAc enolpyruvyltransferase the effect of this and other nucleotides on UDP-GlcNAc pyrophosphorylase was studied (Table 2). Both of the products of the reaction, UDP-GlcNAc and PP_i, inhibited the enzymic activity. Of the other compounds tested only CDP-glycerol, UDP-MurAc-pentapeptide, AMP, ADP and glucose 6-phosphate had any significant effect; these all inhibited the enzyme.

The inhibition by CDP-glycerol was interesting, as this is an intermediate in teichoic acid synthesis, and the effect of this nucleotide was studied further. As

Table 3. *Effect of CDP-glycerol on UDP-N-acetylglucosamine pyrophosphorylase activity*

Incubations were carried out as described in the text with CDP-glycerol added to give the final concentration indicated. Enzyme activity was measured as UDP-*N*-[*acetyl*-^{14}C]acetylglucosamine synthesized.

Concn. of CDP-glycerol (mM)	Relative enzyme activity
0.0	100.0
1.7	102.8
4.2	90.1
8.3	76.3
12.5	72.0
16.7	60.7

Table 4. *Effect of various nucleotides and metabolites on CDP-glycerol pyrophosphorylase from B. licheniformis*

Incubations were carried out as described in the text with effectors added to give the final concentrations indicated. The enzyme activity was measured as CDP-[^{14}C]glycerol synthesized. Each value is the mean of two determinations. With ADP-ribose, ADP-glucose, ADP-mannose, CDP-choline, CDP-glucose, UDP-glucose, UDP-galactose, UDP-glucuronic acid, P_i and phosphoenolpyruvate the relative enzyme activity was 98–103 (2.5mM effector) or 96–103 (20mM effector).

	Relative enzyme activity	
Concn. of effector (mM) ...	2.5	20
Effector		
None	100.0	100.0
PP_i	79.1	23.8
CDP-glycerol	85.9	43.3
UDP-MurAc-pentapeptide	104.8	22.2
UDP-GlcNAc	108.8	103.1
AMP, UMP, CMP	99–101	92–96
ADP, UDP, CDP	88–94	48–69
ATP, UTP	78–83	34–38

shown in Table 3, increasing concentrations of CDP-glycerol resulted in an increased inhibition of the pyrophosphorylase.

CDP-glycerol pyrophosphorylase

The possible interaction of peptidoglycan precursors with an enzyme in teichoic acid precursor synthesis was also studied. The effect of a variety of nucleotides and sugar nucleotides on the activity of CDP-glycerol pyrophosphorylase is shown in Table 4. As was expected, the reaction products, CDP-glycerol and PP_i, inhibited the enzyme. Nucleoside diphosphates and triphosphates also inhibited the reaction. Of particular interest in this study were the effects of UDP-GlcNAc and UDP-MurAc-pentapeptide. UDP-GlcNAc stimulated the enzyme at concentrations up to 5mM. At higher concentrations the activity began to fall below the observed maximum but the enzyme was not strongly inhibited even at concentrations as high as 40mM (Table 4; Fig. 1). UDP-MurAc-pentapeptide at concentrations below 2.5mM also stimulated the enzyme slightly but at concentrations above 2.5mM there was inhibition, which increased with increasing inhibitor concentration (Table 4 and Fig. 1).

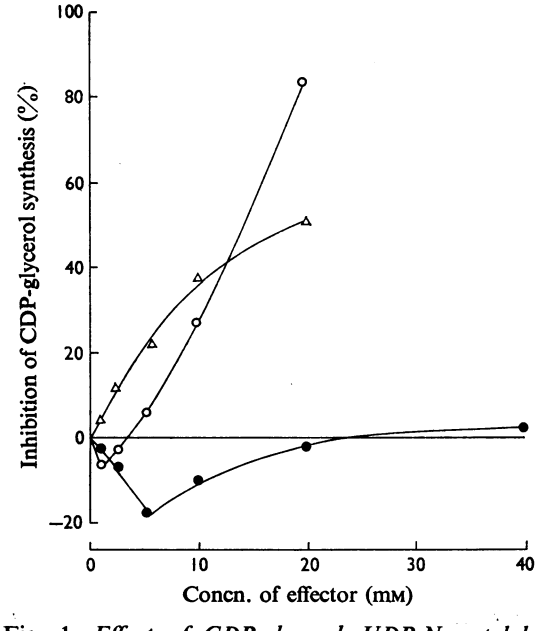


Fig. 1. *Effect of CDP-glycerol, UDP-N-acetylglucosamine and UDP-MurAc-pentapeptide on CDP-glycerol pyrophosphorylase from B. licheniformis*

Incubations were carried out as described in the text with effectors added at the concentrations indicated. The enzyme activity was assayed as CDP-[^{14}C]glycerol synthesized. ●, UDP-GlcNAc; ○, UDP-MurAc-pentapeptide; △, CDP-glycerol.

Discussion

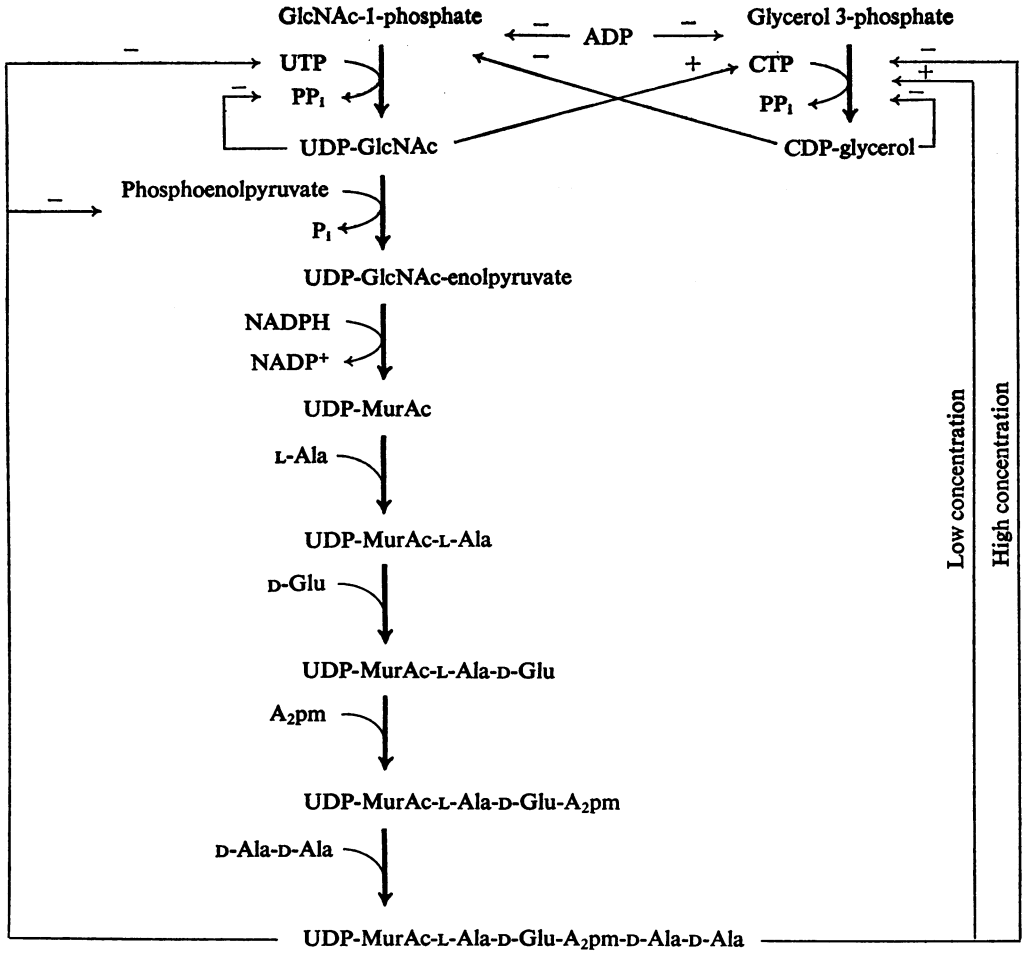
The results presented here indicate that the enzymes, phosphoenolpyruvate–UDP-GlcNAc enolpyruvyltransferase, UDP-GlcNAc pyrophosphorylase and CDP-glycerol pyrophosphorylase are possible control points in the synthesis of the cell wall in *B. licheniformis*.

The pyrophosphorylases investigated were inhibited by the products of the reactions that they catalysed. This may well be a mass-action effect, as the reactions are reversible. Glaser (1965) has investigated the inhibitory effect of CDP-glycerol on CDP-glycerol pyrophosphorylase from *B. subtilis* and has shown that this nucleotide competes with CTP.

The results in Tables 2 and 4 indicate that of all the sugar nucleotides investigated only those specifically involved in peptidoglycan and teichoic acid synthesis affected the enzyme activity. UDP-MurAc-pentapeptide inhibited all three enzymes, thus influencing not only synthesis of the precursors for peptidoglycan but also synthesis of CDP-glycerol, an intermediate for teichoic acid synthesis. CDP-glycerol was shown to inhibit UDP-GlcNAc pyrophosphorylase and therefore affects the synthesis of both peptidoglycan precursors. The only stimulatory effects observed were those of UDP-GlcNAc and UDP-MurAc-pentapeptide, at low concentrations, on CDP-glycerol pyrophosphorylase. It is not possible at this stage to explain the dual effect of UDP-MurAc-pentapeptide. These interactions between the enzymes for the synthesis of the precursors of one polymer and the precursors of the other polymer would ensure the correct balance between the nucleotides required if the concentrations of these at the membrane were to determine the distribution of polyisoprenyl phosphate between the polymerizing enzymes (Anderson *et al.*, 1972).

Cell-wall synthesis must depend on the energy charge of the cell. ADP, AMP and glucose 6-phosphate all inhibited UDP-GlcNAc pyrophosphorylase (Table 2), whereas ADP, UDP and CDP inhibited CDP-glycerol pyrophosphorylase (Table 4). It therefore appears that sugar nucleotide synthesis and synthesis of CDP-glycerol may be influenced by the energy charge of the cell. CDP-glycerol pyrophosphorylase was also inhibited by ATP and UTP. The nature of this inhibition has not yet been established.

The presence in cell extracts of *B. subtilis* and *B. licheniformis* of CDP-glycerol pyrophosphatase was reported by Glaser (1965). This enzyme has a high K_m for CDP-glycerol, and Glaser (1965) proposed that the enzyme participates in the regulation of CDP-glycerol concentration by hydrolysing this nucleotide



Scheme 1. *Inter-relations in the synthesis of UDP-GlcNAc, UDP-MurAc-pentapeptide and CDP-glycerol*

Stimulatory effects are indicated as positive, inhibitory effects as negative. A_2pm, diaminopimelate.

when it reaches high cellular concentrations, a situation that would arise only if CDP-glycerol were to be over-produced. This enzyme was not detected in our extracts, and it is possible that the synthesis of the pyrophosphatase would be dependent on the conditions of growth of the organism. The growth medium used in this work contained a higher concentration of phosphate than did that used by Glaser (1965). As the proportion of teichoic acid in walls can vary according to the availability of phosphate (Ellwood & Tempest, 1969), the phosphate concentration of the medium may be a determining factor in the synthesis of the pyrophosphatase.

The inter-relations between the nucleotide precursors are summarized in Scheme 1. Further work with purified enzymes will be necessary to determine the relative importance of the regulatory processes involved and the mechanism of the inhibitory effects.

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