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The Effect of Magnesium Ion Deprivation on the Synthesis of Mucopeptide and its Precursors in *Bacillus subtilis*

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1. Mg^{2+} or Mn^{2+} starvation causes suspensions of *Bacillus subtilis* strain W 23 to accumulate bound amino sugars that are soluble in trichloroacetic acid. 2. The presence of chloramphenicol or puromycin produces higher intracellular concentrations of amino sugars during Mg^{2+} starvation, but neither compound can stimulate the accumulation when Mg^{2+} is present. 3. The major component of the amino sugar fraction extracted from cells deprived of Mg^{2+} is a nucleotide containing uridine, phosphorus, *N*-acetylmuramic acid, alanine, glutamic acid and $\alpha\epsilon$-diaminopimelic acid in the molar proportions of 1:2:1:3:1:1. This compound represents at least 80% of the bound *N*-acetylhexosamine extracted by trichloroacetic acid. 4. Studies of the binding of this nucleotide with vancomycin support the proposal that it is the mucopeptide precursor UDP-*N*-acetylmuramyl-L-alanyl-D-glutaminyl-$\alpha\epsilon$-diaminopimelyl-D-alanyl-D-alanine. 5. A method is described for the isolation of this material labelled with $[^3H]\alpha\epsilon$-diaminopimelic acid. 6. When Mg^{2+} is supplied to cells previously starved of Mg^{2+}, the accumulated pool of amino sugars rapidly decreases. 7. The biosynthesis of mucopeptide is inhibited by 35–50% under conditions of Mg^{2+} starvation. The presence of EDTA increases this inhibition to 70%. The amount of *N*-acetylhexosamine that accumulates is balanced exactly by the associated fall in mucopeptide synthesis. 8. 'Chase' experiments show that the accumulated *N*-acetylhexosamine compound is utilized in mucopeptide synthesis.

Inhibition of bacterial cell-wall synthesis often causes a concomitant intracellular accumulation of uridine nucleotides that contain *N*-acetylmuramic acid and some, or all, of the amino acids found in the mucopeptide of the wall. Four different ways are known for producing the accumulation of these nucleotides in suspensions of bacteria: (i) by the addition of antibiotics that inhibit cell-wall synthesis, e.g. penicillin (Park, 1952), vancomycin (Jordan, 1961) and cycloserine (Ciak & Hahn, 1959); (ii) by the addition of 5-fluorouracil, which gives the 5-fluorouridine derivatives of the nucleotides produced in the presence of penicillin (Rogers & Perkins, 1960); (iii) by incubating *Staphylococcus aureus* in a medium containing an excess of glycine (Strominger & Birge, 1965); (iv) by depriving the bacteria of an essential amino acid (Strominger & Threnn, 1959). The UDP derivatives that Park (1952) isolated from *S. aureus* H treated with penicillin were identified by Strominger (1959) and Strominger & Threnn (1959) as UDP-*N*-acetylmuramic acid, UDP-*N*-acetylmuramyl-L-alanine and UDP-NAMur†-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (UDP-NAMur-pentapeptide). In some organisms diaminopimelic acid replaces lysine in the largest of these nucleotides and in the mucopeptide of the cell wall. It has been demonstrated that the UDP-NAMur-pentapeptides of *S. aureus*, *Escherichia coli* and *Corynebacterium xerosis* are synthesized by sequential enzymic addition of L-alanine, D-glutamic acid, L-lysine (or DAP) and D-alanyl-D-alanine to UDP-*N*-acetylmuramic acid (Ito & Strominger, 1962*a*,*b*; Comb, 1962; Ito, Nathenson, Dietzler, Anderson & Strominger, 1966). All of these reactions are catalysed by soluble enzymes that are activated by Mg^{2+} or Mn^{2+} ions. The close similarity of the composition of accumulated UDP-NAMur-pentapeptide to that of the mucopeptide in *S. aureus* led to the suggestion that UDP-NAMur-Ala-Glu-Lys-Ala-Ala might be an intracellular precursor of cell-wall mucopeptide (Park & Strominger, 1957). This hypothesis was substantiated by the work of Chatterjee & Park (1964) and Meadow,

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† Abbreviations: NAMur (in sequences), *N*-acetylmuramic acid; DAP, $\alpha\epsilon$-diaminopimelic acid.

Anderson & Strominger (1964), who reported the cell-free synthesis of products resembling mucopeptide from radioactively labelled UDP-NAMur-pentapeptide. Anderson, Matsuhashi, Haskin & Strominger (1965) and Izaki, Matsuhashi & Strominger (1966) have since shown that in the cell-free synthesis of the mucopeptide of *S. aureus*, *E. coli* and *Micrococcus lysodeikticus* the NAMur-pentapeptide moiety is attached first to a polyisoprenyl phosphate present in the membrane preparation, then glycosylated by the addition of *N*-acetylglucosamine and finally transferred to an endogenous acceptor, where cross-linking of the peptide chains takes place. The presence of Mg^{2+} or Mn^{2+} is essential to activate all the enzymes involved in this pathway as well as those required for the biosynthesis of the intracellular precursors. Antibiotics that inhibit cell-wall synthesis inhibit specifically one of the stages in this sequence of reactions. The results presented in this paper show that for *Bacillus subtilis* W23 deprivation of Mg^{2+} causes an inhibition of mucopeptide synthesis and a corresponding increase in the intracellular pool of wall precursors. A preliminary report of some of this work has already appeared (Garrett, 1968).

MATERIALS AND METHODS

Chemicals. Chloramphenicol was obtained from Parke, Davis and Co. (Hounslow, Middx.). D-Cycloserine was from Sigma (London) Chemical Co. (London S.W.6), muramic acid was a gift from Dr C. D. Warren of this Institute and vancomycin was a gift from Eli Lilly and Co. Ltd. (Basingstoke, Hants.). DL-[G-^{3}H]Aspartic acid (specific radioactivity 960mc/m-mole) was obtained from The Radiochemical Centre (Amersham, Bucks.).

Organism. B. subtilis strain W23 was kept as a suspension of spores in distilled water at 0–4°.

Growth of bacteria. Vegetative cells were grown under the conditions described by Janczura, Perkins & Rogers (1961). A casein acid hydrolysate medium of the following composition was used: casamino acids (Difco), 10g.; KH_2PO_4, 2·72g.; water to 900ml. The pH was adjusted to 7·2 with NaOH soln. This medium was sterilized by autoclaving at 120° for 20min. and then supplemented by 100ml. of 17mM-$MgSO_4$, 1·0ml. of a solution containing 0·1mg. each of $ZnSO_4,7H_2O$, $CoCl_2,6H_2O$, ammonium molybdate, $MnCl_2,4H_2O$ and $CuSO_4,5H_2O$/l. and 1ml. of a solution containing 0·156g. of $Fe(NH_4)_2(SO_4)_2,6H_2O$ and 0·168g. of citric acid/100ml. of water. Complete medium (50ml.) supplemented by 3·3mM-$MgSO_4$ and 0·5% of glucose was inoculated with 0·1ml. of a suspension of spores of *B. subtilis* W23 (containing 3×10^8 viable organisms/ml.) and incubated at 35° overnight without shaking. This culture was diluted with 950ml. of the same medium and shaken in a 5l. flask at 35°. Spekker absorptiometer measurements were used to determine cell density in mg. dry wt./ml. The bacteria were harvested early in the exponential phase of growth (0·3–0·4mg. dry wt./ml.) by centrifuging at room temperature and resuspended in water at 35° to give a concentration of 3–4mg. dry wt./ml. Portions of this suspension were transferred as quickly as possible to the medium described below.

Incubation of cells. Micro-organisms were suspended at a final concentration of 1–2·5mg. dry wt./ml. in a medium referred to as 'amino acids–glucose–phosphate solution' containing (final concentrations given): DL-alanine, DL-glutamic acid, DL-aspartic acid, glycine (each at 0·2mg./ml.), glucose (10mg./ml.) and sodium–potassium phosphate buffer, pH 7·0 (0·05M with respect to phosphate). Each suspension was shaken at 35° in a conical flask five times larger than the volume of suspension it contained. Subsequent treatments are described below.

Measurement of mucopeptide synthesis. Tritiated aspartic acid (0·5μc/ml.) was included in the incubation medium. Duplicate samples (5ml. or 10ml.) of the bacterial suspension were transferred to centrifuge tubes containing sufficient ice-cold 50% (w/v) trichloroacetic acid to bring the final acid concentration to 5%. Mucopeptide was prepared by the method of Park & Hancock (1960) and hydrolysed in 6M-HCl in a sealed tube at 105° for 16hr. The HCl was removed *in vacuo* over anhydrous $CaCl_2$ and NaOH, and a portion of redissolved hydrolysate was counted in the Packard Tri-Carb liquid-scintillation spectrometer. The DAP content of a second portion of hydrolysate was determined by the method of Work (1957) or the paper-chromatographic method described below. From these measurements the specific radioactivity of the mucopeptide DAP was determined. When samples of the HCl hydrolysates were run on Whatman no. 4 chromatography paper in solvents A and B (see below), it was shown that [^{3}H]DAP accounted for at least 98% of the total radioactivity in the hydrolysate. Alanine was the only other ^{3}H-labelled compound detected.

Extraction and determination of intracellular N-*acetylhexosamines.* Duplicate samples (5ml. or 10ml.) of the bacterial suspension were transferred to cold tubes and centrifuged at 1000*g* for 10min. The cell pellet was extracted with trichloroacetic acid by the method of Strominger (1957) except that a higher concentration of acid was used. The minimum concentration of trichloroacetic acid required for the maximum extraction of *N*-acetylhexosamine from *B. subtilis* W23 was 8% (w/v). A wet pad containing 10–20mg. dry wt. of cells was finely suspended in ice-cold 10% (w/v) trichloroacetic acid (1·0ml.) and kept at 0° for 1hr. The residue was collected by centrifuging and washed once with 5% trichloroacetic acid (0·5ml.). The extract and washing fluid were combined. The trichloroacetic acid was removed by three extractions with 3ml. of diethyl ether, the aqueous phase was neutralized with 1M-NaOH and the residual ether was evaporated at room temperature in a stream of air. The volume of the extract was finally adjusted to 2·0ml. The free *N*-acetylhexosamine content of this solution was measured by the method of Reissig, Strominger & Leloir (1955). Total *N*-acetylhexosamines were measured by the same method after hydrolysis of a portion of the extract in 0·1M-HCl in a boiling-water bath for 5min. and neutralization of the acid by 1M-NaOH with phenolphthalein as an internal indicator. The difference between the results of these two determinations gives the amount of acid-labile (bound) *N*-acetylhexosamine in the sample.

Derivatives of *N*-acetylhexosamine 1-phosphate can only participate in the Morgan–Elson reaction after the reducing group of the sugar has been exposed by brief acid hydrolysis and subsequent neutralization. In the work presented here, such *N*-acetylhexosamines are described as 'bound' sugars.

Determination of phosphorus. Phosphorus was determined by the method of Chen, Toribara & Warner (1956).

Determination of amino acids. Samples of mucopeptide and mucopeptide precursors were hydrolysed in 6M-HCl in sealed tubes at 105° for 16hr. The HCl was removed *in vacuo* over anhydrous $CaCl_2$ and NaOH and the residue was twice redissolved in water and evaporated to dryness. Amino acids were measured by the paper-chromatographic method of Mandelstam & Rogers (1959) with solvent C (see below) as the developing solvent. DAP was also measured by the method of Work (1957).

Determination of muramic acid. Nucleotide samples that contained *N*-acetylhexosamines were hydrolysed in sealed tunes in 4M-HCl at 105° for 4hr. The HCl was removed *in vacuo* and muramic acid was determined by the method of Rondle & Morgan (1955). Either muramic acid or glucosamine hydrochloride was used as the standard. A 1mg. portion of muramic acid gave 27·5% of the colour given by 1mg. of glucosamine at a wavelength of 530nm.

Measurement of radioactivity. The radioactivity of all samples was counted in a Packard 3000 Tri-Carb liquid-scintillation spectrometer. Aqueous solutions or suspensions (not greater than 0·5ml.) were mixed with 10ml. of a mixture containing naphthalene (180g.), 2,5-diphenyloxazole (4g.) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl) benzene (100mg.) in dioxan (1l.). Paper strips, thoroughly dried, were counted in 10ml. of a mixture containing 2,5-diphenyloxazole (4g.) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (100mg.) in toluene (1l.). At least 1000 counts above background were recorded for all samples.

Paper chromatography. Whatman 3MM paper was used throughout unless otherwise stated. Paper used for preparative separations was washed continuously with 2M-acetic acid for 3 days, and then with distilled water for at least 4 days. The following solvents were used: A, isobutyric acid–0·5M-ammonia (5:3, v/v) (Zetterström & Ljunggren, 1951); B, 1M-ammonium acetate (pH 7·2)–ethanol (2:5, v/v) (Palidini & Leloir, 1952); C, butan-1-ol–pyridine–water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951); D, butan-1-ol–acetic acid–water (4:1:5, by vol.; upper phase). Solvents A and B were used mainly for the separation of nucleotides. Solvent A gives a good separation of DAP, glutamic acid, muramic acid and alanine.

Sephadex chromatography. All the chromatography on Sephadex was conducted at 2–4°. Sephadex G-25 (fine grade) was allowed to swell in at least ten times its volume of 0·5% NaCl and then washed thoroughly with 2mM-acetic acid. The Sephadex was poured into a column (about 90cm. × 2·8cm.). For the initial purification of the *N*-acetylhexosamines in trichloroacetic acid extracts, the crude material was applied to the column in a volume of 2ml. After the sample had been washed into the Sephadex, elution with 2mM-acetic acid proceeded at a rate of 50ml./hr. Fractions of volume 6·0ml. were collected. The extinction at 260nm. was measured for all fractions, and the *N*-acetylhexosamine content was determined for samples of the 20–30 fractions collected after the exclusion volume. When labelled aspartic acid had been incorporated into the bacteria, samples (50μl.) of each fraction were counted in dioxan scintillation fluid.

Purification of nucleotide-N-acetylhexosamine by paper chromatography. The major *N*-acetylhexosamine-containing peak from Sephadex G-25 fractionation was freeze-dried, redissolved in water and applied as a thin streak (about 0·5μmole of *N*-acetylhexosamine/cm.) to washed Whatman 3MM paper. Chromatography in solvent A for 48hr. separated several bands that were located by their absorption in u.v. light. All the areas that absorbed u.v. light were cut out, washed three times with ethanol and eluted with water. Concentrated eluates were streaked on washed Whatman 3MM paper and run in solvent B for 24hr. and u.v.-absorbing bands were eluted as before.

RESULTS

Intracellular accumulation of compounds containing N*-acetylhexosamines*

Fig. 1 shows the intracellular concentrations of bound *N*-acetylhexosamines in *B. subtilis* W23 incubated in amino acids–glucose–phosphate solution plus chloramphenicol; no Mg^{2+} was added. For comparison, vancomycin and cycloserine were added to similar suspensions. In 1hr. the pool of trichloroacetic acid-soluble bound *N*-acetylhexosamines increased more than 15-fold from the initial value of about 0·2μmole/100mg. of cells, whether or not cycloserine was present in the medium. When vancomycin was present, cell lysis occurred at an early stage in the incubation and accounts for the fall in *N*-acetylhexosamine concentration after 30min. When *S. aureus* strain 524/SC is incubated under similar conditions there is no appreciable

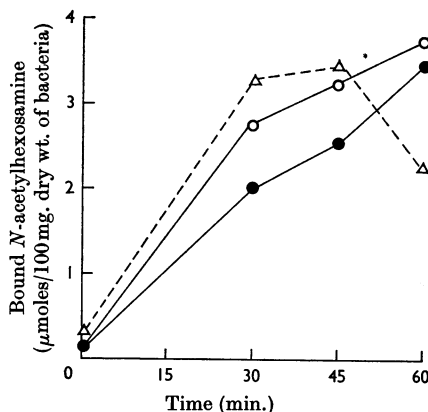


Fig. 1. Time-course for the accumulation of *N*-acetylhexosamines, soluble in trichloroacetic acid, by suspensions of *B. subtilis* W23. Suspensions (2mg./ml.) were prepared in amino acids–glucose–phosphate solution and chloramphenicol (50μg./ml.) was added to each suspension. Further additions: ●, none; △, vancomycin (20μg./ml.); ○, D-cycloserine (50μg./ml.). Incubation was at 35° with shaking. Cells (about 20mg. dry wt.) were harvested at the times indicated and bound *N*-acetylhexosamine soluble in trichloroacetic acid was determined as described in the Materials and Methods section. *N*-Acetylhexosamine is expressed in terms of *N*-acetylglucosamine.

Table 1. *Intracellular concentrations of bound* N-*acetylhexosamines in* B. subtilis *W* 23

Cell suspensions (2mg./ml.) were prepared as described in Fig. 1. Chloramphenicol (50μg./ml.) was present in each incubation mixture. $MgSO_4$, vancomycin and D-cycloserine were included where indicated. Samples (10ml.) of the suspensions were removed after incubation at 35° for 30min. and the cells extracted with trichloroacetic acid. The *N*-acetylhexosamine content of the extract was determined as described in the Materials and Methods section, and is expressed as *N*-acetylglucosamine.

Additions (final concentrations)			Increase in bound *N*-acetylhexosamine in 30min.
$MgSO_4$ (mM)	Vancomycin (μg./ml.)	D-Cycloserine (μg./ml.)	(μmoles/100mg. dry wt. of cells)
None	None	None	2·24
None	20	None	2·85
None	None	50	2·55
1·0	None	None	0·09
1·0	20	None	2·46
1·0	None	50	2·18

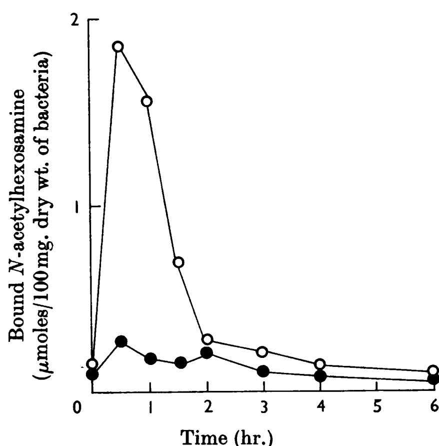


Fig. 2. Effect of Mg^{2+} deficiency on the pool of bound *N*-acetylhexosamines. The preparation of cell suspensions and chemical determinations are described in Fig. 1 and the Materials and Methods section. Chloramphenicol (50μg./ml.) was present in both flasks. ●, $MgSO_4$ (1mM) present; ○, $MgSO_4$ absent.

increase in the content of trichloroacetic acid-soluble bound *N*-acetylhexosamine, unless penicillin or a uridine analogue is included in the medium (Rogers & Perkins, 1960). However, Table 1 shows that for *B. subtilis* W23 supplementation of the medium with Mg^{2+} prevented the accumulation of bound *N*-acetylhexosamines. Chloramphenicol (50μg./ml.) was included in all the incubation mixtures described above, but it is obvious from Table 1 and Fig. 2 that the presence of this antibiotic causes no build-up of the intracellular pool of *N*-acetylhexosamines. Nevertheless chloramphenicol does have a marked effect on the maximum amino sugar concentration attained and the rate at which this concentration decreases. Chloramphenicol was originally included in the system to inhibit the incorporation of the amino acids into protein and, unless otherwise stated, all incubations were carried out in its presence. The influence of chloramphenicol and puromycin on the intracellular content of *N*-acetylhexosamines is described more fully below.

Effect of Mg^{2+} *concentration on the accumulation of* N-*acetylhexosamines*

In a medium devoid of Mg^{2+} the concentration of the bound *N*-acetylhexosamines soluble in trichloroacetic acid increased about 15-fold in 1hr. (Table 2). The inclusion in the medium of Mg^{2+} at a final concentration of 1mM was sufficient to prevent this accumulation and, for a cell density of 2mg./ml., the minimum Mg^{2+} concentration lies between 1mM and 0·5mM (Table 2). Mn^{2+} appears to be more effective than Mg^{2+} in suppressing the accumulation of *N*-acetylhexosamines, and no significant increase could be measured for cells incubated for 1hr. in the presence of 0·1mM-Mn^{2+}. However, approximately the same amount of intracellular *N*-acetylhexosamines accumulated in the presence of 0·01mM-Mg^{2+} or -Mn^{2+}.

Characterization of the material containing amino sugar

N-Acetylglucosamine, *N*-acetylmuramic acid and the peptide derivatives of *N*-acetylmuramic acid give a Morgan–Elson reaction product that has a maximum extinction at 585nm. Therefore it is impossible to decide, from the results given above, which of these compounds accumulate under conditions of Mg^{2+} starvation. Cell extracts were prepared as before but in quantities large enough to

Table 2. *Effect of* Mg^{2+} *and* Mn^{2+} *on the accumulation of bound* N*-acetylhexosamines*

Cell suspensions (2mg./ml.) were prepared in the system described in Fig. 1. Chloramphenicol was present in each incubation mixture. $MgSO_4$ and $MnCl_2$ were included in the medium at the concentrations indicated. Samples (10ml.) were removed after incubation at 35° for 1hr., and intracellular *N*-acetylhexosamine concentrations (expressed as *N*-acetylglucosamine) were determined described as in the Materials and Methods section. The initial concentration of bound *N*-acetylhexosamines was 0·2μmole/100mg. dry wt. of cells.

Concn. of $MgSO_4$ (mM)	Bound *N*-acetylhexosamine (μmoles/100mg. dry wt. of cells)	Concn. of $MnCl_2$ (mM)	Bound *N*-acetylhexosamine (μmoles/100mg. dry wt. of cells)
1·0	0·26	0·1	0·30
0·5	0·47	0·01	2·35
0·1	1·78	0·001	2·83
0·05	2·02	None	2·95
0·01	2·64		
0·005	3·09		
None	2·89		

enable the amino sugar compounds to be characterized. A cell suspension (500ml. at 1·5mg./ml.) was shaken vigorously in the usual medium, lacking Mg^{2+}, for 60min. at 35°. After this time the suspension was cooled in ice and centrifuged at 2°. The cells were washed with water and stirred with at least twice their volume of 15% trichloroacetic acid. After the trichloroacetic acid had been removed by extraction with ether, the aqueous phase was neutralized with sodium hydroxide and concentrated to about 2ml. by freeze-drying. The extract, which contained 37·2μmoles of bound *N*-acetylhexosamine (estimated as *N*-acetylglucosamine), was applied to a Sephadex G-25 column and eluted as described in the Materials and Methods section. The elution pattern is shown in Fig. 3. At least 90% of the *N*-acetylhexosamine put on the column appeared in a single peak (*A*), which emerged soon after the exclusion volume. The leading and trailing edges of this peak (10–15% of the added *N*-acetylhexosamine) and a very small peak (not more than 2% of the added *N*-acetylhexosamine), which appeared 10 fractions (60ml.) after peak *A*, were not examined further. The E_{280}/E_{260} ratio (0·4) indicated that uridine was associated with the *N*-acetylhexosamine and, assuming this, there was an equal molar concentration of nucleotide and amino sugar in each of the fractions included in peak *A*. The fractions were pooled and freeze-dried, and then purified on paper chromatograms run in solvents A and B. Solvent A separated three u.v.-absorbing bands with R_{UMP} values (1) 0·18, (2) 0·25–0·33 (the major band) and (3) 0·43. The major component also gave a positive ninhydrin reaction. All the u.v.-absorbing material was eluted from these three regions and re-run in solvent B for 24hr. Components (1) and (3) ran as single bands with R_{UMP} values 0·28 and 0·57 respectively.

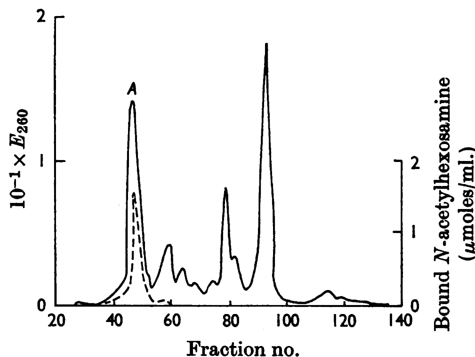


Fig. 3. Fractionation of nucleotides and *N*-acetylhexosamines on Sephadex G-25 after application of the trichloroacetic acid-soluble fraction from cells of *B. subtilis* W23 incubated in the absenceof Mg^{2+} for 1hr. The exclusion volume of the column was 256ml. ——, E_{260}; ----, bound *N*-acetylhexosamine measured in samples (100μl.) from each fraction of the first major nucleotide peak and expressed as *N*-acetylglucosamine.

The material eluted from these bands represented only about 5% of the nucleotides in peak *A*. A small amount of u.v.-absorbing material (R_{UMP} 0·26) was separated from component (2) in solvent B, but the rest ran as a single band with R_{UMP} 0·47. This material (compound 22) represented 80% of the amino sugar that was applied to the Sephadex column. Hydrolysis of compound 22 in 6M-hydrochloric acid at 105° for 18hr. followed by chromatography in solvents A, C and D showed the presence of alanine, glutamic acid, DAP, muramic acid and uracil. Quantitative analyses are given in Table 3. At pH 7 the E_{280}/E_{260} ratio was 0·40 and the E_{250}/E_{260} ratio was 0·75, confirming the

Table 3. *Quantitative analysis of the major* N-*acetylhexosamine fraction isolated from trichloroacetic acid extracts of* B. subtilis *W* 23 *incubated in a* Mg^{2+}-*deficient medium*

Details of the isolation of the material are given in the text. Analytical procedures are described in the Materials and Methods section.

Component	Molar proportions
Uridine	1·00
Phosphorus	2·19
Alanine	3·20
DAP	1·01
Glutamic acid	1·01
Muramic acid	1·10

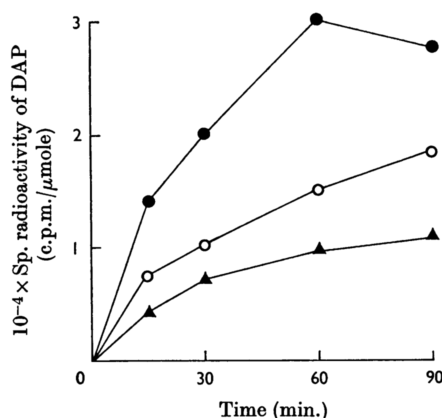


Fig. 4. Influence of Mg^{2+} on mucopeptide synthesis. Suspensions of bacteria (1·4mg./ml.) were incubated in conical flasks in the system described in Fig. 1. Chloramphenicol (50μg./ml.) and [3H]aspartic acid (0·5μc/ml.) were present in each mixture. Further additions: ○, none; ●, $MgSO_4$ (final concn. 1mM); ▲, EDTA (final concn. 1mM). Samples (5ml.) of the suspensions were removed at intervals, mucopeptide was prepared and the specific radioactivity of the DAP was determined as described in the Materials and Methods section.

presence of uridine. The identity of the hexosamine was confirmed by the spectrum of the Rondle–Morgan reaction product, which corresponded exactly to that of the product from muramic acid. Thus it seems probable that the material that accumulated under conditions of Mg^{2+} starvation was UDP-*N*-acetylmuramic acid attached to the pentapeptide Ala-Glu-DAP-Ala-Ala.

Binding of vancomycin to compound 22. Under the conditions described by Perkins (1969), compound 22 combined with vancomycin and provided additional evidence for the isolated nucleotide being UDP-NAMur-pentapeptide. Vancomycin forms a tight association with compounds of the general structure UDP-NAMur-X-D-Glu-Y-D-Ala-D-Ala when X is L-alanine and Y is L-lysine or *meso*-DAP, or when X is glycine and Y is homoserine. The specificity of this binding is associated with the structure of the D-Ala-D-Ala terminus of the peptide.

Isolation of UDP-NAMur-pentapeptide labelled with [3H]*DAP*. The accumulation of amino sugars was induced as before in a Mg^{2+}-deficient medium in which unlabelled aspartic acid was replaced with [3H]aspartic acid (100μg./ml.; specific radioactivity 26·7μc/μmole). About 400mg. dry wt. of cells was incubated with 4mc of [3H]aspartic acid for 1hr. at 35°, and after the cells had been harvested the supernatant fluid was reincubated with another 400mg. of fresh cells. The trichloroacetic acid extracts from both batches of cells, containing a total of 14μmoles of *N*-acetylhexosamine, were combined, and the UDP-*N*-acetylmuramic acid fraction was purified exactly as for compound 22. Amino acid analysis showed the DAP:glutamic acid:alanine molar proportions to be 1·09:1·00:3·07. The specific radioactivity of the nucleotide was 2·63μc/μmole. Material of higher specific radioactivity could be obtained by decreasing the concentrations of the amino acids in the incubation medium. Samples hydrolysed in 6M-hydrochloric acid (at 105° for 18hr.) were examined by paper chromatography. Development on Whatman no. 1 paper in solvent A showed one u.v.-absorbing spot that corresponded with a marker of uracil. Squares (1cm. × 1cm.) of the chromatogram and of one run in solvent D were cut out and counted in the Packard spectrometer. More than 90% of the radioactivity detected on each chromatogram was in DAP. Alanine was the only other compound labelled and accounted for the remainder of the radioactivity. No radioactive uracil was found.

Inhibition of mucopeptide synthesis

As a mucopeptide precursor accumulated when *B. subtilis* W23 was deprived of Mg^{2+}, it seemed probable that cell-wall mucopeptide synthesis would be affected under such conditions. Cell suspensions were incubated in the amino acids–glucose–phosphate solution plus chloramphenicol. Fig. 4 shows the results obtained. The omission of Mg^{2+} from the incubation medium caused an inhibition of about 50% in the incorporation of DAP for at least 1hr., and the addition of EDTA (final concentration 1mM) increased this inhibition to 70%. The influence of EDTA could be interpreted as an extension of the effects produced by Mg^{2+} starvation alone. If EDTA enters the cell it would be available to form chelation complexes with any free Mg^{2+} in the cytoplasm.

Utilization of accumulated amino sugars by the cell

Having established that the accumulated amino sugar compound had the composition of the expected mucopeptide precursor and that there was an accompanying inhibition of mucopeptide synthesis, it remained to be shown whether or not the accumulated material could be used by the cell if the conditions promoting its accumulation were removed.

Addition of Mg^{2+} *to* Mg^{2+}*-starved cells.* The results in Fig. 5 show that, although the pool of amino sugars remained at about 2·5 μmoles/100 mg. of cells for more than 2 hr. when no Mg^{2+} was present, the inclusion of this cation produced an immediate decrease in the pool, and 30 min. after the addition the amino sugar concentration was comparable with that of a suspension incubated in the presence of Mg^{2+} from the beginning of the experiment.

Addition of EDTA to cells incubated with Mg^{2+}. This experiment reversed the conditions of the one above. Fig. 5 shows that the addition of EDTA caused an immediate increase in the concentration of intracellular amino sugars, and in 30 min. cells treated with EDTA contained about 10 times the amount of bound *N*-acetylhexosamine found in the control cells suspended in a medium containing free Mg^{2+}.

Correlation of inhibition of mucopeptide synthesis with accumulation of precursor. If the *N*-acetylhexosamine that accumulated in cells in a Mg^{2+}-deficient medium would normally have been utilized in mucopeptide synthesis, there should be a close relationship between the amount of intracellular precursor found and the amount by which DAP incorporation into mucopeptide synthesis was inhibited. To determine whether this relationship existed, two suspensions of cells were incubated at 35° in the amino acids–glucose–phosphate medium, one with Mg^{2+} added to a final concentration of 1 mM and the other with no further addition. The results in Table 4 show that the amount of *N*-acetylhexosamine that accumulated in the absence of added Mg^{2+} balanced exactly the amount by which DAP incorporation into mucopeptide was inhibited. It has been assumed that all

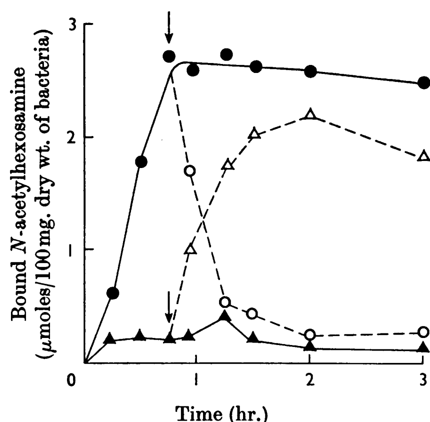


Fig. 5. Influence of Mg^{2+} on the intracellular concentrations of bound *N*-acetylhexosamines. Two cell suspensions (2·2 mg./ml.) were incubated in the system described in the legend to Fig. 1. One (●) contained no Mg^{2+}, the other contained 1 mM-$MgSO_4$ (▲). After 45 min., half of the first suspension was transferred to a flask containing sufficient $MgSO_4$ to give a final concentration of 1 mM-Mg^{2+} (○) and half of the second suspension was transferred to a flask containing EDTA to give a final concentration of 5 mM (△). Samples (5 ml. or 10 ml.) of each suspension were taken at intervals for determination of intracellular *N*-acetylhexosamines, as described in the Materials and Methods section.

Table 4. *Relationship between mucopeptide synthesis and accumulation of intracellular* N*-acetylhexosamines*

Two cell suspensions were prepared in the system described in Fig. 1. One contained $MgSO_4$ (1 mM). No further addition was made to the other suspension. Chloramphenicol (50 μg./ml.) and [^{3}H]aspartic acid (0·5 μc/ml.) were present in both mixtures, which were incubated, with shaking, at 35°. Quadruplicate samples (5 ml. for mucopeptide determination, 10 ml. for *N*-acetylhexosamine determination) were removed at the beginning of the experiment and after 60 min. incubation. The DAP of the mucopeptide and the intracellular *N*-acetylhexosamines were determined as described in the Materials and Methods section.

Addition	Increase in mucopeptide DAP in 1 hr. (μmole/5 ml. suspension)	Increase in bound *N*-acetylhexosamines in 1 hr. (μmole/5 ml. suspension)	Inhibition of mucopeptide incorporation (%)	
			(*a*)*	(*b*)†
$MgSO_4$	0·58	0·03	—	—
None	0·31	0·29	35	39

* From chemical determination of DAP.
† From specific radioactivity of [^{3}H]DAP.

the bound N-acetylhexosamine in the trichloroacetic acid extract is UDP-NAMur-Ala-Glu-DAP-Ala-Ala.

'Chase' of DAP from UDP-NAMur-pentapeptide into mucopeptide

Dilution of the low-molecular-weight constituents of the cytoplasm. To follow the metabolism of a labelled UDP-NAMur-pentapeptide that had accumulated under the stimulus of Mg^{2+} starvation, it was necessary to dilute the radioactive pool of low-molecular-weight metabolites without seriously affecting the concentration of the nucleotide to be studied. In preliminary experiments *B. subtilis* W23 was incubated with [^{3}H]aspartic acid ($0{\cdot}5\mu c$/ml.) in a Mg^{2+}-deficient medium for 30min. The cells (80mg. dry wt.) were harvested, and it was shown that one wash in 30ml. of an ice-cold solution of 0·05M-sodium–potassium phosphate buffer, pH 7, containing alanine, aspartic acid and DAP (each 1mg./ml.) was sufficient to remove 83% of the intracellular radioactive material. Of the remaining 17%, about half could be adsorbed on acid-washed Norit A charcoal. A second wash in the amino acids–phosphate solution decreased the intracellular radioactivity to 9% of its original value, and 84% of this could be adsorbed on charcoal. The specific radioactivity of the material eluted from the charcoal with 50% ethanol–0·05M-ammonia was calculated by assuming that all the eluted radioactivity and the N-acetylhexosamine were associated with mucopeptide precursor, and on this basis the specific radioactivity of N-acetylhexosamine-containing nucleotides remained constant during two washings, and after this treatment 88% of the bound N-acetylhexosamine originally found in the cells was still present. Chromatography in solvents A and B showed that 80% of the radioactivity in the eluted material ran, in positions corresponding with markers of UDP-NAMur-pentapeptide. When radioactively labelled cells were washed twice with the alanine–aspartic acid–DAP–phosphate solution at 35°, no significant loss of nucleotide-N-acetylhexosamine occurred and less than 2% of the original radioactive amino acid pool remained.

Metabolism of accumulated UDP-NAMur-pentapeptide. In this experiment a pool of [^{3}H]DAP-labelled UDP-NAMur-pentapeptide was allowed to accumulate in a suspension of *B. subtilis* W23 in the presence of chloramphenicol ($50\mu g$./ml.), and the cells were washed twice with a solution of alanine, aspartic acid and DAP (each 1mg./ml.) in 0·05M-sodium–potassium phosphate buffer, pH 7, and then resuspended in water. *B. subtilis* is susceptible to autolysis caused by cold shock, so all operations were carried out as quickly as possible and at 35° to avoid loss of cells in this way. To check the mucopeptide-synthesizing activity of the cells, a portion of the suspension was reincubated in a medium identical with the original one used to induce accumulation of amino sugar. A second portion was resuspended in the same medium supplemented with Mg^{2+}. To follow the fate of the labelled nucleotides, two more portions of resuspended cells were incubated in flasks containing the usual amino acids–glucose–phosphate medium, which contained no ^{3}H-labelled aspartic acid. One of these mixtures contained Mg^{2+} (1mM); the other contained none. The specific radioactivity of DAP in the mucopeptide was determined as before. From a similar series of samples UDP-NAMur-pentapeptide was isolated from the trichloroacetic acid extracts by adsorption on Norit A and by paper chromatography. Mucopeptide synthesis continued after the cells had been washed and resuspended in fresh medium, and the degree of inhibition caused by Mg^{2+} starvation (35–40%) was similar to that measured for cells that had not been subjected to the centrifuging and washing operations required in this experiment. After the cells had been shaken for 15min. in a medium containing Mg^{2+} and unlabelled aspartic acid, the specific radioactivity of mucopeptide DAP increased by 33%; a steady decline in specific radioactivity ensued after this point. In a medium containing unlabelled aspartic acid and no Mg^{2+} the specific radioactivity of mucopeptide DAP increased by only 10% in 15min., by 13% in 30min. and decreased 30min. later to the value recorded at the beginning of the second incubation period. The results presented in Fig. 6 indicate that [^{3}H]DAP is transferred from UDP-NAMur-Ala-Glu-DAP-Ala-Ala to the mucopeptide. The increase in total radioactivity in the mucopeptide fraction balanced almost exactly the decrease in the precursor radioactivity in all the samples analysed. When Mg^{2+} was present 93% of the mucopeptide precursor disappeared during the first 20min. of the second incubation and an equal amount of radioactivity appeared in the mucopeptide. In the same period cells reincubated in an Mg^{2+}-deficient medium retained more than 70% of the labelled precursor and even after 2hr. about 50% remained in this fraction.

Measurement of the amino sugars soluble in trichloroacetic acid showed that at the start of the reincubation of the cells $1{\cdot}5$–$2{\cdot}0\mu$moles of bound N-acetylhexosamine were present in 100mg. dry wt. of cells. This pool was depleted within 1hr. when Mg^{2+} was present, but if the cells continued to be deprived of Mg^{2+} the bound N-acetylhexosamine content rose to a maximum of 5μmoles/100mg. dry wt. of cells. The N-acetylhexosamine concentrations that were ultimately reached in cells that were reincubated for 1hr. in fresh Mg^{2+}-deficient medium were

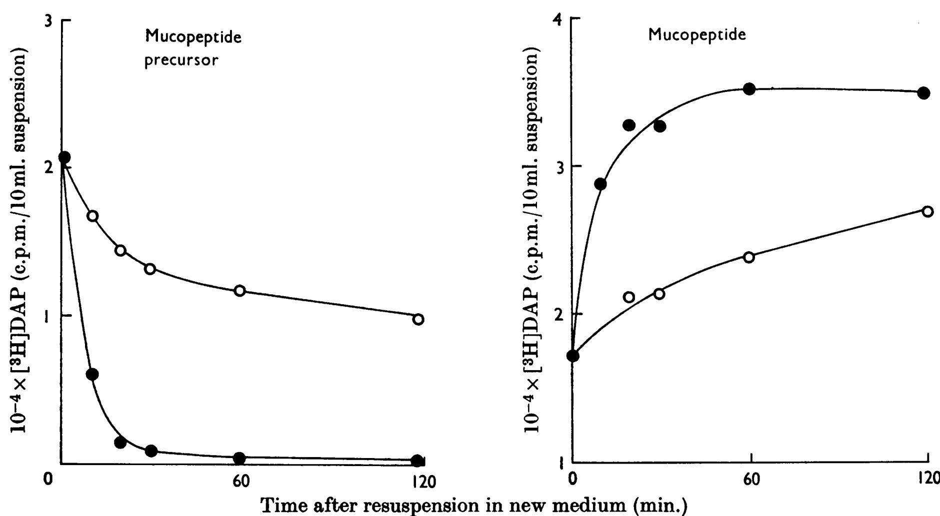


Fig. 6. 'Chase' of radioactivity from UDP-NAMur-Ala-Glu-[^{3}H]DAP-Ala-Ala to mucopeptide. A suspension (0·5mg./ml.) of *B. subtilis* W 23 was incubated in the system described in Fig. 1. Chloramphenicol (50μg./ml.) and [^{3}H]aspartic acid (0·5μc/ml.) were present. The cells were harvested after 30min. incubation, washed in a solution containing alanine, aspartic acid, DAP (each 1mg./ml.) and 0·05M-sodium–potassium phosphate buffer, pH 7, and resuspended in water. All operations were performed at 35°. Portions of the resuspended cells were incubated in fresh amino acids–glucose–phosphate solution containing chloramphenicol (50μg./ml.) and the following additions (final concentrations given): ●, $MgSO_4$ (1mM); ○, none. No [^{3}H]aspartic acid was added. Samples (5ml. and 10ml.) were taken at intervals. Mucopeptide was prepared from the smaller sample, and the specific radioactivity of the DAP was determined as described in the Materials and Methods section. The cells from the 10ml. sample were extracted with trichloroacetic acid; UDP-NAMur-pentapeptide was isolated by paper chromatography of the extracts, and the specific radioactivity of the nucleotide-DAP was determined.

higher than any measured for cells incubated for one single period of 90min. This may not be due entirely to replenishment of cell nutrients because, even though cells suspended at 0·5mg./ml. gave a higher yield of bound *N*-acetylhexosamine per cell than did denser suspensions (Table 5), the recovery of bound *N*-acetylhexosamine from 100mg. of cells never reached 5μmoles. Aspartic acid at 0·2mg./ml. is at least 20 times the amount needed in 1hr. for mucopeptide DAP synthesis in a 2mg./ml. cell suspension, but to allow for any possible deficiency in amino acids the concentrations of alanine, glutamic acid and glycine were doubled. The maximum concentration the *N*-acetylhexosamine pool reached was then 3·3μmoles/100mg. of cells.

Table 5. *Effect of cell concentrations on intracellular concentrations of* N*-acetylhexosamines*

Cell suspensions were prepared, at the concentrations indicated, in the system described in Fig. 1. Samples were removed after incubation at 35° for 90min., and *N*-acetylhexosamine concentrations were determined as described in the Materials and Methods section.

Cell concentration (mg. dry wt./ml.)	Bound *N*-acetylhexosamine (μmoles/100mg. dry wt. of cells)
0·56	3·54
1·20	3·24
2·08	2·90
3·84	1·20

Effects of chloramphenicol and puromycin on accumulation of amino sugars

Chloramphenicol was used in most of the experiments described above to inhibit protein synthesis. It is known that, when *S. aureus* is suspended at a density of 1mg./ml. in a similar system to the one used here, mucopeptide synthesis is unaffected by the presence of growth-inhibiting concentrations of chloramphenicol (70μg./ml.) (Mandelstam & Rogers, 1958; Hancock & Park, 1958). However, after incubation for 60–90min. under virtually the same conditions, chloramphenicol (50μg./ml.) inhibited by 10–20% the incorporation of [^{3}H]aspartic acid into *B. subtilis* mucopeptide DAP, whether or not Mg^{2+} was present. The presence of chloramphenicol does not promote the accumulation of trichloroacetic acid-soluble amino sugars (see Fig. 2), but it allows a higher concentration of these compounds to be

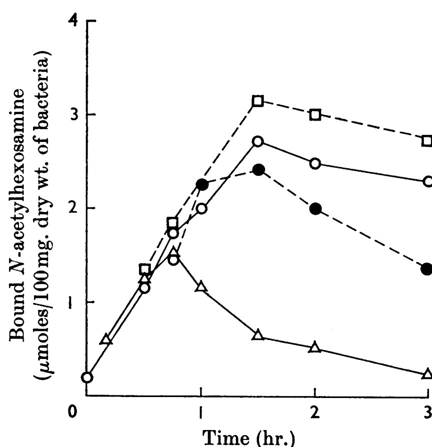


Fig. 7. Effect of chloramphenicol on the bound *N*-acetylhexosamines found in trichloroacetic acid extracts of *B. subtilis* W23. Two cell suspensions (1·4mg./ml.) were prepared as described in Fig. 1. No Mg^{2+} was added to either suspension. Chloramphenicol (50µg./ml.) was included in one incubation mixture (○) from the beginning of the experiment. No chloramphenicol was added to the other mixture (△). After 10min. (□) and 30min. (●), portions of the incubation mixture containing no chloramphenicol were transferred to flasks containing sufficient chloramphenicol to give a final concentration of 50µg./ml. Samples were withdrawn at intervals for the determination of bound *N*-acetylhexosamine, as described in the Materials and Methods section. *N*-Acetylhexosamine values are expressed as *N*-acetylglucosamine.

reached when the cells are deprived of Mg^{2+} (Fig. 7). If chloramphenicol was introduced into the incubation mixtures at any time up to about 30min. after the accumulation of nucleotides had begun, the effect was almost the same as adding it at the beginning of the experiment, and the concentration of bound *N*-acetylhexosamines remained higher than that of the control suspension, to which no chloramphenicol had been added (Fig. 7). Once the maximum concentration of the amino sugar pool had been passed, subsequent addition of chloramphenicol served only to diminish the rate at which the pool decayed. Puromycin (50µg./ml.) had the same effect as chloramphenicol. One explanation of this phenomenon could be that protein inhibitors prevent the formation of degradative enzymes, which are induced by high concentrations of mucopeptide precursors. To test this hypothesis, a search was made for degradative enzymes in cell-free extracts from *B. subtilis* W23.

Exponentially growing cells were harvested and resuspended in two batches in amino acids–glucose–phosphate solution. Chloramphenicol (50µg./ml.) was added to one and no addition was made to the other. After incubation for 70min. the cells were collected, resuspended in 0·05M-sodium–potassium phosphate buffer, pH 7, and crushed in the Hughes press. Any whole cells remaining were removed by centrifugation at 1000***g*** for 10min. at 4°. The cell extracts and appropriate controls were then incubated at 35°. The accumulated nucleotide-*N*-acetylhexosamine was used as substrate and purified UDP-NAMur-pentapeptide was added only to the control tubes. No diminution in the *N*-acetylhexosamine concentration of any extract was recorded. The addition of Mg^{2+}, ATP or UTP to the incubation mixtures had no effect. Therefore no degradative process similar to that observed in whole cells could be demonstrated. If, indeed, an enzyme system is involved, the procedures used for cell disintegration and fractionation may have destroyed it or an important activator.

To determine whether nucleotide-bound *N*-acetylhexosamine had leaked from cells during a 3hr. incubation in the absence of chloramphenicol and Mg^{2+}, the extracellular fluid was collected, acidified with trichloroacetic acid and treated with Norit A. About 40% of the nucleotides adsorbed on the charcoal were subsequently eluted with 50% ethanol–0·05M-ammonia, but no *N*-acetylhexosamine could be detected in the eluate.

Effects of bivalent ion deprivation on other bacteria

The accumulation of *N*-acetylhexosamines during bivalent ion deprivation is not peculiar to *B. subtilis* W23, but this organism showed the most noticeable reaction within the small group examined. *B. subtilis* 168 initially possessed an amino sugar pool about four times as great as that in exponentially growing cells of strain W23, but incubation in the Mg^{2+}-deficient medium increased the concentration only twofold in 1hr. *B. megaterium* KM showed no significant rise in amino sugar concentration in the absence of Mg^{2+}. The Gram-positive cocci presented an interesting series of results: the amino sugar pools of *M. lysodeikticus* 2665 and several strains of *S. aureus* were not affected appreciably by incubation in Mg^{2+}-deficient media, but the addition of EDTA (final concentration 1mM) caused an increase in 1hr. of about twofold in *S. aureus* strain H and strain 20P, and about fourfold in strain Oxford. *M. lysodeikticus* 2665 gave the highest yield of bound *N*-acetylhexosamines after 1hr. incubation in a solution containing phosphate buffer, 'wall' amino acids, glucose and EDTA. The yield (4–5µmoles/100mg. dry wt. of cells) was 10–15 times that of cells incubated in the medium with no EDTA. 'Staphylococcus strain 11', a strain that is probably more closely related to micrococci than staphylococci (Rogers & Perkins, 1959), reacted to EDTA in a similar way.

Another feature of the response of *Micrococcus lysodeikticus* to bivalent ion deprivation was revealed on examination of the pool of *N*-acetylhexosamines. By using the same techniques of column and paper chromatography as described for the characterization of UDP-NAMur-Ala-Glu-DAP-Ala-Ala from *B. subtilis*, the trichloroacetic acid-soluble material from *M. lysodeikticus* was found to contain both mucopeptide precursors (UDP-NAMur-Ala-Glu-Lys-Ala-Ala and UDP-*N*-acetylglucosamine) and UDP-*N*-acetylaminomannuronic acid, the expected precursor, of the glucose–aminomannuronic acid polymer found in the cell walls of this organism (Perkins, 1963). 'Staphylococcus strain 11' also contains three UDP derivatives that have been characterized qualitatively as UDP-NAMur-pentapeptide, UDP-*N*-acetylglucosamine and UDP-*N*-acetylaminohexuronic acid, another observation relating this organism to micrococci. A brief account of these findings has already been given (Garrett, 1969).

No UDP-*N*-acetylglucosamine has been detected in *B. subtilis* W23 deprived of Mg^{2+}. If it were present in the small amino sugar-containing peak from Sephadex G-25 chromatography (Fig. 3), it could only account for about 2% of the *N*-acetylhexosamines in the trichloroacetic acid extract. Rogers & Perkins (1960), who described the accumulation of UDP-*N*-acetylglucosamine and its fluorinated derivative in *S. aureus* treated with 5-fluorouracil, Glaser (1964) and Ito & Saito (1963) are the only workers who have reported abnormally high concentrations of UDP-*N*-acetylglucosamine in micro-organisms. In the last case, a rapid increase in UDP-*N*-acetylglucosamine was measured during the first 10min. incubation of *S. aureus* 209P with benzylpenicillin, and an equally rapid decrease followed, so that no appreciable accumulation was recorded after 30min. incubation. However, the accumulation of UDP-*N*-acetylglucosamine on treatment with antibiotics is not as rare as might be inferred from a survey of the literature. P. E. Reynolds (personal communication) has observed an increase in intracellular UDP-*N*-acetylglucosamine and UDP-NAMur-pentapeptide in vancomycin-treated *M. lysodeikticus* and *Sarcina flava*, but none in *B. cereus*, *B. subtilis* or *S. aureus* treated in the same way. Vancomycin also induces the accumulation of both mucopeptide precursors in corynebacteria (H. R. Perkins, personal communication).

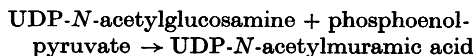
DISCUSSION

It would not have been expected that Mg^{2+} deficiency would cause an accumulation of UDP-NAMur-pentapeptide. However, qualitative and quantitative analyses show that at least 80% of the *N*-acetylhexosamine that accumulates is UDP-NAMur-Ala-Glu-DAP-Ala-Ala, and inhibition of mucopeptide synthesis caused by Mg^{2+} starvation is balanced exactly by the amount of precursor accumulating. It therefore appears that Mg^{2+} deficiency may affect mucopeptide synthesis by *B. subtilis* W23 primarily by interfering with the activities of the enzymes associated with the synthesis of mucopeptide at the cytoplasmic membrane. Several steps in the biosynthesis of *S. aureus*, *E. coli* and *B. megaterium* mucopeptide are known to be catalysed by membrane-bound enzymes. For example, if transfer of NAG-NAMur-Ala-Glu-DAP-Ala-Ala from the isoprenoid alcohol phosphate carrier to the 'acceptor' were inhibited by a low Mg^{2+} concentration, the lipid intermediate would be prevented from recycling, UDP-NAMur-pentapeptide would then be unable to enter the cycle and an abnormally high pool of this nucleotide would appear unless feedback inhibition stopped its synthesis. The membrane must retain a Mg^{2+} concentration high enough to allow this and other enzymes to work efficiently.

Many workers have emphasized the role played by Mg^{2+} in maintaining the structural and functional integrity of bacterial membranes. For example, Weibull (1956) showed that 0·01M-Mg^{2+} preserved the protoplast membranes of *B. megaterium*, and 0·02–0·03M-Mg^{2+} was required to prevent leakage of enzymes from protoplasts of *B. licheniformis* (Rogers, Reaveley & Burdett, 1967) or *B. subtilis* (Reaveley & Rogers, 1969). A concentration of 0·02M-Mg^{2+} was required in all solutions used for washing preparations of cytoplasmic membranes from *B. licheniformis* to prevent disaggregation (Reaveley & Rogers, 1969). Thus the membranes can readily be damaged and the enzymes essential for mucopeptide synthesis could be denied the conditions required for their maximum activity when the cells are starved of Mg^{2+}. Despite this effect, the intracellular pool of Mg^{2+} must remain large enough for synthesis of soluble mucopeptide intermediates to continue for at least 1hr. at approximately the same rate as in the presence of an extracellular source of Mg^{2+}. Evidence from Webb (1966, 1968) and Tempest, Dicks & Meers (1967) shows that Mg^{2+} is concentrated within bacterial cells when they are suspended or grown under conditions of Mg^{2+} starvation. Tempest *et al.* (1967) reported that *B. subtilis* var. *niger* when growing in a chemostat at a density of approx. 0·17mg. dry wt./ml. with a limiting concentration of 0·1mM-Mg^{2+} absorbed more than 95% of the total Mg^{2+} content of its environment in 24hr. None of this Mg^{2+} was bound to the cell surface. Tempest, Dicks & Ellwood (1968) suggested that most of the Mg^{2+} was associated with ribosomes. In the present work the amount of Mg^{2+} in the culture fluid

trapped within the cell pellet would give a final Mg^{2+} concentration no greater than 0·04mM when cells are resuspended in a Mg^{2+}-free incubation mixture to give a density of 2mg. dry wt./ml. It should perhaps be noted that Webb (1968) claims that *B. subtilis* var. *niger* is uniquely efficient in the concentrating of Mg^{2+}.

Any interference with cell-wall synthesis might be expected to lead to increased intracellular concentrations of both UDP-NAMur-pentapeptide and UDP-*N*-acetylglucosamine. It is more common, however, for UDP-NAMur-pentapeptide to accumulate alone. The synthesis of UDP-NAMur-pentapeptide from UDP-*N*-acetylglucosamine may be unaffected by situations inhibiting mucopeptide synthesis in all organisms, but in some the pathway may not function efficiently enough to cope with the extra UDP-*N*-acetylglucosamine made available. This implies, of course, that in cells that can accumulate UDP-*N*-acetylglucosamine the enzymes catalysing the reaction



UDP-*N*-acetylglucosamine + phosphoenol-pyruvate → UDP-*N*-acetylmuramic acid

are saturated at the normal steady-state concentrations of UDP-*N*-acetylglucosamine.

I thank Dr H. J. Rogers for his advice and encouragement throughout this work and Miss Shlomith Hampel for excellent assistance. I am also indebted to Dr H. R. Perkins for the analysis of the mucopeptide precursor by its combination with vancomycin and for many helpful discussions.

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