The Bactericidal Action of β-Lactam Antibiotics on an Autolysin-deficient Strain of Bacillus subtilis

By HOWARD J. ROGERS*, PAUL F. THURMAN AND IAN D. J. BURDETT

Division of Microbiology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

(Received 20 May 1982; revised 23 July 1982)

An autolysin-deficient mutant of Bacillus subtilis was completely tolerant to 5 h incubation with 50–100 μg cycloserine ml⁻¹ whereas the wild-type was rapidly lysed and killed by 12 μg ml⁻¹. Lysis also did not occur when low concentrations of β-lactams were added to exponentially growing cultures of the mutant, but over 90% of the bacteria were killed within 90–120 min. Protein, lipid and peptidoglycan synthesis as well as growth were inhibited after about 60 min. At this time, but not earlier, small amounts of these three cell components appeared in culture supernatants. Earlier, at about 20–30 min, the intracellular pools of amino acids started to decline rapidly and there was a temporary apparent increase in the rate of lipid synthesis. Neither of the latter phenomena occurred with cycloserine, with which protein and lipid synthesis declined only slowly and the rate of peptidoglycan synthesis was 80% inhibited within 30 min. Only occasional cells with damaged walls were seen 30–90 min after addition of either β-lactams or cycloserine to the cultures. It thus seems unlikely that wall hydrolysis or penetration by residual autolysins in the mutant are responsible for mass cell death caused by the β-lactams.

INTRODUCTION

The bactericidal action of penicillin upon rapidly growing cultures of susceptible bacteria and the concomitant lysis that frequently occurs were known soon after the discovery of the antibiotic (Chain & Duthie, 1945). Other antibiotics (e.g. tetracycline and chloramphenicol) do not cause lysis, are only bacteriostatic and can antagonize the lethal and lytic effects of penicillin (Jarwetz et al., 1951; Prestidge & Pardee, 1957; Rogers, 1967). The only known specific biochemical action of penicillin upon bacteria is the inhibition of the formation of mature peptidoglycan in their walls and this provides an excellent rationale for bacteriostatic action but not so obvious a rationale for a lethal effect. The possible secondary role for autolysins in destroying the existing bacterial wall thereby leading to bacterial lysis and death was envisaged many years ago (Rogers, 1962, 1964; Shockman, 1963; Weidel & Pelzer, 1964) as well as more recently (Tomasz, 1974, 1979). Subsequent to the earlier ideas it was shown (Rogers, 1967; Rogers & Forsberg, 1971; Tomasz et al., 1970; Ayusawa et al., 1975; Best et al., 1974; Fein & Rogers, 1976) that strains of various organisms, deprived of autolysins either phenotypically or genotypically, are lysed and killed less rapidly by antibiotics that inhibit wall synthesis than are the parent organisms. The term ‘tolerant’ was used to describe such strains (Tomasz et al., 1970). Considerable additional support has been obtained for the role of autolysins in the lethal effects of antibiotics that inhibit wall synthesis (Holtje & Tomasz, 1975; Tomasz & Waks, 1975; Suginaka et al., 1978; Shockman et al., 1979; Lopez et al., 1976) and possible clinical significance has been given to the hypothesis (Best et al., 1974; Sabath et al., 1977). There are difficulties, however, in its universal application. For example, some streptococci such as

Abbreviations: CHY, casein hydrolysate yeast extract medium; MSM, minimal salts/glucose medium; PAB, Penassay Broth.
Streptococcus pyogenes are killed by penicillin despite the absence of detectable autolysin (Horne & Tomasz, 1977; Tomasz, 1979). Others that are naturally very low in autolytic activity (Sund & Linder, 1976), such as, for example, Streptococcus mutans, are somewhat tolerant to $\beta$-lactams but protein and RNA synthesis are rapidly inhibited (Mattingly et al., 1977; Mychajlonka et al., 1980; Shockman et al., 1981). The $\beta$-lactams have no similar inhibitory effects on protoplasts (McQuillen, 1960; Shockman & Lampen, 1962; Reynolds, 1971; Rosenthal et al., 1975; Mychajlonka et al., 1980; Shockman et al., 1981).

**METHODS**

**Bacterial strains.** The autolysin-deficient strain used was Bacillus subtilis 168 FJ6 (metC3 lyt-2) (Fein & Rogers, 1976) which was 90–95% deficient in both known autolytic enzymes. The wild-type parent strain used for comparison was B. subtilis 168 MB21 (lew-8 metC3 tal-1).

**Growth media.** The following media were used: minimal salts/glucose medium (MSM; Sargent, 1973), which for some experiments was supplemented with 0.3% (w/v) sodium glutamate; casein hydrolysatye yeast extract medium (CHY; Hughes, 1968); a meat broth medium, Penassay Broth (PAB; Antibiotic Medium no. 3, Difco); Tryptone medium (0.8% (w/v) Tryptone (Difco), 0.5% (w/v) NaCl and 0.5% (w/v) glucose). Viable counts were made on LA agar (Luria & Burroughs, 1957). For studying the uptake of amino acids the bacteria were suspended in the PRE medium of Cundliffe (1968) supplemented by maleic acid as in McCleod et al. (1973).

**Standard cultural conditions.** A loopful of bacteria from a fresh LA agar plate culture was transferred to 5 ml PAB or CHY medium warmed to 35 °C in a 50 ml flask. The culture was incubated at 35 °C for 3–4 h after which a 1/10 dilution in MSM was made and incubated for 3 h at 35 °C. Dilutions were then prepared and each inoculated into 10 ml MSM medium. These cultures were incubated overnight at 35 °C and the following day the culture that had reached the early exponential phase of growth (corresponding to an A(675) of between 0.2 and 0.4) was diluted to an absorbance value of 0.10. It was then divided between two flasks, antibiotic was added to one and incubation at 35 °C continued. When the experiment involved the use of radioactively labelled compounds, these were usually included in the media used for the overnight incubation of the dilutions. Occasionally the radioactivity was added to dilutions of the overnight cultures. In all cases, the organisms were allowed to multiply for four to five generations in the presence of the radioactive precursor. The purpose of this was to ensure uniform labelling of the bacteria in the culture. When the rates of biosynthesis were to be measured no radioactivity was present at this stage. Cultures were aerated by shaking the flasks which contained one-tenth or one-fifth of their volumes as being equal to about half the volume of the original culture. The bacteria were then suspended in fresh, warm growth medium and incubation continued.

**Measurement of growth.** The A675 was measured using the appropriate medium as a blank. The mutant strain, FJ6, grows as very long chains of unseparated cells (Fein & Rogers, 1976) leading to agglutination, especially when the rate of growth is high, as in rich media. In order to obtain reproducible readings the cultures were shaken vigorously immediately before the measurements were made.

**Measurement of c.f.u.** The interpretation of determinations of c.f.u. in cultures of bacteria which do not separate from each other is difficult. Methods such as ultrasonic treatment also damage the bacteria. In the present work cultures of the mutant were incubated for a short time with very low concentrations of lysozyme. The concentration (0.75 µg ml\(^{-1}\)) was the minimum that would break up the chains without affecting the growth rate in MSM medium at 35 °C over a period of 3 h. Lysozyme was added to the culture 30 min before plating. Most of the long chains gave very short ones. The c.f.u. values of cultures of FJ6 after lysozyme treatment were about ten times those of the untreated ones. Cell death did not occur during the lysozyme treatment and lysis of penicillin-treated cultures was minimal. Nevertheless, c.f.u. values are regarded only as rough indications of the number of viable cells and more attention was paid to the re-growth of cultures after removal of the antibiotic.

**Re-growth experiments.** Removal of wild-type autolytic bacilli from exponentially growing cultures usually leads to some lag in growth when they are suspended in fresh medium of the same composition. Autolysin-deficient strains do not suffer such lags and grow again immediately at the same rate as in the original cultures (H. J. Rogers and C. W. Forsberg, unpublished work). If the bacteria are damaged, or a proportion of them is dead in the primary cultures, then lags occur in the secondary ones. The primary cultures were filtered rapidly through sterile 0.45 µm Millipore filters and the bacteria washed on the filters with two samples of warm medium, each sample being equal to about half the volume of the original culture. The bacteria were then suspended in fresh, warm growth medium and incubation continued.

**Peptidoglycan synthesis.** The incorporation of $N$-acetyl[\(\text{[1-\text{14C}}\)glucosamine (1 µCi µmol\(^{-1}\); 0.1 mM) (1 µCi = 37 kBq) was used as a method for the determination of peptidoglycan synthesis. As it is known that a considerable incorporation of radioactivity into protein takes place when minimal, as distinct from complex, media are used (Rogers et al., 1974), it was necessary to prepare peptidoglycan rather than to study incorporation of the amino sugar into whole organisms. Samples which were made up to contain 5% (w/v) TCA were treated as described by Rogers et al. (1974) for studying mucopeptide synthesis. The dried discs bearing the peptidoglycan were used for determination of radioactivity.
Lipid synthesis. The incorporation of [2-14C]glycerol (0.08 μCi μmol⁻¹; 0.6 mM), [2-3H]glycerol (0.33 μCi μmol⁻¹; 0.6 mM) and [1-14C]acetate (0.08 μCi μmol⁻¹; 0.6 mM) into material that could be extracted from the dried organisms by chloroform/methanol was studied. Samples (2 ml) of the cultures were measured into an equal volume of 10% TCA, mixed and stored overnight at 2 °C. The next day, the organisms were removed by filtering through 0.45 μm Millipore filters and the deposits on the discs were washed with 5.0 ml 5% TCA containing unlabelled glycerol or sodium acetate (10 mg ml⁻¹), pH 6-6, according to the precursors used. The discs were then washed with 5 ml 75% (v/v) ethanol added very slowly, transferred to vials and allowed to dry at room temperature overnight or at 60 °C for 30 min. The next day, 1 ml methanol was added to each sample and the vials heated to 65 °C for 5 min. Chloroform (2 ml) was added, the contents mixed and heated to 45 °C for 20 min. The mixture was then filtered through Whatman no. 1 filter paper. Three successive extractions at 45 °C were made. The filtrates were combined, evaporated to dryness and finally dried at 100 °C for 30 min. Radioactivity was measured in the remaining lipid. Separate experiments showed that washing organisms treated with TCA with 75% ethanol did not remove significant amounts of lipid.

Protein synthesis. The incorporation of L-[4,5-3H]leucine (1 μCi μmol⁻¹; 0.3 mM) was studied. The samples were taken into an equal volume of 10% TCA, stored overnight at 2 °C and the next day they were heated to 90 °C for 20 min, cooled and passed through a filter of pore size 0.45 μm. The residue was washed very slowly with 5 ml 5% TCA containing 10 mg unlabelled L-leucine ml⁻¹, followed by 10 × 0.5 ml amounts of 75% ethanol. The dried filters were then transferred to glass vials and the deposit suspended in 0.1 M-phosphate buffer, pH 8.0, containing 0.5 mg trypsin ml⁻¹. A drop of toluene was added and the vials were incubated at 35 °C overnight. The next day, the digest was passed through a filter of pore size 0.45 μm and a sample of the filtrate estimated for radioactivity.

The rates of formation of cell constituents. Antibiotics were added as usual to exponentially growing cultures with turbidities of 0-1. At various times afterwards the radioactively labelled precursors described above were added (at the specific radioactivities stated in the legend to Table 1) and incubation was continued at 35 °C for 15 min. Samples were then removed into equal volumes of 10% TCA. The subsequent preparation of cell fractions was as described above. Results were calculated as radioactivity incorporated into a given cell fraction per A₆₅₀ unit of the culture.

Secretion of materials into the culture supernatant. A volume (50 ml) of cultures of completely radioactively labelled cells with turbidity values of 0-1 were passed through Millipore filters of pore size 0.45 μm. The organisms remaining on the discs were washed with 2 × 20 ml medium at 35 °C and resuspended in 50 ml medium. Neither the medium used for washing or for resuspension contained radioactively labelled precursor. The suspension was then divided into two, and 0.05 μg cephalothin ml⁻¹ added to one culture. Both were incubated at 35 °C. Samples (3 ml) were taken at intervals and passed through 0.45 μm filters. One sample of the filtrate was estimated for radioactivity (0-3 ml) and another (2-5 ml) dialysed for 48 h against 2 × 5 litres 0-1 M-phosphate buffer, pH 7-2, at 2 °C. The material in the dialysis sac was then washed out, made up to 5 ml and estimated for radioactivity. When necessary, the remainder of the sample was dried mō vacuo and lipids extracted with chloroform/methanol as above. This method was compared with precipitation by TCA as used by Horne et al. (1977) and Hakenbeck et al. (1978). The results obtained were similar.

Intracellular pool sizes. The sizes of the intracellular pools of glutamic acid and alanine were measured by growing the organisms with either sodium [1-14C]glutamate (0.33 μCi μmol⁻¹; 1.8 mM) or with [1-14C]alanine (0.08 μCi μmol⁻¹; 0-6 mM). The basal medium used was MSM. Two samples (1 ml) were taken at various intervals, filtered through 0.45 μm Millipore discs and both discs washed with 5 ml medium which did not contain radioactively labelled amino acid. One of the samples was then washed with 5 ml boiling water to remove the pool of low molecular weight substances. Both samples were estimated for radioactivity by the usual procedure. The difference between the samples was taken as a measure of the intracellular pool.

Uptake of glutamic acid. Tryptone medium was used instead of MSM because it was intended eventually to explore the behaviour of membrane vesicles; such studies require the higher yields of bacteria obtainable from rich medium. No radioactively labelled compound was present during growth; otherwise, standard culture conditions were used. To study uptake, the cells were suspended at a turbidity of 0.1. At various times afterwards the radioactively labelled precursors described above were added (at the specific radioactivities stated in the legend to Table 1) and incubation was continued at 35 °C for 15 min. Samples were then removed into equal volumes of 10% TCA. The subsequent preparation of cell fractions was as described above. Results were calculated as radioactivity incorporated into a given cell fraction per A₆₅₀ unit of the culture.

Degree of cross-linkage in peptidoglycan. Walls were isolated after various periods of treatment with antibiotic by the method described by Fein & Rogers (1976). Treatment with SDS was used to remove membranes and inactivate autolysin. The proportion of 2,6-diaminopimelic acid in the samples that was engaged in cross-linking was then measured by the method of Fordham & Gilvarg (1974). The concentration of 2,6-diaminopimelic acid was measured in acid-hydrolysed walls (6 M-HCl at 105 °C for 16 h) by using an amino acid analyser.

Radioactivity determinations. Toluene biosolve emulsifier scintillator 299 (United Technologies, Packard Instruments) was added to dry discs bearing the radioactive material contained in vials or inserts and counted for 14C or 3H in a Packard scintillation counter. The counting efficiency for 14C was 86% and for 3H, 27%.
Electron microscopy. (i) Fixation and embedding. Samples (5-10 ml) were fixed with formaldehyde/glutaraldehyde by methods previously described (Burdeett, 1980) except that the concentrations were adjusted to 0.5% (v/v) formaldehyde and 1.0% (v/v) glutaraldehyde. The fixative also contained KCl (0.08 M, final concentration) and magnesium acetate (0.01 M). After fixation at room temperature for 2 h the samples were washed in 0.05 M-sodium cacodylate buffer, pH 6.2, containing 0.08 M-KCl and 0.01 M-magnesium acetate. OsO₄-postfixation and embedding procedures have been described (Burdeett, 1980). Sections were examined with a Jeol JEM 100 CX microscope at 60 kV.

(ii) Cationized ferritin. Control samples, as well as samples of strain FJ6 which had been grown in the presence of cephalothin (0.1 μg ml⁻¹, final concentration) for 30 min or 90 min, were treated with cationized ferritin. All samples were washed three times in 100 mM-phosphate buffer, pH 7.0, and resuspended, to give the same turbidity value, in phosphate buffer containing cationized ferritin at a final concentration of 1 mg ml⁻¹ (Tsien et al., 1978). The samples were exposed to ferritin for 15 min in an ice-bath to minimize residual autolysin activity, and resuspended by occasional mixing on a vortex mixer. All samples were thoroughly washed in 100 mM-phosphate buffer, pH 7.0, and fixed for electron microscopy as described above. Sections were generally examined without further poststaining.

Chemicals. All radioactively labelled compounds were obtained from Amersham. Trypsin was of the highest grade (type X-1; Sigma). D-Cycloserine was from Sigma, cephalothin from Eli Lilly, Basingstoke, U.K., cloxacillin from Sigma, methicillin from Beecham Research Laboratories, Brentford, U.K., and cephaloridine from Glaxo. Carbonyl cyanide m-chlorophenylhydrazone was from Sigma. Dicyclocarbodi-imide was from BDH. All other chemicals used were of Analar standard.

RESULTS

Bactericidal and bacteriostatic effects

The lyt-2 mutant of B. subtilis, FJ6, growing on MSM medium was almost completely tolerant to 50–100 μg cycloserine ml⁻¹. Concentrations could be found such that no autolysis or growth was demonstrable for 6 h after treatment of exponentially growing cultures. There was some variation from experiment to experiment in the precise concentration within the range necessary to achieve this. When growing in tryptone medium the necessary concentration was about 20 μg ml⁻¹. Rapid re-growth without lag was obtained when the antibiotic was removed after 5 h treatment (Fig. 1). The parent, fully autolytic, strain began to lyse rapidly about 1.5 h after the addition of 12.5 μg antibiotic ml⁻¹ to cultures in MSM medium or of 2 μg ml⁻¹ to cultures in Tryptone. Viable counts after treatment with 75 μg antibiotic ml⁻¹ on MSM medium showed that whereas the c.f.u. values in the parent strain MB21 were reduced in 6 h from 5 × 10⁷ ml⁻¹ to 2 × 10⁴ ml⁻¹, cultures of FJ6 lost only 30% of c.f.u. in the same period.

Thus, grossly reduced autolysin activity of FJ6 leads to almost complete protection against the bactericidal effects of cycloserine during an incubation for at least 5–6 generations. The mutant strain was thus almost completely tolerant to cycloserine. Results with penicillins and cephalosporins were more complicated. Strain FJ6 lysed very slowly, or not at all, with concentrations of the antibiotics greater than those required to lyse the parent rapidly (Fig. 2). Attempts to re-grow the organisms, however, after treatment with relatively low doses and for short times of exposure, were unsuccessful (Fig. 2) despite the absence of lysis. Higher concentrations for rather short times of exposure (e.g. 90 min) or longer times with lower concentrations were lethal to a high proportion of the population. For example, after treatment for 2–3 h with 1 μg cloxacillin or cephaloridine ml⁻¹, growth or only very slow re-growth occurred. There was almost no re-growth with 0.6 μg cloxacillin ml⁻¹ after 5 h treatment and after 4 h treatment with 0.05 μg cephalothin ml⁻¹ slow accelerating growth was obtained on removal of the antibiotic. In none of these experiments was there any detectable lysis. After the latter treatment the c.f.u. value corresponded to only about 20–30% of that in the control culture having the same cell density as the antibiotic-treated one. Results with methicillin were similar to those with cloxacillin, cephaloridine and cephalothin.

The lethal effects of the β-lactams upon macromolecular biosynthesis under conditions where lysis did not occur were examined. Most of the experiments have been done with the cephalosporin, cephalothin, although unreported experiments with cloxacillin, cephaloridine or methicillin gave similar results.
Bactericidal action of β-lactams

469

Peptidoglycan synthesis

The incorporation of N-acetyl[1-14C]glucosamine into the hot TCA trypsin-insoluble fraction prepared from the mutant strain FJ6 proceeded normally in the presence of either 0·05, or 0·1 μg cephalothin ml⁻¹ for about 40–50 min, representing two-thirds of a generation of growth on MSM medium. Incorporation then slowly came to a halt in parallel with the cessation of bacterial mass increase (Fig. 3). The most important, perhaps the only, site of action known for β-lactams is upon the transpeptidases that link together the peptide side chains of peptidoglycan. The extent of inhibition of cross-linking of the peptidoglycan in the presence of cephalothin was examined. It decreased from a value of 41% at zero time to 34% at 30 min. Thereafter, there was little change up to 2 h incubation with 0·05 μg cephalothin ml⁻¹. The period of incorporation of amino sugar immediately following the addition of antibiotic therefore reflects the addition of un-cross-linked material.

In order to examine the loss of wall material from the bacteria during inhibition of transpeptidation by 0·05 μg cephalothin ml⁻¹, the amount of radioactivity present in culture filtrates was measured during incubation of steady-state labelled bacteria with and without the antibiotic. Amounts equivalent to 5–8% of the incorporated radioactivity were found in the nondialysable fraction of the filtrates from control cultures. This proportion remained constant over two generations of growth. In the antibiotic-treated cultures a slightly higher proportion of 13–14% was liberated towards the end of the two generations growth (Fig. 4). The material in such a sample was of high molecular weight (> 5 × 10⁵) and would only just enter Sephadex G-100. Of the total radioactivity present in the filtrate, 80–90% entered Sephadex G-25 and moved on columns at a rate similar to that of N-acetylglucosamine itself. This amounted to about 20% of
Fig. 3. Effect of cephalothin upon (a) peptidoglycan, (b) lipid and (c) protein synthesis by strain FJ6. Cephalothin (0.05 pg ml⁻¹, final concentration) was added to one of two cultures growing exponentially in MSM. Samples were taken into TCA and treated as described in Methods. ●, Control cultures not treated with antibiotic; ○, cultures treated with 0.05 pg cephalothin ml⁻¹.

Fig. 4. Excretion of non-dialysable radioactively labelled material during incubation with cephalothin. Appropriately radioactively labelled cultures of FJ6 were grown exponentially in MSM. Bacteria were removed by filtration and resuspended in the same medium without radioactively labelled compounds added. Samples were removed, filtered and the filtrates dialysed and estimated after growing with the following labelled compounds in the presence of 0.05 pg cephalothin ml⁻¹: ○, N-acetyl[1-¹⁴C]glucosamine; □, [2-¹⁴C]glycerol; and ■, l-[4,5-²H]leucine. ●, Controls without antibiotic.
the incorporated material in the control and 30% in the antibiotic-treated cultures and presumably represents unincorporated precursor.

**Peptidoglycan turnover**

Previous results (Pooley, 1976; Fein & Rogers, 1976) have suggested that the turnover of peptidoglycan in the autolysin-deficient strains was either very slow or did not occur. To test this in the presence of cephalothin, bacteria with their walls completely labelled with \(N\)-acetyl[1-\(^{14}\)C]glucosamine by the standard techniques were removed from the medium and resuspended in MSM containing 100 \(\mu\)M unlabelled \(N\)-acetylglucosamine, either with or without the addition of 0.05 \(\mu\)g cephalothin ml\(^{-1}\). There seemed to be no detectable loss of incorporated radioactivity from the control bacteria. Those in cultures containing cephalothin lost about 10% of the radioactivity that had already been incorporated during 2 h incubation. Turnover both in the presence and absence of antibiotic was thus very low but just detectable in the presence of cephalothin. This result confirms the study of the excretion of material from \(N\)-acetylglucosamine-labelled cells, since the small amount of soluble labelled material (Fig. 4) produced by the control cells is within the experimental error for determinations of the total radioactivity incorporated into the peptidoglycan fraction.

**Lipid biosynthesis**

A study of the effect of \(\beta\)-lactams upon bacterial lipid synthesis has become particularly important since the claim (Horne et al., 1977) that inhibition of peptidoglycan synthesis by antibiotics leads to rapid secretion of lipids by streptococci and a variety of other bacterial species, including wild-type \(B.\ subtilis\). The effect of cephalothin on the incorporation of [1-\(^{14}\)C]acetate, [2-\(^{14}\)C]glycerol or [2-\(^3\)H]glycerol into the chloroform/methanol soluble fraction of exponentially growing cultures of the autolysin-deficient strain of \(B.\ subtilis\), FJ6, was studied. All three labels gave essentially the same result in that their incorporation appeared to stop about 40–50 min after the addition of the cephalosporin, and there was a loss from the bacteria during the second generation of growth (Fig. 3). Of the total radioactivity incorporated from either of the three precursors, only a proportion was into lipid material. For example, using [1-\(^{14}\)C]acetate, 13.7% of the label could be extracted by chloroform/methanol from the TCA-precipitated control cells whereas 44% was solubilized by treatment with lysozyme. Using [2-\(^3\)H]glycerol, a higher proportion (about 40%) was extracted by chloroform/methanol and 13% was solubilized by lysozyme. The radioactivity in the lysozyme-soluble fraction presumably arises, with the use of [1-\(^{14}\)C]acetate, from the \(N\)-acetyl substituents of the wall peptidoglycan and in one of the wall teichoic acids, and with labelled glycerol, from the teichoic acids.

Examination of the culture filtrates from control and antibiotic-treated cultures showed that using [2-\(^{14}\)C]glycerol as a precursor, for example, the control filtrate contained 2–3% of the total counts incorporated into the cells, and the dialysed fraction only 1–2%. In the presence of the antibiotic more was present, amounting to 20% in the unfractionated filtrates and 8% in the dialysed samples from cultures incubated for 1 h (Fig. 4). If the whole of the non-dialysable material was assumed to be lipid, this formed about 35% of the lipid in the cells at the time lipid synthesis stopped. However, of this higher molecular weight non-dialysable material, only 15% was soluble in chloroform/methanol and 44% was solubilized by lysozyme. The proportions exactly reflect those in the whole cell. Of the remainder, 16% was solubilized in 50% aqueous methanol by treatment with 0.2 \(\text{m-NaOH}\) and might, therefore, have represented lipoteichoic acid. Using [2-\(^3\)H]glycerol as a precursor, the proportion of the total radioactivity incorporated into the soluble non-dialysable material as chloroform/methanol-soluble material was higher, being about 30%, again reflecting the proportion in the bacteria. It would thus appear that a considerable proportion of the soluble material in antibiotic-treated cultures is wall material, which would be expected to be labelled from either glycerol (into both teichoic acids) or acetate (into peptidoglycan and one teichoic acid).

If the amounts of radioactivity present in the dialysed culture filtrates from the antibiotic-treated cultures were added to those in the chloroform/methanol soluble fraction from the cells, they exactly compensated for the loss from the latter that occurred during continued incubation.
However, the significance of this is doubtful because of the preponderance of wall material rather than lipid in the dialysed filtrate fraction.

It would seem that about 5–10% of the lipid of strain FJ6 had been excreted from the cells into culture fluid after 1 h treatment with cephalothin, that is after growth had stopped completely. After 2 h incubation about 30% of the cell lipid was found in the culture supernatants.

**Protein biosynthesis**

This was measured by the incorporation of L-[4,5-3H]leucine into the trypsin-solubilized part of the TCA-precipitated bacteria. Cephalothin (0·05 µg ml⁻¹) was added to one culture at zero time. Protein biosynthesis reduced in rate at about 40 min and stopped at 80 min, which represents about 1–2 generations of growth in the control culture (Fig. 3). No significant radioactively labelled non-dialysable material was present in the culture filtrate from the control culture. In the antibiotic-treated culture excretion of non-dialysable labelled material started 20–30 min after addition of the cephalothin and rose exponentially for a brief period. It then stopped and remained constant from 80 min until 120 min (Fig. 4). There was no evidence that, in the presence of the antibiotic, protein synthesis continued normally in the whole population but that a high proportion of protein synthesized was excreted as soluble material.

**Rates of biosynthesis with cephalothin and cycloserine**

The rates of synthesis of peptidoglycan, protein and lipid were measured at various times after the addition of cephalothin and cycloserine to exponentially growing cultures. The incorporation of 15 min pulses of the radioactive precursors was studied.

The results (Table 1) for the rates of synthesis of protein and peptidoglycan during inactivation with cephalothin were as would be expected from the results for the accumulation of the macromolecule. Protein synthesis, for example, was reduced to about 30–40% after 60 min and to about 10–15% after 2 h. Cycloserine had a much less drastic effect and protein synthesis was only reduced to 50–60% of its initial value after 4 h incubation, but peptidoglycan synthesis was reduced by 80% after 30 min incubation. The effects of cephalothin on lipid synthesis were unexpected from the earlier results. An increase in the rate occurred during the first 20–40 min followed by a decline to about 30% of the initial rate during 2 h. The increase was reproducible in time though somewhat variable in extent, irrespective of whether radioactively labelled glycerol or acetate was used as precursor. Since no increased accumulation of lipid in the bacteria has been found, but rather a small loss, the present results may indicate an increase in turnover of membrane lipids. It should be noted that pool sizes of precursors have not yet been measured. No similar increase occurred with cycloserine.

**Leakage of intracellular amino acid pools**

The pool sizes of two amino acids, glutamic acid and alanine were measured. The amounts of both that could be extracted from the cells by boiling water, a commonly accepted way of measuring pool sizes, declined rapidly after about 20 min incubation with 0·05 µg cephalothin ml⁻¹. Eventually, after 2 h incubation, they were reduced to about 20–30% of the initial value. The pool size in the control culture was constant (Fig. 5). No distinction was made between free glutamic acid and peptide-bound material. The small rise in the pool in the very early stages of incubation with antibiotic was reproducible despite some scatter of the points. It might have represented the accumulation of peptidoglycan precursors. In the above experiments, the organisms were grown on MSM medium. When the experiments were repeated using a rich growth medium (Tryptone), the results were even more impressive. After incubation with 0·1 µg cephalothin ml⁻¹, the pool was reduced to 40% of its initial value in 1 h and after 90 min it was undetectable. No evidence of lysis of the bacteria was seen. Similar results were obtained when the pool size for alanine was measured.

The size of the intracellular pool of glutamate in FJ6 during incubation with non-lytic concentrations of cycloserine showed no loss during 2 h. The initial increase that also occurred with cephalothin was rather greater with cycloserine. The pool size then declined to a level slightly greater than its initial value during 30–40 min, and stayed constant thereafter.
**Table 1. Rates of macromolecular synthesis during incubation with cycloserine and cephalothin**

The rates of synthesis of peptidoglycan, protein and lipid per unit of 4675 were measured by using 15 min pulses of the following radioactive precursors: N-acetyl[1-14C]glucosamine (1 μCi μmol⁻¹; 0-1 mM), L-[4,5-3H]leucine (4 μCi μmol⁻¹; 0-3 mM), and [3H]acetate (1.7 μCi μmol⁻¹; 1.3 mM). The growth medium was MSM and the concentrations of the antibiotics were 50 μg ml⁻¹ for cycloserine and 0.05 μg ml⁻¹ for cephalothin. Growth is indicated by the 4675, and the zero time samples taken immediately before the addition of antibiotic are given the arbitrary value of 100.

<table>
<thead>
<tr>
<th>Time after addition of antibiotic (min)</th>
<th>Cycloserine added</th>
<th>Cephalothin added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth (4675)</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>0</td>
<td>0.100</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>0.125</td>
<td>22.2</td>
</tr>
<tr>
<td>60</td>
<td>0.130</td>
<td>19.1</td>
</tr>
<tr>
<td>120</td>
<td>0.130</td>
<td>22.7</td>
</tr>
<tr>
<td>240</td>
<td>0.135</td>
<td>11.2</td>
</tr>
</tbody>
</table>

ND, Not done.

Fig. 5. Effect of cephalothin upon the size of the pool of glutamic acid in FJ6. Bacteria growing exponentially and fully labelled with L-[1-14C]glutamic acid were treated with 0.05 μg cephalothin ml⁻¹. Two samples were taken at the indicated times. One was washed with warm medium, the other with distilled water at 100 °C. The difference between the amounts of radioactivity remaining associated with the bacteria represents the L-glutamic acid pool together with any peptide containing the amino acid. The results are expressed as c.p.m. per 0.1 4675 unit of the cultures. ●, No antibiotic (control); ○, with antibiotic.

**Active transport of amino acids**

Bacteria growing in Tryptone medium were incubated for varying times with 0.1 μg cephalothin ml⁻¹, removed from the medium and resuspended in the absence of antibiotic but in the presence of L-[1-14C]glutamic acid for 15 min under conditions known to be favourable for accumulation of free amino acids by bacilli (McCleod et al., 1973). The uptake capacity of the bacteria started to fall after about 15–20 min and was undetectable after 90–100 min.

The kinetics of the decline in apparent uptake of glutamate were exactly the same as those for the emptying of the pool itself. It seemed probable, therefore, that an exchange was occurring rather than active transport. A preliminary study of the effect of inhibitors on the rate of uptake of glutamic acid during incubation with cephalothin showed that it rapidly became resistant to inhibitors of electron transport such as KCN, but not to uncouplers such as carbonyl cyanide m-chlorophenylhydrazone or dicyclohexylcarbodi-imide.
Fig. 6. Sections of *B. subtilis* FJ6 grown in Tryptone medium. (a) Control showing unseparated cells and roughened outer layers of wall; (b) and (c) organisms exposed to 10 μg cloxacillin ml⁻¹ for 1 h (b) or 3 h (c). The arrows in (b) show thickening of the cross-wall and peripheral wall, and the arrow in (c) indicates a perforated wall. The bar markers represent 0.5 μm.

Electron microscopy

The fine structure of *B. subtilis* FJ6 when grown in rich media has been described (Burdett, 1980). Briefly, the cells grow as long chains with very little evidence of separation at the cross-walls. Unlike the wild-type strain, the outer surface of the walls appeared very ragged (Fig. 6a). After growth in the presence of 10 μg cloxacillin ml⁻¹ for 1 h, the most obvious change was in
Fig. 7. Sections of *B. subtilis* FJ6 exposed to cationized ferritin after growth in the presence of cephalothin (0.1 μg ml⁻¹) for 30 min (a) and for 90 min (b–e). In (a) note the uniform coating of ferritin on the outer surface of the wall, but excluded from the inner layer and cross-wall. (b) A lysed cell with ferritin (f) on both the outer and inner face of the wall. (c) Ferritin particles located on the outer face of both the wall (w) and 'unit' membrane (m); the cross-wall (cw) is not labelled. In (d) and (e) the arrows show ferritin localized beneath the membrane (d) and cross-wall (e). In (a) and (b) the bar markers represent 0.5 μm and in (c–e) 0.1 μm.

the thickening of the cross-walls, where multiple layers were observed. The peripheral wall did not appear conspicuously thickened at this stage. At 3 h some thickening of the side walls, possibly originating at the cross-walls or presumptive division sites, was observed (Fig. 6b).
There was some distortion of cell shape in samples treated with cloxacillin, and lysed organisms were also present. As noted previously (Burdett, 1980), specific sites of lysis were difficult to detect but, where present, the wall was entirely perforated (Fig. 6c). Such sites appeared to be associated with the wall immediately adjacent to completed cross-walls or with presumptive division sites.

Similar observations were made with cultures grown in the presence of cephalothin (0.1 \( \mu \text{g ml}^{-1} \)). Cationized ferritin was added to the cultures to test whether the iron core could be visualized on the inner face of the wall. Ferritin will not normally penetrate the intact wall but will only bind to the outer surface; the presence of cationized ferritin on the inner edge was therefore taken to indicate damage to the wall. In the control cultures, the ferritin was observed as clusters over the entire outer surface of the wall. Samples grown in the presence of cephalothin (0.1 \( \mu \text{g ml}^{-1} \) for 30 or 90 min) presented the same appearance (Fig. 7a).

Visible evidence for wall or membrane damage was most readily seen in organisms incubated with cephalothin for 90 min. After 30 min incubation the walls appeared thickened and covered uniformly with ferritin; lysed cells were very rare. Only in completely lysed organisms, amounting to perhaps 1% of the total number of bacteria observed in the 90 min sample, were ferritin particles seen on both faces of the wall (Fig. 7b), and along the outer face of the cytoplasmic membrane (Fig. 7c). The particles were notably absent from the inner edge of the wall except, as noted above, in completely lysed organisms (Fig. 7b). In very rare instances, clusters of ferritin particles were noted either within, or enclosed by, folds of the cytoplasmic membrane (Fig. 7d). In organisms treated with cephalothin for 90 min, ferritin particles were observed occasionally between layers of the cross-wall (Fig. 7e).

**DISCUSSION**

\( \beta \)-Lactams that inhibit a 'late' stage in the pathway of peptidoglycan synthesis damaged the autolysin-deficient mutant FJ6 much more than did cycloserine, an 'early' inhibitor. Un-cross-linked peptidoglycan was added to the walls during the first 30–40 min in the presence of cephalothin rather than being liberated into the culture filtrates (Tynecka & Ward, 1975). The immediate secondary effects occurred at about 20–30 min when the intracellular pool started to empty and an early increase in the rate of lipid synthesis occurred which was not reflected in lipid accumulation in the bacteria. Leakage of intracellular low molecular weight metabolites might well explain the later cessation of macromolecular syntheses and growth. The bacteria also ceased to transport amino acids actively as soon as, or probably before, the pool started to empty. Cycloserine caused neither of these effects. Inhibition by penicillin of the uptake of amino acids by *Staphylococcus aureus* was observed over thirty years ago (Gale & Taylor, 1947) but the phenomenon was then studied over longer periods in an autolytic organism which made interpretation of the results difficult.

Large amounts of lipids rapidly leave the cells and appear in the supernatant fluids of cultures of streptococci treated with \( \beta \)-lactams (Horne & Tomasz, 1977; Horne et al., 1977). This did not happen with the autolysin-deficient mutant of *B. subtilis*. Cell lipids and proteins were found in the culture supernatants but these did not start to appear until 40–50 min after addition of the \( \beta \)-lactam when growth had stopped, and much later than when membrane damage occurred. Considerable amounts of lipid only accumulated after 90 min–2 h which would seem more likely to be a consequence of cell death rather than of an immediate secondary action of the antibiotic. Thus, the early damage to membrane function cannot be directly related to loss of membrane lipids and proteins as in streptococci (Horne et al., 1977). In this latter work membrane vesicles were liberated from the antibiotic-treated bacteria otherwise the very early loss of lipid might have been simulated by the increased rate of lipid turnover possibly illustrated in the present work.

Interpretation of the causes of the rapid secondary membrane damage by \( \beta \)-lactams turns on the importance of the small residual autolytic activity in the mutant. Rather large amounts of soluble high molecular weight wall polymers were found in the culture supernatants from pneumococci (Hakenbeck et al., 1978) despite the autolysin-deficiency of the bacteria. In the
present experiments, only very small amounts of soluble larger wall material appeared 50-
60 min after the addition of cephalothin. If wall damage by the small residual amounts of active
autolysins in the mutant occurred, it could not be demonstrated by early formation of soluble
wall polymers. Alone, this does not completely exclude damage since insufficient peptidoglycan
bonds may be broken in the right places to allow the loss of soluble products. Nevertheless, only
very occasional penetration by ferritin through the walls occurred after 30 min incubation and a
very few empty cell husks were seen. The walls of the cells grew thicker not thinner during the
first hour’s incubation with β-lactams, and later the cytoplasmic membrane tended to shrink
away from the walls. For over 90% of the bacteria this picture would be consistent with the early
deposition of partially un-cross-linked material on the inner face of undamaged fully cross-
linked walls followed by shrinking of the cytoplasm as a consequence of the membrane damage,
causing sufficient reduction in the osmotic pressure to lead to plasmolysis. There was no
evidence of expansion of the wall due to the incorporation of un-cross-linked material.
Nevertheless, some wall damage after 90 min but not 30 min incubation with cephalothin must
have occurred to a few cells to allow ferritin particles to appear occasionally on the outer surface
of the cytoplasmic membrane.

The rate of lipid synthesis increased almost simultaneously with the start of leakage from the
intracellular pool. It is attractive to think that these events are causally connected. The absence
of evidence for early membrane damage during cycloserine treatment of the bacteria makes it
more likely to be a cause of death with β-lactams, since cycloserine was truly bacteriostatic
inhibiting all synthesis. In contrast, the cytoplasmic membrane is usually rapidly lysed in a
few minutes by β-lactams. This could explain the rapid death in cycloserine treated cultures.

REFERENCES


Best, G. K., Best, N. H. & Korai, A. V. (1974). Evidence for participation of autolysins in the bac-

General Microbiology 120, 35-49.

Chain, E. & Duthie, E. S. (1945). Bacterial and bacterioytic action of penicillin on Staphylococcus.
Lancet i, 652.


Bacteriology 127, 1427-1442.

Fordham, W. D. & Gilvarg, C. (1974). Kinetics of cross-linking of peptidoglycan in Bacillus megater-
ium. Journal of Biological Chemistry 249, 2478-2482.

Gale, E. F. & Taylor, E. S. (1947). The assimilation of amino-acids by bacteria. 5. The action of penicillin in preventing the assimilation of glutamic acid by Staphylococcus aureus. Journal of General
Microbiology 1, 314-326.

the growth medium of lysis-defective pneumococci during treatment with penicillin and other inhibitors
of cell wall synthesis. Antimicrobial Agents and Chemotherapy 13, 302-311.

Holt, J. V. & Tomasz, A. (1975). Lipoteichoic acid. A specific inhibitor of autolysin activity in Pneumo-
coccus. Proceedings of the National Academy of Sciences of the United States of America 72, 1690-
1694.

wall inhibitors. Antimicrobial Agents and Chemotherapy 11, 888-896.

Horne, D., Hakenbeck, R. & Tomasz, A. (1977). Secretion of lipids induced by inhibition of peptido-

Hughes, R. C. (1968). The cell wall of Bacillus licheniformis NCTC 6346: isolation of low molecular

synergism and antagonism. The interference of chloramphenicol with the action of penicillin.
Archives of Internal Medicine 87, 349-359.

tericidal effects of cell wall inhibitory antibiotics. Antimicrobial Agents and Chemotherapy 10, 697-706.

Bacteriology 74, 461-476.

intra-cellular iodophilic polysaccharide storage.
in Streptococcus mutans. Infection and Immunity 16, 967–973.


