

## Effect of mecillinam on peptidoglycan synthesis during the division cycle of *Salmonella typhimurium* 2616

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### SUMMARY

The effects of mecillinam, ampicillin and cephalixin on peptidoglycan synthesis in *Salmonella typhimurium* 2616 have been studied at equivalent concentrations or "isoactivities". Using antibiotics at isoactivities allows a direct comparison of the biochemical effects of different antibiotics. When mecillinam was added at different times during the division cycle at a concentration that produced 50 % inhibition of peptidoglycan synthesis in an exponential culture over a short period of time, the inhibition of synthesis was greatest in the newborn cells and least in the dividing cells. Antibiotic competition experiments showed that mecillinam preferentially bound to penicillin-binding protein 2 in *S. typhimurium* 2616. High performance liquid chromatography analysis of the residual peptidoglycan synthesized in the presence of mecillinam showed an unexpected increase in pentapeptides and a significant increase in cross-linking. Other antibiotics added at equivalent activities did not show an increase in cross-linking.

**Key-words:** Peptidoglycan, *Salmonella typhimurium*, Mecillinam, Ampicillin, Cephalixin;  $\beta$ -Lactam, MIC, Diaminopimelic acid, PBP, Division cycle.

### INTRODUCTION

Mecillinam preferentially binds to penicillin-binding protein 2 (PBP 2) in *Escherichia coli*. Other  $\beta$ -lactam antibiotics do not exhibit such a singularity of binding to one PBP (Jacoby and Young, 1988). In conjunction with *rodA*, PBP-2 appears to be required for the maintenance of

the rod shape in Gram-negative bacteria (Spratt, 1975). The role of PBP-2 in peptidoglycan synthesis during the division cycle has not been clearly defined. It has been suggested that PBP-2 is specifically involved in cell elongation and does not act throughout the division cycle (Satta *et al.*, 1979). More recently it was proposed that PBP-2 acts throughout the division cycle, but

Submitted May 10, 1993, accepted July 2, 1993.

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is only utilized to extend cylindrical wall in response to the priming action of PBP-1 (Wientjes and Nanninga, 1991).

Wientjes and Nanninga (1991) studied the effect of mecillinam on peptidoglycan synthesis in *E. coli* by measuring the incorporation of tritiated diaminopimelic acid (DAP) during the division cycle. Centrifugal elutriation was used to obtain synchronized cultures. The concentration of mecillinam used, combined with the lag in DAP uptake by *E. coli*, resulted in a 9-10 min delay before any effect could be observed. As a consequence, the determinations of inhibition were measured between 10 and 30 min following the addition of the antibiotic. This raises questions regarding the resolution of the experiments, *i.e.* the ability of the experiment to determine when, during the division cycle, there could be a specific effect of an antibiotic. Furthermore, the use of centrifugal elutriation to produce synchronized cultures has been questioned on both theoretical, experimental, and historical grounds (Cooper, 1991a). Wientjes and Nanninga (1991) observed inhibition by mecillinam throughout the cell cycle. Although the data was interpreted as indicating a slight increase in sensitivity in the newborn cells, the data could also be interpreted as showing that sensitivity is invariant during the division cycle.

We have been using *Salmonella typhimurium* strain 2616 to study peptidoglycan synthesis because of its efficient incorporation of radioactive DAP into peptidoglycan (Gally and Cooper, 1993) with essentially no lag. In short-term labelling experiments, strain 2616 was 30-50 times more efficient than *E. coli* strains at incorporating DAP (Cooper and Metzger, 1986). This result has been confirmed (Wientjes *et al.*, 1991), although it was also suggested that there may be some conversion of DAP to lysine, diminishing the utility of DAP labelling in *Salmonella*. We have rigorously excluded the possibility of leakage of DAP to lysine (Cooper *et al.*, 1992).

Here we study the effect of mecillinam on peptidoglycan synthesis during the division cycle using a concentration of mecillinam that gives an immediate effect on peptidoglycan synthesis. We have used short-term labelling assays to determine the concentration of antibiotic that gives a 50 % inhibition of peptidoglycan synthesis over a short period of labelling. This compares different antibiotics at equivalent "activities" with regard to peptidoglycan synthesis (isoactivities). We have combined the concept of antibiotic isoactivities with the membrane-elution method for cell-cycle analysis to study the effect of different antibiotics on the rate of peptidoglycan synthesis during the division cycle. In addition, we have used high-performance liquid chromatography (HPLC) to analyse the composition of the residual peptidoglycan after inhibition of synthesis by mecillinam, ampicillin and cephalixin.

## MATERIALS AND METHODS

### Bacterial strain and growth conditions

*S. typhimurium* 2616 (LysA<sup>-</sup>) (from Dr. K.E. Sanderson, the University of Calgary) was grown in C medium as described previously (Gally and Cooper, 1993) supplemented with lysine (40 µg/ml). Glycerol was added to a final concentration of 0.4 %. All cells were grown at 37°C with rotary shaking.

### Determination of antibiotic activity

Exponentially growing cells were added to a dilution series of an antibiotic and the mixture was incubated for 1.5 min. Radioactive DAP was added (*meso*-2,6-diamino-3,4,5-<sup>3</sup>H-pimelic acid, 23 Ci/mM, to a final concentration of 0.22 µCi/ml) and incubation was continued for 8 min. The pre-incubation and the 8 min labelling period were determined to be optimal for measuring the short-term effect of an antibiotic on peptidoglycan synthesis. The radioactivity incorporated into peptidoglycan was then determined as described below.

DAP = diaminopimelic acid.  
HPLC = high-performance liquid chromatography.  
PBP = penicillin-binding protein.

SDS-PAGE = sodium dodecyl sulphate/polyacrylamide gel electrophoresis.  
TCA = trichloroacetic acid.

### Isolation of sacculi for radioactivity analysis

After labelling for 8 min, samples were placed in a boiling water bath, and an equal volume of hot sodium dodecyl sulphate (SDS, 8 % w/v) was added, and boiling was continued for 30 min. Samples (4 ml) were then cooled and filtered through a nitrocellulose membrane (0.45  $\mu$ M, 0.25 cm) and washed with 10 volumes of distilled water.

### Membrane-elution technique

The membrane-elution apparatus and theory have been discussed in detail elsewhere (Cooper, 1991a, b). In these experiments, this method was used only to produce synchronized cultures. An exponentially growing culture was filtered onto a nitrocellulose membrane, the membrane was inverted and fresh medium was pumped through the membrane. Newborn cells were released by division from cells bound to membrane. Thus there was a continuous elution of newborn cells from the membrane-elution apparatus. After 1 h of elution, newborn cells were collected into a series of separate tubes and incubated at 37°C. After a period of collection greater than one generation time, a series of tubes was obtained with cells of different ages in each tube. The last tube collected contained the youngest cells and the first tube contained the oldest cells which will have gone through more than one division cycle since elution. The intermediate tubes contained cells at different stages during the division cycle. The concentration of bacteria in each sample was determined immediately after collection and again at the time of radiolabelling in order to monitor the degree of synchronization. The ratio of the final cell count to the initial cell count is a measure of the degree of division that occurred in the samples since elution from the membrane-elution apparatus.

### Determination of antibiotic action during the division cycle

Each fraction eluted from the membrane-elution apparatus was divided into three tubes and placed in a shaking water bath. Three sets of tubes were therefore collected. The first set of tubes was for the addition of the antibiotic, the second set as a control without antibiotic, and the third set of tubes was used to determine the change in cell number during the incubation period. A multipipeter was used to add the antibiotic to the first set of tubes and an equal volume of C medium to the second set. After 1.5 min,  $^3$ H-DAP was added to these two sets of tubes. After 8 min of incubation, the incorporated radioactivity was determined. Tubes containing bacteria from a non-synchronized exponential culture (of

similar volume and cell concentration) were interspersed among the synchronized samples as controls.

### HPLC analysis of peptidoglycan

The concentration of the antibiotic giving 50 % inhibition of DAP incorporation was added to an exponential culture (30 ml) and then labelled with  $^3$ H-DAP. Muropeptides were prepared and separated by reverse-phase HPLC exactly as described previously (Glauner, 1988; Gally and Cooper, 1993). Control samples (no antibiotic) from the same culture were also analysed. Radioactivity in the individual peaks was measured by an on-line scintillation counter (Radiomatic, Packard Instrument Co., Inc., Rockville, MD). Integration of peaks was performed using Maxima 820 software (Waters Associates, Inc., Milford, MA).

### Determination of PBP profiles for mecillinam, cephalixin and ampicillin in *S. typhimurium* by competition with $^{125}$ I-penicillin-X

Bacteria were prepared by first selecting for the Lys<sup>-</sup> phenotype on M9-glucose. An overnight culture was used to inoculate two 400-ml M9-glucose-lysine broth cultures at a starting OD<sub>550</sub> = 0.02, which were grown to OD<sub>550</sub> = 0.4. The cells were harvested by centrifugation (10,000 g) and the pellets were suspended in 4 ml of 100 mM Tris-HCl pH 8.0 + 10 mM MgCl<sub>2</sub>. Samples (3 ml) were placed into a French press minicell (4°C) and pressed twice at 16,000 psi. This produced 70-80 % cell breakage as determined by phase contrast microscopy.

PBP were labelled with reagents prepared as described (Masson and Labia 1983) according to procedures previously reported (Jacoby and Young, 1988). Antibiotic competition experiments were carried out with whole cells and French press lysates. Aliquots (15  $\mu$ l) of the whole cells (150  $\mu$ g protein) and of lysate (150  $\mu$ g protein) were prepared by addition of 3  $\mu$ l of 100 mM 2-mercaptoethanol and 3  $\mu$ l of competing antibiotic. The mixture was vortexed and incubated for 15 min at 37°C. A range of final antibiotic concentrations was prepared by making 1/10 dilutions to give a series of concentrations that bracketed the 50 % inhibition concentration determined as described above. Mecillinam was present at concentration from 100x to 0.001x (where x is the concentration giving 50 % inhibition of DAP incorporation). Ampicillin was present over a range of 1000x-0.001x and cephalixin from 100x-0.001x. After the 37°C incubation the samples were vortexed, 3  $\mu$ l of 83  $\mu$ g/ml  $^{125}$ I-penicillin-X (833  $\mu$ Ci/ml) added, the samples were vortexed again and then incubated at 37°C for 15 min. After the 37°C

incubation with the unlabelled competing antibiotic, the samples were vortexed, and 3  $\mu$ l of 100 mg/ml penicillin G stock solution was added, the mixture was vortexed, and 9  $\mu$ l of SDS-PAGE sample buffer (4-fold concentration) was added. The samples were boiled for 4 min, vortexed, cooled on ice and electrophoresed on a 15 % SDS-PAGE gel. The gels were dried and placed into a cassette with "Kodak X-OMAT" autoradiography film for 4 days.

## RESULTS

### Determination of antibiotic activity

The effect of different concentrations of mecillinam on the incorporation of DAP into peptidoglycan is shown in figure 1. The 1.5 min pre-incubation with antibiotic and the 8 min  $^3$ H-DAP labelling period were determined to be the optimal conditions for assaying the effect of antibiotics on peptidoglycan synthesis in *S. typhimurium* 2616. Under the conditions used, the uptake of DAP was linear for the first 15 min after addition when no antibiotic was present.

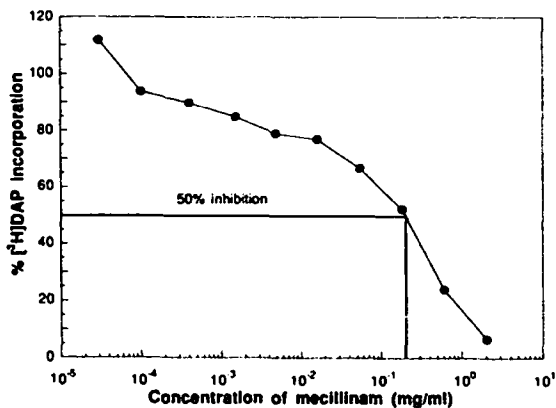


Fig. 1. Determination of sensitivity of *Salmonella typhimurium* 2616 to mecillinam.

Cells growing exponentially in glucose-C medium were added to a dilution series of mecillinam for 1.5 min, then radioactive DAP was added. After 8 additional min of incubation, the radioactivity was determined as described in the text. The percentage of DAP incorporation at each antibiotic concentration was calculated relative to the mean of three control tubes with no antibiotic (100 % incorporation). The 50 % inhibition concentration was determined by interpolation.

The concentration of mecillinam for 50 % inhibition of incorporation was 0.2 mg/ml (fig. 1). The 50 % inhibition concentrations for cephalixin and ampicillin were 2 mg/ml and 0.02 mg/ml respectively.

It should be noted that the antibiotic inhibition curve does not show a plateau or shoulder, *i.e.*, inhibition of peptidoglycan synthesis rises continuously from 0 to 100 % (or conversely, incorporation decreases from 100 to 0 %) with increasing concentration of antibiotic. The same curve shape has been demonstrated for ampicillin, cephalixin and piperacillin (Cooper *et al.*, 1992).

As discussed in detail below, the 50 % inhibition concentration is the most sensitive for determining differences during the division cycle. By utilizing the same "activity" of different antibiotics, rather than comparing equal weights or concentrations, we are eliminating extraneous factors from consideration and comparing only the immediate effects of different antibiotics. The ability of *S. typhimurium* 2616 to produce results with a short-term labelling protocol means that the resolution of our assay is good enough to determine the effect of different antibiotics during the division cycle. In order to determine whether the concentrations of antibiotics used had other effects on the bacteria,  $^{14}$ C-leucine incorporation into TCA precipitable material was also studied under the antibiotic assay conditions. At antibiotic concentrations giving 50 % inhibition of peptidoglycan synthesis there was no effect observed on  $^{14}$ C-leucine incorporation with mecillinam and ampicillin. However, the high concentration of cephalixin (2 mg/ml) also lowered  $^{14}$ C-leucine incorporation into the cell by 50 %. The assay is clearly less applicable in cases where considerable resistance to an antibiotic is shown, resulting in the need for relatively high concentrations of antibiotic. It is not known whether the effect of cephalixin on  $^{14}$ C-leucine incorporation is due to cessation of cell growth or an effect on uptake processes. In any case, this shows that the results with cephalixin are complicated by the multiplicity of affects on biosynthesis. We therefore present the cephalixin results with the

caveat that the results are not clearly due to specific peptidoglycan inhibition. This may serve as a cautionary note for further investigations into isoactivity analysis.

### **Binding of mecillinam, cephalixin and ampicillin to the PBP of *S. typhimurium* 2616**

Whole cell and French press lysates were used in competition experiments with either mecillinam, cephalixin and ampicillin against  $^{125}\text{I}$ -penicillin-X. The results are shown in figure 2. The amount of inhibition of peptidoglycan synthesis for each final concentration of antibiotic used for the competition assay is also shown. Ampicillin exhibits a general binding profile and appears to bind to all of the PBP from *S. typhimurium* 2616 at the higher concentrations used. Cephalixin, which is reported to be specific for PBP-3 in *E. coli*, appears to bind preferentially to PBP-3 and -4, and at higher concentrations, to all PBP. Mecillinam demonstrates the most distinct binding profile, preferentially binding only to PBP-2 at all but the highest concentrations. The same PBP binding profiles were observed whether whole cells or lysates were used in the assay. These results show that the binding profile of mecillinam is different from the other two antibiotics tested and predominantly inhibits PBP-2 in *S. typhimurium* 2616.

It is of interest to compare percentage of inhibition of peptidoglycan synthesis with the PBP binding profile. For example, even when ampicillin is bound to all of PBP-2 and -3, there is essentially no inhibition of peptidoglycan synthesis (fig. 2). This shows the importance of noting both the physiological concentration of an antibiotic and its binding activity in the same conditions. In all cases the most dramatic fall in the rate of peptidoglycan synthesis is associated with inhibition of PBP-1a/b and PBP-5 and -6, not to PBP-2, -3, -4, -7 or -8.

We emphasize that the experiments presented in figure 2 enable one to compare the PBP binding activities with the observed physiological effect of the antibiotic (*i.e.* inhibition of DAP incorporation, which is an indicator of inhibition of peptidoglycan synthesis) and at isoactivity

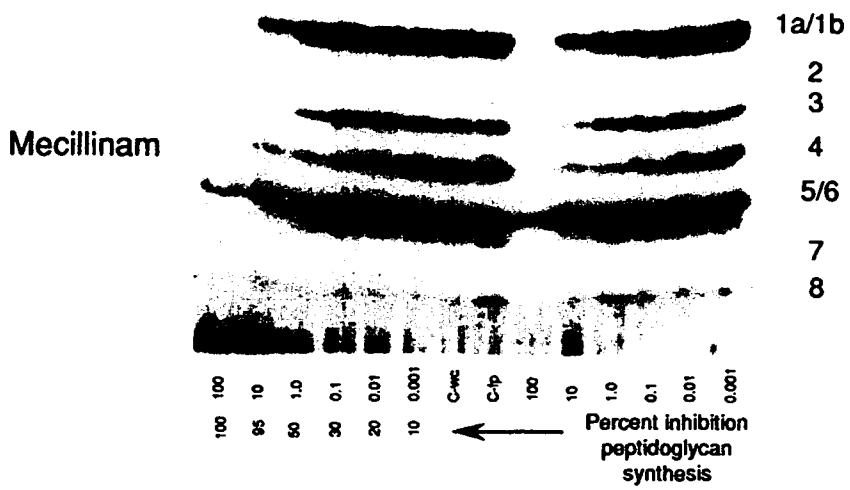
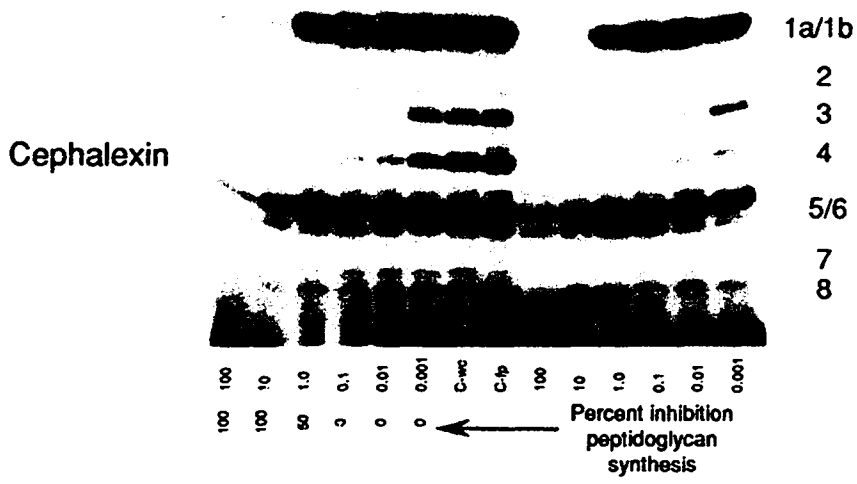
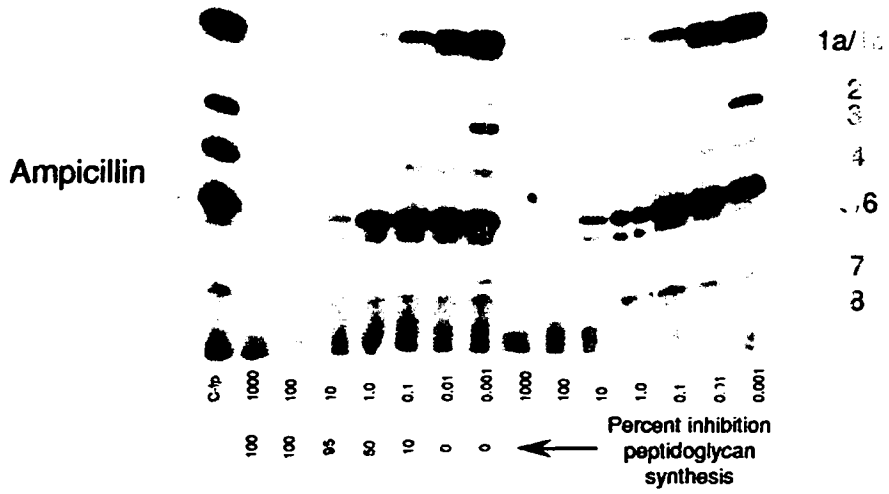
concentrations when comparing different antibiotics. Rather than choosing concentrations for different antibiotics and comparing these irrespective of the effect on cells, our experiments allow one to compare the PBP binding profiles at concentrations exhibiting a precise biochemical effect. It should be cautioned that this experiment does not allow one to conclude that there is a relationship between PBP binding and biosynthesis inhibition.

### **Mecillinam action during the division cycle of *S. typhimurium* 2616**

The effect of mecillinam on DAP incorporation during the division cycle is shown in figure 3. The percentage of DAP incorporated into peptidoglycan in the presence of mecillinam is expressed relative to its corresponding control (no antibiotic). This percentage of DAP incorporation is lowest during the first half of the cell cycle (0-40 min), rises significantly at the time of cell invagination (indicated by the subsequent increase in cell number) and then drops to a lower level in the following division cycle. This demonstrates that mecillinam's action is maximal during the first part of the division cycle and that there is less inhibition by mecillinam during the latter part of the division cycle. The degree of inhibition in the exponentially growing cells suggests that there is no major change in the sensitivity of the synchronized population since the inhibition is equivalent to the mean (average) of the separate synchronized cell populations.

Mecillinam's effect on DAP incorporation into unselected bacteria (*i.e.* cells of all ages) is also shown in figure 3 (dashed line). Since the inhibition of the unselected population is similar to the mean of inhibition of the selected cells, we conclude that the synchronized cells are not altered in their sensitivity to the mecillinam. The ratio of the cell number determined at the time of labelling to the cell number determined at the time of collection is shown in figure 3, upper panel. The cell number remains constant for the first 40 min and then increases sharply, indicating that the bacteria were synchronized. Analy-

Whole cells    French press extracts    PBP



sis of the size distribution of the different samples at the time of labelling (using a Coulter Counter Channelyzer, Coulter Electronics, Inc., Hialeah, FLA) confirmed the degree of synchrony. A similar result has been obtained in two other experiments. Similar experiments with ampicillin and cephalixin at this isoactivity indicate no preferential action during the division cycle. As noted above, the results with cephalixin are not as clear since there is an affect of the antibiotic on total protein synthesis as well as on cell wall synthesis.

#### Composition of residual peptidoglycan after inhibition by antibiotics

The HPLC profiles from muropeptides prepared from exponential cultures treated with (a) no antibiotic, (b) ampicillin (0.02 mg/ml), (c) mecillinam (0.2 mg/ml) and (d) cephalixin (2 mg/ml) are shown in figure 4. The antibiotics were used at concentrations measured to give 50 % inhibition of DAP uptake. Bacteria were incubated for 1.5 min with the antibiotic and then labelled with  $^3\text{H}$ -DAP for 8 min. Ampicillin, which does not have a marked binding preference to any PBP, does not effect any observable change in peptidoglycan composition relative to the control (fig. 4a, b). Cross-linking was 20.2 % in the control sample and 21.6 % from ampicillin-treated cells. (Percentage values are based on total labelled peptidoglycan.) Cephalixin produced a profile similar to the control (fig. 4c) and that produced by ampicillin, with a slightly lower percentage of cross-linking, 18.3 %. However, the percentage of cross-linking from mecillinam-treated cells was sub-

stantially higher at 30.9 % (fig. 4d). Further analysis of the effect of mecillinam on peptidoglycan structure, by examining the incorporation of  $^3\text{H}$ -DAP at a series of short labelling times (30 sec to 15 min), revealed that the proportion of pentapeptide monomers was higher in mecillinam-treated bacteria. The percentage of cross-linking from mecillinam-treated bacteria was consistently higher at all time points analysed compared to the controls. It was also noted that the proportion of muropeptide trimer was significantly reduced in cephalixin-treated bacteria (tetra-tetra-tetra, fig. 4c).

#### DISCUSSION

In these studies we have used the concentration of a particular antibiotic that causes an immediate 50 % inhibition of peptidoglycan synthesis. This was taken as the concentration of antibiotic that caused a 50 % reduction of  $^3\text{H}$ -DAP incorporation into an exponentially growing culture of *S. typhimurium*. Consequently, all antibiotics that have as their immediate site of action the inhibition of peptidoglycan synthesis could theoretically be compared at equivalent activities within the cell. We introduce the term "isoactivity analysis" for this type of investigation. The 50 % inhibition point was chosen as it allows for a degree of variability between different stocks of the antibiotic and best permits detection of increased or decreased sensitivity during the division cycle. It can be argued that other physiological parameters could also be used to study the effect of antibiotics, and thus the inhibition of peptidoglycan synthesis should not be proposed to be the exclusive

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Fig. 2. Antibiotic competition experiments with mecillinam, ampicillin and cephalixin.

Whole cells or French press extracts were assayed for binding activity to different PBP as described in the text. Ampicillin, cephalixin and mecillinam were assayed at concentrations bracketing the 50 % inhibition point (*i.e.*, 1 means a concentration equal to the 50 % inhibition point, 10 is 10 times that concentration, etc.). Below the concentrations we have indicated the percentage of inhibition of DAP uptake for each antibiotic concentration used. C-fp = control French press extract with no antibiotic; C-wc = control whole cells with no antibiotic.

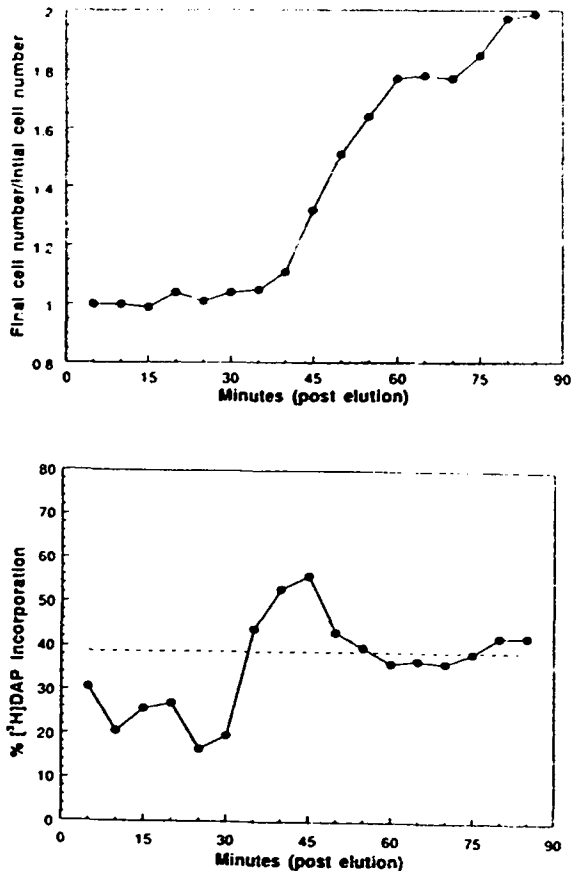


Fig. 3. Inhibition of peptidoglycan by mecillinam during the division cycle of *S. typhimurium* 2616.

Newborn cells were collected for 2 min intervals separated by a 3 min period (*i.e.*, a window of newborn cells was collected for 2 min at 5 min intervals). The collected cells were incubated until antibiotic was added. After 80 min of collection, a series of 16 tubes were obtained with cells at different stages of the division cycle. The different fractions were divided into 3 separate tubes. To one series of tubes, mecillinam was added to a final concentration of 0.2 mg/ml. To another series, a control addition was made without antibiotic. These additions took less than 1 min.

*Upper panel.* — At the time of addition the cell concentration in the third series of tubes was measured and compared to the original eluted cell concentration in order to determine the relative change in cell number during the period of incubation. The relative change in cell number indicates that there was no significant cell division for approximately 40 min followed by a narrow period of cell division.

*Lower panel.* — The ratio of the radioactivity in the samples with mecillinam to the radioactivity in the control samples (no antibiotic) was calculated and is plotted as the percentage of residual DAP incorporation in the presence of mecillinam (●). The effect of mecillinam on an exponentially growing population of cells (*i.e.* cells of all ages, adjusted to the same concentration as the eluted cells) is also shown (---).

measure of activity. Among such parameters are changes in cell division, cell death, shape, or the activity on PBP. The following points should be noted. Observations of cell shape, cell death and cell division require long periods of observation to record an activity. Thus, over the period of a division cycle the cell actually is in the presence of the inhibitor for many different cell cycle ages. This makes it difficult to distinguish short windows of sensitivity during the division cycle.

One further criticism of our approach is the observation that different  $\beta$ -lactams do not interfere with only one target, but recognize at least six to eight different targets, the PBP of the cell. Each  $\beta$ -lactam has a different affinity for each PBP, and the same PBP may have different affinities for each  $\beta$ -lactam (Lorian, 1991). In addition, it has been proposed that different PBP play different roles in cell physiology. Therefore it is argued, if one takes as a reference the concentration that inhibits 50 % of peptidoglycan synthesis, the antibiotic may saturate only one, or a few or all the targets depending on the role the PBP with the highest affinity has in peptidoglycan synthesis. This critique, of course, applies to all measures of antibiotic activity. The difference in the experiments presented here is that we have attempted to compare the antibiotic affinities to different PBP and relate this to the immediate effect of an antibiotic on the growth and biochemical activity of a cell. If the 50 % inhibition of peptidoglycan synthesis is the result of the sum of, for example, 35 % inhibition of PBP-a, and 10 % inhibition of PBP-b, and 90 % inhibition of PBP-c, at least we now have a comparative basis for looking at the activity of different antibiotics, relating the different levels of interaction with different PBP, and comparing this interaction at a constant and reproducible physiological effect.

Mecillinam was shown to be specific for PBP-2 at all but the highest concentrations. Mecillinam appears to act differentially during the division cycle. We find that the greatest sensitivity is in cells at the start of the division cycle, with cells in the later phase of the division cycle being more resistant to the inhibition by mecillinam. These results are in agreement with PBP-2 acting throughout the cell cycle, but with



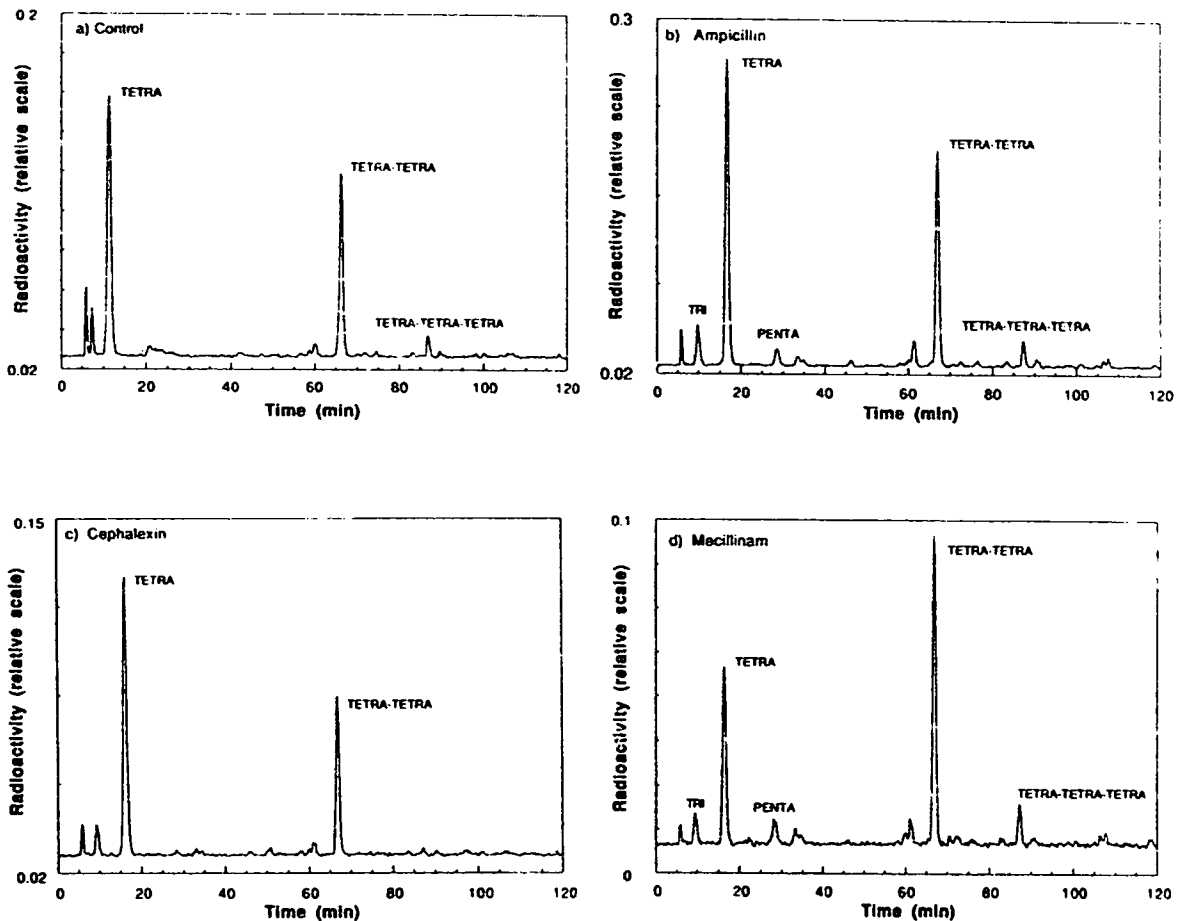


Fig. 4. HPLC analysis of peptidoglycan synthesized in the presence or absence of mecillinam, ampicillin and cephalosporin.

The abbreviations used follow the nomenclature used by Glauner (1988); tri, tetra, penta are N-acetylglucosamine and N-acetylmuramyl tri, tetra and pentapeptides. The tripeptide consists of L-alanine, D-glutamic acid, and *meso*-diaminopimelic acid. The tetrapeptide consists of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid and D-alanine. The pentapeptide has an additional D-alanine unit. Other mucopeptides described are cross-linked combinations of the above monomer units; a = control; concentrations are (b) ampicillin: 0.02 mg/ml; (c) cephalosporin: 2.0 mg/ml; (d) mecillinam: 0.2 mg/ml.

its activity reduced during constriction. It has been previously demonstrated that there is a reduction in the rate of side wall synthesis in the latter half of the division cycle (Gally *et al.*, 1983) and during constriction (Woldringh *et al.*, 1987). Consequently, if PBP-2 is specific for cell wall elongation, then a reduction in its activity would be expected during constriction.

There was no cell cycle-specific inhibition by ampicillin or cephalosporin. The result with cephalosporin is different from previous findings in *E. coli* (Wientjes and Nanninga, 1991) where cephalosporin acted only during septation. The relatively high resistance of *S. typhimurium* 2616 to cephalosporin and the generalized PBP binding profile at the 50% inhibition point may account

for this difference. The generalized PBP binding profile of ampicillin (especially at the 50 % inhibition concentration) may account for our observations with ampicillin.

The preferential binding of certain  $\beta$ -lactam antibiotics to specific PBP should theoretically lead to the inhibition of the particular PBP at one (low) concentration of the antibiotic and then to the inhibition of the other PBP at higher concentrations. The resulting inhibition curve against increasing antibiotic concentration would therefore be bimodal. This curve shape was not found experimentally for any antibiotic tested (e.g., fig. 1). This argues against an all or nothing requirement for a particular PBP and reaffirms the duplication of functions within a set of PBP.

The cell cycle-specificity of PBP-2 may be effected in two different ways. It may be that the major activity of PBP-2 is required primarily for elongation and not constriction or that its activity is replaced by another PBP during constriction. HPLC analysis of the residual peptidoglycan provides an indication of the biochemical activity of the inhibited enzyme. Ampicillin, which did not exhibit preferential binding, gave an HPLC profile very similar to that of a control (no antibiotic). However, the effect of inhibiting primarily PBP-2 was a marked increase in cross-linking, coupled with an increased percentage of pentapeptides at short labelling times. One possible conclusion from these results is that PBP-2 acts as a carboxypeptidase, although this activity for PBP-2 has not been reported. Alternatively, it may be that binding to this enzyme upsets the equilibrium of a tightly controlled assembly system; a system that requires a highly regulated level of cross-linking to meet the contrasting requirements of expansion and rigidity. PBP-2 may effect cross-linking reactions (by transpeptidation, carboxypeptidase activity, or even cleavage) that maintain the level of cross-linking within limits that are optimal to allow the safe elongation of the cylindrical wall. An increase in cross-linking in the presence of mecillinam has been previously reported (Essig *et al.*, 1982). One implication of these results may be that peptidoglycan synthesized during con-

striction (with PBP-2 less active) may be more cross-linked. We have found this not to be the case (Gally and Cooper, 1993), implying that the reduced activity of PBP-2 is compensated for by another enzyme (potentially PBP-3). We hope that further studies utilizing the inhibition of peptidoglycan synthesis with isoactivity concentrations of antibiotics will provide further insight into the biochemical roles of PBP during the division cycle.

We would note one important experimental point regarding the alignment of PBP binding activity and the physiological affect of an antibiotic. Since the assay of inhibition of peptidoglycan synthesis by the measurement of incorporation of DAP is done on living, growing cells, and the PBP binding assay is performed on washed cells or fragments of cells with a slightly different timing, there is a possibility that there may be differences that are ascribable to the experimental procedures. We note this point with the proposal that it may be necessary, in future work, to perform the PBP assays on living cells under the exact same conditions as required for uptake and incorporation assays. We have presented the PBP binding data using the standard experimental conditions so that our results can be compared in different laboratories.

Aside from the experimental findings, we cannot emphasize too strongly the two items of theoretical importance: (i) using isoactivities for comparing different antibiotics and (ii) using the 50 % inhibition concentration as a marker. These proposals are independent of the antibiotic used and should not be restricted to penicillins but affect the study of all antibiotics which have a specific and recognizable measure of inhibition by the antibiotic. The proposal to approach antibiotic studies this way was made two decades ago (Cooper, 1974), and we hope that its relevance is apparent in the work presented here.

#### Acknowledgements

The preparation of this article and the experiments within were supported by National Institutes of Health Grant R01 GM44022-01A1 (S.C.) and R01 GM40947 (K.Y.).

**Action du mécilliname sur la synthèse du peptidoglycane au cours du cycle de division de *Salmonella typhimurium* 2616**

L'action du mécilliname, de l'ampicilline et de la céphalexine sur la synthèse du peptidoglycane de *Salmonella typhimurium* 2616 a été étudiée à concentrations équivalentes ou « isoactivité ». L'utilisation des antibiotiques à l'isoactivité permet de comparer directement les effets biochimiques des différents antibiotiques. Quand le mécilliname est ajouté à différents moments au cours de la division cellulaire, à la concentration produisant 50 % d'inhibition de la synthèse du peptidoglycane dans une culture en phase exponentielle sur une courte période de temps, l'inhibition de la synthèse du peptidoglycane est la plus forte dans les cellules naissantes, et elle est la plus faible dans les cellules en division. Des antibiotiques mis en compétition permettent d'observer que le mécilliname se lie préférentiellement à la PBP-2 (penicillin-binding protein 2) chez *Salmonella typhimurium* 2616. L'analyse par HPLC (high-performance liquid chromatography) du peptidoglycane résiduel synthétisé en présence du mécilliname met en évidence une augmentation imprévue des pentapeptides et une augmentation significative du nombre de liaisons croisées. Avec les autres antibiotiques ajoutés à isoactivité, il n'y a pas d'augmentation du nombre des liaisons croisées.

**Mots-clés:** Mécilliname, Ampicilline, Céphalexine, Peptidoglycane, *Salmonella typhimurium*;  $\beta$ -Lactames, CMI, Acide diaminopimélique, PBP, Division cellulaire.

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