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Separation and Quantification of Murein and Precursors from *Enterobacter cloacae* after Treatment with Trimethoprim and Sulphadiazine

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Abstract—The intracellular concentrations of the soluble murein precursors UDP-Mur-NAc-pentapeptide in the cytoplasm, the membrane-bound lipid precursor disaccharide pentapeptide and the muropeptides of *Enterobacter cloacae* cultures treated with trimethoprim (12·5 μg mL^{-1}) and sulphadiazine (250 μg mL^{-1}) were determined by using capillary zone electrophoresis analysis. In the presence of trimethoprim, UDP-Mur-NAc-pentapeptide as well as disaccharide pentapeptide accumulated. In the case of sulphadiazine-treated cells, the concentration of UDP-Mur-NAc-pentapeptide roughly paralleled the control cells but sulphadiazine caused a slow incremental accumulation of disaccharide pentapeptide. The muropeptide composition of the murein indicated that the differences between the peptidoglycans produced by the control cells and the cells grown in the presence of either trimethoprim or sulphadiazine alone or in combination were quite marked. The results suggest that the enhanced activity of trimethoprim plus sulphadiazine against *E. cloacae* is caused by an additional effect on the inhibition of the bacterial peptidoglycan biosynthesis and that this additional effect is a fundamental part of the antibacterial action of the antimetabolites. This effect leads to changes of cell morphology and resultant changes in bacterial cell permeability.

Recent work has indicated that the enhanced activity of combinations of trimethoprim and sulphonamides at sub-inhibitory concentrations is related to mutually increased uptakes of both antibacterials, even when the bacteria are resistant to one member of the combination (Richards & Xing 1991, 1992; Richards et al 1991, 1993a). It has been proposed that the sequential blockade of folinic acid synthesis imposed by trimethoprim plus a sulphonamide results in changes to the bacterial cell permeability barrier which, in turn, leads to increased bacterial uptake of both antibacterial agents (Richards & Xing 1991, 1992; Richards et al 1991, 1993a, b). Electron microscopy indicated that the morphological changes produced by the action of trimethoprim and sulphadiazine on *Enterobacter cloacae* (Richards et al 1993a) and *Enterococcus faecalis* (unpublished results) involved damage to the peptidoglycan of each organism and had an effect on septum completion in *E. faecalis*. This provided an explanation of how trimethoprim and sulphadiazine affect cell permeability and leads to increased uptake of both antibacterials.

The present investigation was to determine the molecular events of inhibition of peptidoglycan biosynthesis induced by trimethoprim and sulphadiazine by using capillary zone electrophoresis (CZE) analysis, and analysing the intracellular concentrations of the soluble murein precursor (UDP-Mur-NAc-pentapeptide) in the cytoplasm, the membrane-bound lipid precursor (disaccharide pentapeptide) and the quantification of muropeptides of *E. cloacae* cultures treated with trimethoprim and sulphadiazine.

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Materials and Methods

Materials

E. cloacae NCTC 10005 was obtained from the National Collection of Type Cultures, Colindale, London, UK. Isosensitest broth was obtained from Oxoid, Basingstoke, UK. Unless otherwise stated, all the reagents used were of commercial analytical grade and obtained from Sigma, Poole, UK. *n*-Butanol was obtained from FSA Laboratory Supplies, UK. Dibromopropamidine isethionate was a gift from Rhone-Poulenc Rorer, UK. Glass beads (1·5–2·0 mm) were obtained from BDH Ltd, Poole, UK. The UDP-Mur-NAc peptide precursor and the disaccharide pentapeptide used as standards were gifts from Dr Holtje, Abteilung Biochemie, Max-Planck-Institute fur Entwicklungsbiologie, Germany. HPLC-grade methanol was obtained from Rathburn Chemicals Ltd, Walkerburn, UK, and water was glass-distilled and then further purified by a Millipore Milli-Q system.

Electropherograph equipment

The capillary zone electrophoresis apparatus used for these experiments was the Model 3850 electropherograph from Isco (Nebraska, USA). Fused-silica capillaries 50 μm i.d., had a total length of 65 cm and a separation length of 45 cm. The sample was introduced into the column by hydrodynamic loading.

Determination of murein precursors

Extraction and determination of UDP-Mur-NAc-pentapeptide and disaccharide pentapeptide from *E. cloacae* was based on the procedures described by Kohlrausch & Holtje (1991), Kohlrausch et al (1989) and Richards & Xing (1994). Briefly, 4 mL 18-h culture was inoculated into a flask containing 96 mL Isosensitest broth prewarmed to 37°C and

incubated in a shaking water-bath for 4 h. Subsequently, 50 mL of the exponentially growing cells was transferred to 50 mL of plain, prewarmed Isosentitest broth (control) or broth containing either of the antibacterials alone, or in combination, for further incubation at 37°C. *E. cloacae* was found fairly resistant to trimethoprim (minimum inhibitory concentration (MIC) 15 $\mu g\,mL^{-1}$) and sulphadiazine (MIC, $> 3000\,\mu g\,mL^{-1}$) (Richards et al 1991). In this investigation the final antibacterial concentrations in Isosensitest broth were: trimethoprim 12·5 $\mu g\,mL^{-1}$, sodium sulphadiazine 250 $\mu g\,mL^{-1}$, and trimethoprim 12·5 $\mu g\,mL^{-1}$ plus sodium sulphadiazine 250 $\mu g\,mL^{-1}$. At 10-min intervals, viable counts were performed using the overdried agar plate method and at the same time 1-mL samples of culture were withdrawn and added to 2·5 mL ice-cold solvent (*n*-butanol/6 M–pyridinium acetate pH 4; 4:1, v/v) to extract the murein precursor. After vigorous shaking with 0·5 g glass beads in a shaking bath for 30 min at 4°C, the samples were centrifuged for 10 min at 70 rev min^{-1} using an IEC Centra-4B centrifuge and portions from both phases were withdrawn for quantification of the murein precursors. UDP-Mur-NAc-pentapeptide in the aqueous phase was separated by CZE. Dibromopropamidine isethionate (100 $\mu g\,mL^{-1}$) was added as internal standard. The separations were carried out with running buffer 50 mM sodium hydroxide adjusted to pH 3·0 with phosphoric acid containing 15% methanol. The sample was loaded over a period of 1 s and the electrophoresis was at 18 kV. UV absorbance was at a wavelength of 205 nm. The lipid-linked disaccharide pentapeptide in the organic extraction phase was hydrolysed with 0·1 M HCl for 15 min in a boiling-water bath to release the disaccharide pentapeptide. The muramyl residues were reduced to the muramitol derivatives with sodium borohydride and adjusted to pH 4·5 with phosphoric acid. Pyrimethamine (200 $\mu g\,mL^{-1}$) was used as internal standard for disaccharide pentapeptide. The CZE assay was carried out as described above except that the running buffer was pH 4·0, the sample loading time was 10 s and the applied voltage was 20 kV. Both UDP-Mur-NAc-pentapeptide and disaccharide pentapeptide were identified by running the authentic standards kindly provided by Dr Holtje, under the same electrophoresis conditions. The ratios of the peak heights of these compounds were calculated with reference to the peak heights of the internal standards. The concentrations of UDP-Mur-NAc-pentapeptide and disaccharide pentapeptide in the control culture at time zero was used as 100% and the subsequent concentrations are represented as percentages of this concentration of control culture.

Analysis of composition of muropeptides

Preparation of muropeptides. Extraction of murein from *E. cloacae* was based on the procedures described by Glauner et al (1988) and Hoyle & Beveridge (1984). Cells were grown exponentially at 37°C in plain Isosensitest broth or in broth containing trimethoprim (12·5 $\mu g\,mL^{-1}$) or sodium sulphadiazine (250 $\mu g\,mL^{-1}$) alone or in combination and harvested by centrifugation (15 min, 6000 *g*, 4°C) and resuspension in HEPES buffer (10 mM pH 6·8). This suspension contained approximately 150 mg dry weight of cells. The cells were washed twice with HEPES buffer and resuspended in 15 mL buffer. The cell suspension was then added dropwise to 185 mL boiling 4% w/v sodium dodecyl sulphate (SDS) over a 15-min period with vigorous stirring. The suspension was kept boiling for 45 min and then allowed to cool and remain at room temperature (21°C) overnight. The murein was collected by centrifugation (70 000 *g*, 40 min, 20°C). SDS was removed from the pellet by four washings with 10 mL water. DNAse (50 $\mu g\,mL^{-1}$), RNAse (100 $\mu g\,mL^{-1}$) and $MgCl_2$ (20·3 $\mu g\,mL^{-1}$) were added to the suspension and allowed to react for 60 min at 23°C. The suspension was centrifuged as above and after extensive washing the pellet was then resuspended in 30 mL 10 mM HEPES buffer. Trypsin (1·0 mg dry weight) was added and incubated for 45 min at 23°C to cleave bound lipoprotein. After centrifugation the sample was washed three times with water and then resuspended in 0·5 mL 20 mM sodium phosphate (pH 4·8) containing 0·02% w/v sodium azide. Lysozyme was added to produce a final concentration of 20 $\mu g\,mL^{-1}$ and incubated overnight at 37°C. The enzyme reaction was stopped by boiling the sample for 3 min. Insoluble material was removed by centrifugation (13 000 rev min^{-1}, 5 min). The resulting samples were lyophilized, dissolved in 0·5 mL water and stored frozen (−20°C).

Reduction of muropeptides. Five hundred microlitres of the above muropeptide solution was mixed with 0·5 mL 0·5 M sodium borate pH 9·0 (boric acid titrated with sodium hydroxide). Solid sodium borohydride (10 mg mL^{-1}) was added immediately and the sample was incubated for 30 min at room temperature. Excess borohydride was eliminated with phosphoric acid by adjusting the sample to pH 3–4.

Electrophoresis conditions. The conditions for the CZE separation of the muropeptides were as follows. The column was given a 1-min wash with 0·1 M sodium hydroxide and a 1-min wash with methanol, followed by a 5-min rinse with running buffer. The sample was then loaded for 3 s. The separation was carried out with 50 mM phosphate buffer containing 15% v/v methanol at pH 2·1, at 12·5 kV. The column eluant was monitored for UV absorbance at a wavelength of 200 nm.

Results

Intracellular concentration of murein precursors

The quantities of soluble murein precursor UDP-Mur-NAc-pentapeptide and the disaccharide pentapeptide for cultures treated with the antibacterials are presented in Fig. 1. The level of UDP-Mur-NAc-pentapeptide and disaccharide pentapeptide in the absence of antibacterials was constant during the 3-h experimental period, but gave different overall levels from the cells grown in the presence of the antibacterials. Inhibition of murein synthesis by trimethoprim (12·5 $\mu g\,mL^{-1}$) caused a slow increase in the concentration of UDP-Mur-NAc-pentapeptide. The intracellular concentration of disaccharide pentapeptide markedly increased after 40 min of the addition of trimethoprim. A different pattern in the intracellular concentration levels of the precursor was observed for sulphadiazine (250 $\mu g\,mL^{-1}$)-treated cells. The concentration of UDP-Mur-NAc-penta-

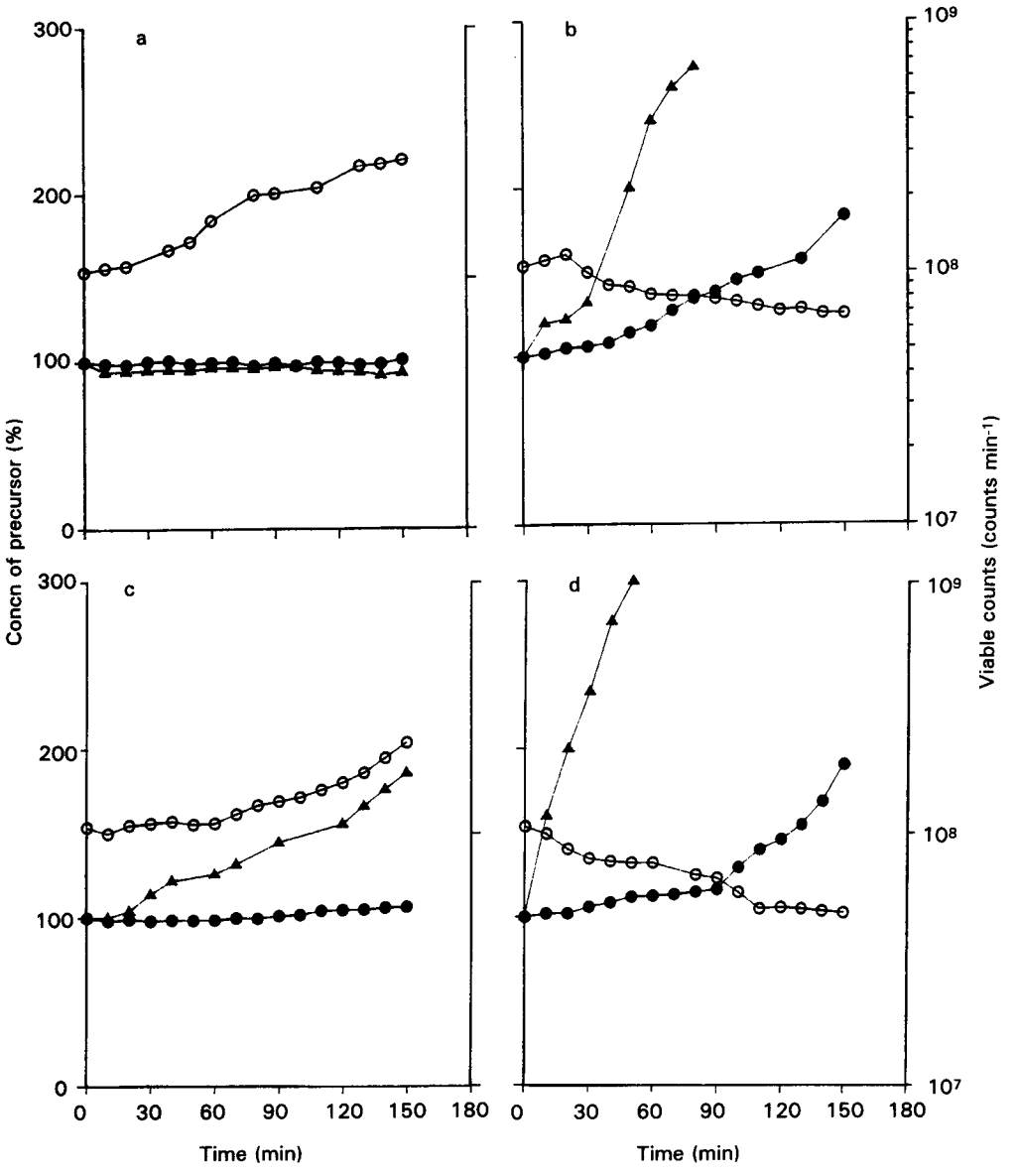

FIG. 1. Changes in the concentration of murein precursors during antibacterial treatment. ● UDP-Mur-NAc-pentapeptide and ▲ disaccharide pentapeptide were determined in the aqueous and organic phases after extraction of 1-mL culture samples with *n*-butanol-pyridinium acetate. a. Control culture; b. trimethoprim (12·5 μg mL^{-1}); c. sulphadiazine (250 μg mL^{-1}); d. a combination of trimethoprim (12·5 μg mL^{-1}) and sulphadiazine (250 μg mL^{-1}). ○ Represents the viable counts for each culture.

peptide kept almost constant and was similar to the control cells throughout the cell cycle. However, the concentration of disaccharide pentapeptide initially remained constant but after 30 min sulphadiazine caused a slow incremental accumulation of this precursor roughly in parallel with the increase in the viable counts. The combination of trimethoprim (12·5 μg mL^{-1}) plus sulphadiazine (250 μg mL^{-1}), produced intracellular concentrations of UDP-Mur-NAc-pentapeptide which were slightly greater than for trimethoprim-treated cells and an even greater increase in the concentration of disaccharide pentapeptide.

Changes in muropeptide composition

The CZE method allowed identification of the changes in peptidoglycan composition in terms of the particular distinct muropeptides involved. The reproducibility of migration times and the relative percentages of the ten major peptides present in the total muropeptides from the control cells were determined by five replicate determinations (Table 1). The relative standard deviations for the reproducibility of migration times are within 2% for all the ten peaks. The standard deviations for the relative percentages of the ten major peptides present in the total muropeptides were shown to be less than 1·0 for the five replicates.

There is no report on the muropeptide composition of the cell wall of *E. cloacae* as far as we know. Since it is difficult to prepare material separated by the CZE instrument, and there are very few muropeptide standards available, the

Table 1. Reproducibility of migration times and relative amounts of ten major muropeptides.

Peptide no.	Mean value of migration time (min) (n = 5)	Relative standard deviation (%) for migration time	Mean value of amount (%) (n = 5)	Standard deviation for amount
1	18·3	1·88	33·62	0·12
2	19·1	1·58	47·63	0·23
3	20·5	1·55	1·95	0·19
4	21·4	0·61	0·87	0·24
5	22·0	1·10	2·35	0·84
6	22·4	1·50	1·20	0·58
7	23·6	1·68	3·93	0·13
8	24·8	1·52	4·84	0·64
9	25·6	2·02	3·07	0·14
10	26·4	1·60	0·93	0·24

ten peptides were not identified. Nevertheless, the differences between the peptidoglycans produced by the control cells and by the cells grown in the presence of either trimethoprim or sulphadiazine alone or in combination were quite marked and are illustrated in Table 2. The same overall changes were detected when the antibacterials were used alone and when they were used in combination, although some changes were less pronounced in the case of sulphadiazine used alone.

New peaks in the organic phase extracts

There were no new peaks found with the water-phase extracts of cells treated with the antibacterials, but with the organic phase a total of three new peaks were seen after 30 min in treated cells. The separation of the extraction with *n*-butanol-pyridinium acetate gave the peaks of new compounds formed in the presence of the antibacterials (Fig. 2). These new compounds have not been identified at present, but they were formed by new substances present in the antibacterial-treated cells. No new peak was seen in the control cells. Peak I was formed by a new substance present in the trimethoprim-treated cells and new peaks II and III represented new substances present in the sulphadiazine-treated cells. In addition, these new peaks were all produced by the extracts from the cells treated with the antibacterial combination. Fig. 3 shows the increase in concentration with time of these new substances after treatment of the cultures with either trimethoprim or sulphadiazine. The data are represented as the ratio of the peak heights to the internal standard.

Discussion

It has been speculated that changes in the relative amounts of the murein precursor represent a general effect of murein synthesis inhibition (Tomasz & Waks 1975; Tomasz & Holtje 1977). The biosynthesis of murein may be conveniently divided into four stages (Franklin & Snow 1989); stage 1, formation of UDP-Mur-NAc; stage 2, formation of UDP-Mur-NAc-pentapeptide; stage 3, formation of the linear peptidoglycan; and stage 4, cross-linking of two linear peptidoglycan chains. Different bacteria show variations in peptidoglycan structure. The biosynthesis of peptidoglycan was first determined for *Staphylococcus aureus* and it is this biosynthetic sequence which is used here to illustrate the general features of the process. Fig. 4 gives the structural formulae and provides an overall diagram of the relevant part of the biosynthetic pathways involved in murein synthesis.

The results presented here show that characteristic changes in the levels of the murein precursors occur when murein synthesis is inhibited by trimethoprim or sulphadiazine. Treatment with trimethoprim caused a marked accumulation in UDP-Mur-NAc-pentapeptide. Further metabolism seems to be blocked. Zemell & Anwar (1975) suggested that UDP-Mur-NAc-pentapeptide acted as a negative modulator of pyruvate-UDP-Glc-NAc transferase (the enzyme which catalyses the first committed step in the biosynthesis of bacterial cell wall peptidoglycan) and that there was no accumulation of UDP-Mur-NAc-pentapeptide on treatment with penicillin or D-cycloserine (Zemell &

Table 2. Relative amounts (%) of ten major muropeptides in control cells compared with trimethoprim-sulphadiazine-treated cells.

Peptide no.	Control	Trimethoprim		Sulphadiazine		Trimethoprim/ sulphadiazine	
	Relative amount	Relative amount	Increase (%)	Relative amount	Increase (%)	Relative amount	Increase (%)
1	34·17	41·41	+21·17	33·32	−2·48	39·31	+15·20
2	48·01	45·42	−5·40	45.59	−5·05	50·49	+5·16
3	2·34	1·13	−51·63	2·29	−1·93	1·11	−52·44
4	0·98	1·98	+101·0	1·97	+100·0	2·18	+121·97
5	2·37	3·71	+56·34	3·41	+43·87	3·91	+64·60
6	1·11	1·89	+70·95	1·56	+41·36	1·88	+70·23
7	3·65	1·86	−49·20	4·75	+30·07	0·56	−84·61
8	4·79	1·80	−62·56	4·05	−15·50	0·56	−88·28
9	2·58	0·61	−76·31	2·21	−14·21	0·01	−99·61
10	0·95	0·21	−75·71	0·85	−10·56	0·007	−98·94

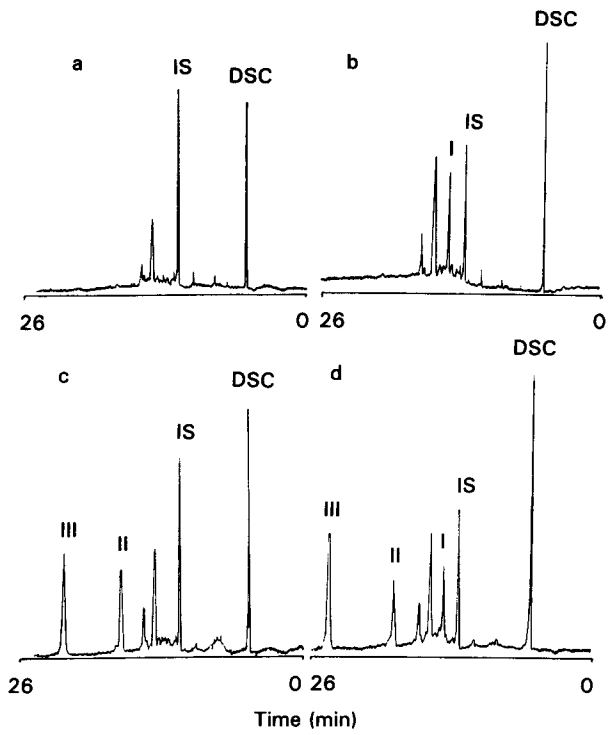

FIG. 2. Electropherogram of the separation of the disaccharide pentapeptide by CZE. a. Control culture; b. trimethoprim (12·5 $\mu g\,mL^{-1}$); c. sulphadiazine (250 $\mu g\,mL^{-1}$); d. trimethoprim (12·5 $\mu g\,mL^{-1}$) plus sulphadiazine (250 $\mu g\,mL^{-1}$). DSC = disaccharide pentapeptide, IS = internal standard (pyrimethamine), I, II, III = new peaks in the extracts of the trimethoprim- or sulphadiazine-treated cells.

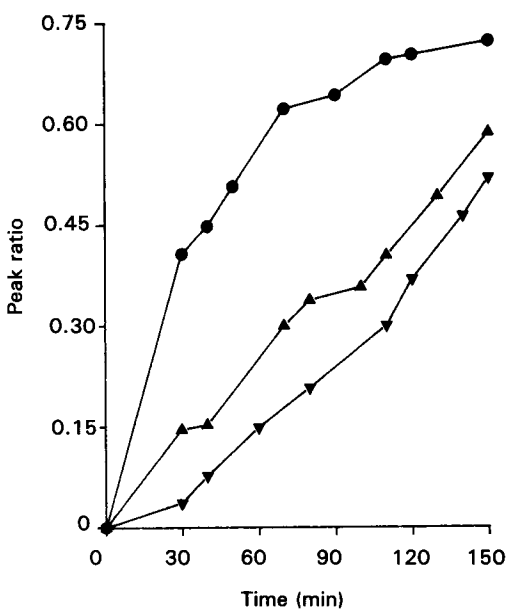

FIG. 3. The concentrations of the new material found in the aqueous and organic phase extracts after trimethoprim and sulphadiazine treatment. The data are presented as the ratio of the peak heights to the internal standard. ● Peak I, a new substance produced by the trimethoprim-treated cells; ▲ peaks II and ▼ III, new substances produced by the sulphadiazine-treated cells.

Anwar 1975; Kohlrausch & Holtje 1991). The results presented here indicate a different mechanism for trimethoprim from that of penicillin or D-cycloserine.

Marked increases in the disaccharide pentapeptide after treatment with trimethoprim and also to a lesser extent sulphadiazine were observed. This could be the result of the blocking of its incorporation into peptidoglycan or the recycling of the undecaprenylphosphate. It has been found that moenomycin induces accumulation of UDP-Mur-NAc-pentapeptide and disaccharide pentapeptide in *Escherichia coli* (Franklin & Snow 1989; Kohlrausch & Holtje 1991), similar to the *E. cloacae* cells treated with trimethoprim here. Moenomycin has been shown to inhibit a glycan transglycosidase reaction in a cell-free system that utilized the substrate undecaprenyl-pyrophosphate-disaccharide pentapeptide (Heijenoort et al 1978; Heijenoort & Heijenoort 1980). The results presented here suggest that trimethoprim may have a similar direct or indirect effect on this biosynthetic stage. It seems that sulphadiazine may also have an effect on this stage by slowing down the transglycosidase reaction. The enhanced magnitude of the accumulated effect on UDP-Mur-NAc-pentapeptide and disaccharide pentapeptide produced by a combination of trimethoprim plus sulphadiazine provides evidence of an increased effect by the combination on the inhibition of peptidoglycan biosynthesis by *E. cloacae*.

Morphological changes observed in *E. cloacae* cells, which resulted from the action of trimethoprim or sulphadiazine, indicated that the peptidoglycan layer was damaged and that this damage was similar to that caused by an EDTA plus lysozyme-induced hydrolysis of the peptidoglycan layer (Richards et al 1993a). Trimethoprim also affects septum completion in *E. faecalis* (unpublished results). Since the morphological changes of a bacterial cell during growth and division involve rearrangements within the sacculus molecule and one of the roles of the novel transglycosylase is the local rearrangement of murein (Holtje et al 1975), the results presented here could provide an explanation for the previously reported morphological observations; that is, a direct or indirect effect of trimethoprim and sulphadiazine on the transglycosylation of bacterial peptidoglycan biosynthesis may be responsible for the damage to the peptidoglycan layer and the incomplete septum formation observed in previous investigations (Richards et al 1993a).

The new compounds found in the organic extract are the result of an effect of the antibacterials on cell metabolism since the concentration of the peak materials increased with increased length of incubation. The composition of these products of trimethoprim- and sulphadiazine-affected cell metabolism is not known.

Since there are several factors involved in the bacterial peptidoglycan biosynthesis, e.g. transglycosidase, the substrate (Tomasz & Waks 1975; Heijenoort et al 1978; Heijenoort & Heijenoort 1980) and the penicillin binding proteins (PBP) 1A, 1B, 2 and 3 which are involved in the polymerization steps of the biosynthesis of peptidoglycan (Suzuki et al 1980; Matsuhashi et al 1990; Kohlrausch & Holtje 1991), the modes of action of the two antimetabolites on this biosynthetic step are not clear. It is well known that both trimethoprim and sulphadiazine are antifolate drugs and it is likely that the inhibition of biosynthesis of

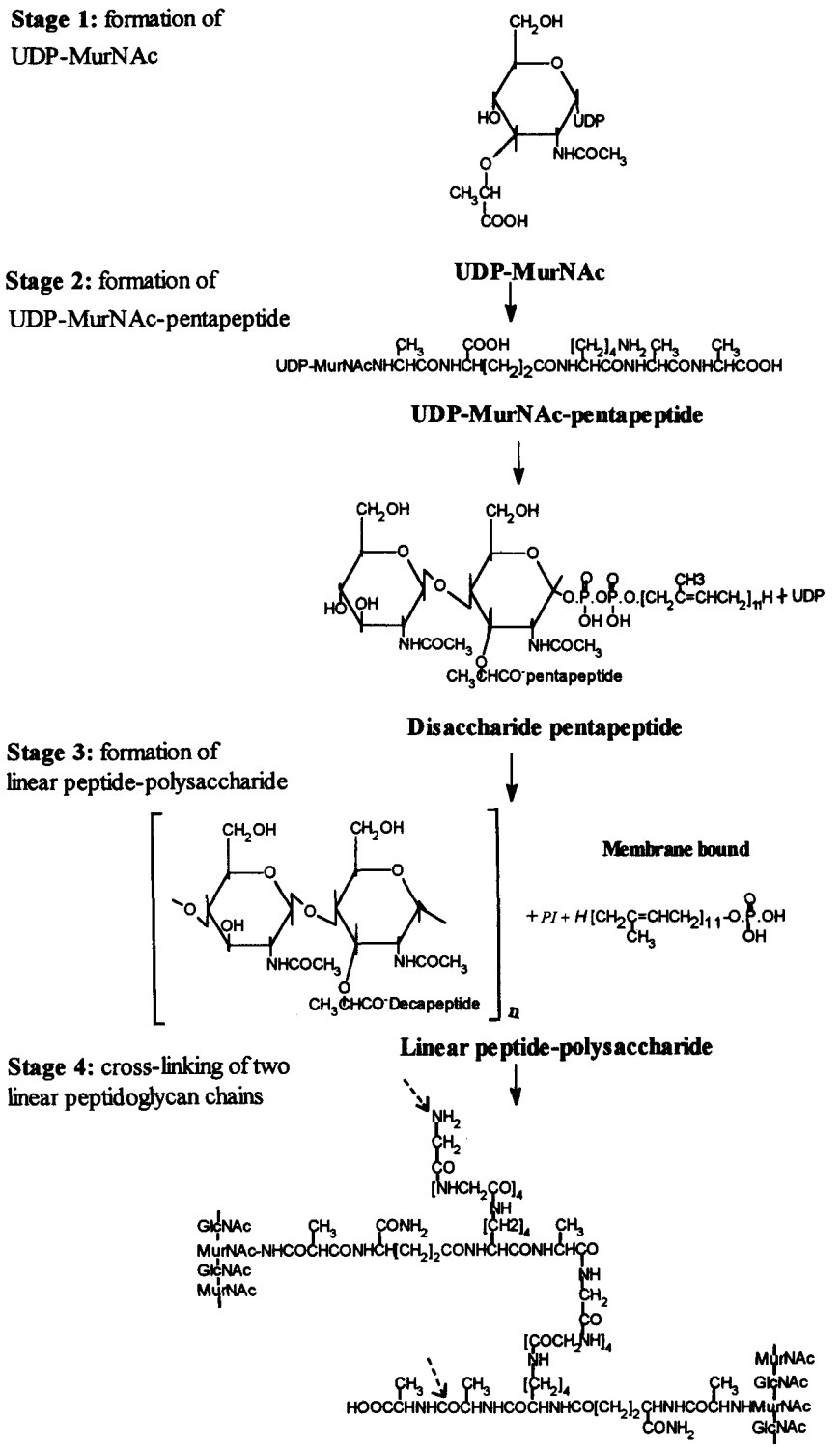

FIG. 4. Biosynthetic pathways involved in murein synthesis (adapted from Franklin & Snow (1989)). Broken arrows show points at which further cross-links may be formed with other polymer chains.

peptidoglycan caused by these agents is a consequence of their effect on the folate pathway.

The results presented here in conjunction with previously reported results (Richards & Xing 1991, 1992; Richards et al 1991, 1993a, b) indicate that the enhanced activity of trimethoprim plus sulphadiazine against *E. cloacae* is due to an additional effect on the inhibition of the bacterial peptidoglycan biosynthesis (possibly an inhibition of the transglycosylation step), which leads to changes of cell morphology and resultant change in bacterial cell permeability. This additional effect is considered to be a fundamental part of the antibacterial action of these antimetabolites.

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