Reversible inhibition of bacterial growth after specific inhibition of spermidine synthase by dicyclohexylamine

Tiina MATTILA,* Tuula HONKANEN-BUZALSKI† and Hannu PÖSÖ*

*Department of Pharmacology and Toxicology and †Department of Internal Medicine, College of Veterinary Medicine, SF-00551, Helsinki 55, Finland

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The effect of dicyclohexylamine on seven freshly isolated bacterial strains of mastitis pathogens was studied. *Streptococcus uberis* was the most sensitive strain investigated, since 5 mM-dicyclohexylamine totally arrested its growth and 1.25 mM of the drug caused 60% growth inhibition. The *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* strains were also sensitive to the drug, but less so than *Strep. uberis*, since 5 mM drug caused only partial inhibition of growth. *Micrococcus* sp. and *Klebsiella* sp. grew in the presence of 10.0 mM-dicyclohexylamine, and, finally the growth of *Streptococcus agalactiae* was not at all affected by dicyclohexylamine. These different sensitivities towards dicyclohexylamine *in vivo* were paralleled by different sensitivities of the bacteria's spermidine synthase to the drug *in vitro*, and also by the ability of the drug to lower spermidine concentration in bacterial cells. Spermidine synthase from sensitive bacteria was inhibited by more than 90% by 50 μ M-dicyclohexylamine *in vitro*, and the concentration of spermidine was decreased in *E. coli* and *Ps. aeruginosa* by 70% and in *Strep. uberis* by 95%, whereas in *Strep. agalactiae* 5 mM-dicyclohexylamine did not affect the concentration of spermidine at all. Dicyclohexylamine treatment led to the accumulation of putrescine in *Strep. uberis*. Spermidine synthesis catalysed by the extracts of *Micrococcus* sp. required 500 μ M-dicyclohexylamine for 90% inhibition, and *Strep. agalactiae* contained a spermidine synthase that was still active at 1000 μ M-dicyclohexylamine. The observed inhibition of growth was totally reversed by adding 50 μ M-spermidine (final concentration) to the medium. Putrescine reversed the inhibition only when bacteria had a spermidine synthase activity insensitive to dicyclohexylamine. Spermine did not overcome the inhibition of growth caused by dicyclohexylamine, probably because it was not taken up by the bacterial cells used in this study. The inhibition of the growth by dicyclohexylamine (even in the case of *Strep. uberis*) was reversible in the sense that addition of 50 μ M-spermidine 18 h after dicyclohexylamine still restored the growth rate of untreated controls.

The importance of natural polyamines (putrescine and spermidine) is clearly seen in the case of bacteria, because *Escherichia coli* mutants lacking detectable amounts of putrescine (Morris & Jorstad, 1973), spermidine (Tabor *et al.*, 1978) or both polyamines (Hafner *et al.*, 1979) grow very slowly or not at all (Tabor *et al.*, 1981). The exact role of polyamines in these mutants is not known, although numerous studies have shown that the polyamines have a large variety of effects *in vitro* at the levels of protein and nucleic acid metabolism (Sakai & Cohen, 1976; Tabor & Tabor, 1976; Jänne *et al.*, 1978; Pösö & Kuosmanen, 1983). The

attempts to determine which of these effects are relevant to the physiological function of the polyamines *in vivo* have given conflicting results, but work with an *E. coli* mutant having an absolute need for polyamines (Tabor & Tabor, 1982) indicates that polyamines are needed to maintain a ribosomal structure suitable for protein synthesis, a proposal that has also been suggested in other studies (Echandi & Algranati, 1975; Igarashi *et al.*, 1981).

Apart from some other work with yeast mutants lacking polyamines (Cohn *et al.*, 1978, 1980), evidence for the absolute need for polyamines in

the growth of eukaryotes comes mainly from studies with inhibitors of polyamine biosynthesis (Pösö & Jänne, 1976; Jänne *et al.*, 1978; Sjoerdsma, 1981; Pösö & Pegg, 1982; Pegg & McCann, 1982). These inhibitors cause effective blocks of cell division, which can be reversed by adding exogenous polyamines.

The discovery by Bitonti *et al.* (1982) that dicyclohexylamine, a competitive inhibitor of mammalian (Hibasami *et al.*, 1980) and bacterial spermidine synthase (Paulin *et al.*, 1983*b*; Pegg *et al.*, 1983; Pösö *et al.*, 1983), together with DL- α -monofluoromethylornithine (an irreversible inhibitor of bacterial ornithine decarboxylase; Bitonti *et al.*, 1982) and DL- α -difluoromethyl-arginine (an irreversible inhibitor of bacterial arginine decarboxylase; Kallio & McCann, 1981; Paulin & Pösö, 1983), inhibits bacterial growth, most probably by lowering the concentration of spermidine in bacteria, has opened up a new way to study the metabolic consequences of the lack of polyamines in bacteria. In the present paper we show that dicyclohexylamine alone is able to inhibit bacterial growth (completely, in the case of one type of bacteria), and that the extent of the inhibition is reflected in the ability of the drug to inhibit spermidine synthase *in vitro* and to lower the concentration of spermidine in growing bacteria.

Experimental

Bacterial growth

Freshly isolated strains of mastitic pathogenic *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus uberis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Micrococcus* sp. and *Klebsiella* sp. were used in this investigation.

The bacteria were harvested from blood agar plates and then grown in 500 μ l of meat broth (Difco) in γ -ray-sterilized cuvettes with intermittent shaking (15 min in every hour). Growth was measured turbidimetrically with a nine-cuvette multichannel FP-9 system (Suovaniemi & Järnefelt, 1982; Mattila *et al.*, 1984), which measures the absorbance vertically. Blanks were handled identically, but contained 0.1% NaN₃ to prevent growth. The absorbance values were stored in a desk computer (Olivetti LI M-20) connected to an FP-9 analyser (Suovaniemi & Järnefelt, 1982). When 50 h incubation was completed, the growth curves were automatically recorded.

Cells for enzymic studies were grown overnight in 500 ml of meat broth, harvested by centrifugation (10000 *g* for 5 min) and washed once with ice-cold 20 mM-sodium phosphate buffer, pH 8.2, containing 2.5 mM-dithiothreitol and 0.1 mM-EDTA

(standard buffer). The pellet was suspended in ice-cold standard buffer and disrupted by sonication with an MSE PG-742 cell disruptor (3 $\times$ 5 s with half-maximal power). The supernatant after centrifugation at 42000 *g* for 45 min was dialysed against 500 vol. of standard buffer at 4°C overnight to remove small molecules and then used as the source of spermidine synthase.

Spermidine synthase (putrescine aminopropyl-transferase, EC 2.5.1.22)

Spermidine synthase was assayed by measuring the production of [¹⁴C]methylthioadenosine from decarboxylated S-adenosyl-L-[Me-¹⁴C]methionine at 37°C and pH 8.2 (Bowman *et al.*, 1973; Paulin *et al.*, 1983*a*) by the method of Raina *et al.* (1983). The assay medium (0.2 ml) contained 150–250 μ g of protein, 0.5 mM-putrescine, 100 mM-sodium phosphate buffer, pH 8.2, and 3 μ M decarboxylated S-adenosyl[Me-¹⁴C]methionine. After 15 min incubation the reaction was stopped with 0.5 ml of 25 mM-HCl, and [¹⁴C]methylthioadenosine was isolated from 0.5 ml portions of the mixture as described by Raina *et al.* (1983) and its radioactivity counted by liquid scintillation.

Protein was measured by the method of Bradford (1976), with bovine serum albumin as standard.

Measurement of intracellular polyamines

Cells were grown in 250 ml of meat broth for 24 h with and without 5 mM-dicyclohexylamine. After these had been washed twice with ice-cold standard buffer, the polyamines were analysed directly from the homogenates by the method of Raina & Cohen (1966).

Uptake of [³H]spermidine and [³H]spermine by *E. coli* and *Ps. aeruginosa*

Bacteria were grown to the middle of the exponential growth phase in 2 ml of the broth. [³H]Polyamines (5 μ M) were then added to the medium. The radioactive cultures were sampled after 30 and 60 min by pelleting them by centrifugation (10000 *g* for 5 min). The pellets were washed twice with 10 ml of ice-cold standard buffer containing 10 mM-spermidine or -spermine to remove all radioactive polyamines bound to the outer membrane of the bacteria. The washed pellets were placed in 2 ml of 10% (w/v) trichloroacetic acid and the radioactivity in the acid supernatants was taken to represent the intracellular pool of radioactive polyamine.

Chemicals

S-Adenosyl-L-[Me-¹⁴C]methionine (62 Ci/mol), [methylene-³H(n)]spermidine (26 Ci/mol) and [3-aminopropyl-³H(n)]spermine (28 Ci/mol) were purchased from New England Nuclear (Drei-

eichenhain, West Germany). Unlabelled polyamines (as their hydrochlorides) and dicyclohexylamine (as dicyclohexylammonium sulphate) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Unlabelled and ^{14}C -labelled decarboxylated *S*-adenosylmethionine were prepared by decarboxylation with bacterial adenosylmethionine decarboxylase and purified further as described by Pösö *et al.* (1976).

Results

The effects of various concentrations of dicyclohexylamine on the growth of the seven mastitic pathogenic bacteria are shown in Figs. 1–3. The bacteria can be grouped according to their sensitivity. *Strep. uberis* did not grow at all at 5 mM-dicyclohexylamine, and both the rate of the growth during the initial phase and the final cell mass (after 50 h) were greatly decreased by 1.25 mM-dicyclohexylamine (Fig. 1*a*). Addition of 50 μM -spermidine (final concentration) overcame the inhibition by 1.25 mM-dicyclohexylamine, but only partially reversed the effects of higher concentrations of the drug (Fig. 1*a*). Increasing the spermi-

dine concentration to 100 μM caused no further increase in growth (results not shown). Although *Staph. aureus* (Fig. 1*b*), *E. coli* (Fig. 2*a*) and *Ps. aeruginosa* (Fig. 2*b*), in that order, were able to grow increasingly well at 5 mM-dicyclohexylamine, for all three bacteria both rates of growth and final cell masses were clearly decreased by 5 mM or 2.5 mM of the drug. However, addition of 50 mM-spermidine (final concentration) completely (*E. coli* and *Ps. aeruginosa*) or nearly completely (*Staph. aureus*) overcame the effects of dicyclohexylamine (Figs. 1 and 2), but putrescine (1 mM) and spermine (25–100 μM) did not cause a similar protection (results not shown). The *Micrococcus* sp. and *Klebsiella* sp. both showed rapid initial growth rates in the presence of 10 mM-dicyclohexylamine, although the final cell masses were somewhat decreased, and for these two strains the effects of dicyclohexylamine could be completely overcome by 100 μM -putrescine as well as by 50 μM -spermine (Figs. 3*a* and 3*b*). Finally, the growth of *Strep. agalactiae* was unaffected by 10 mM-dicyclohexylamine (Fig. 3*c*).

As shown in Table 1, dicyclohexylamine lowered the spermidine concentration by 70% in *E.*

Fig. 1. Inhibition of growth of *Strep. uberis* (a) and *Staph. aureus* (b) by dicyclohexylamine, and reversal by spermidine. Bacteria were grown without additions (∇) or with 5 mM-dicyclohexylamine ($\bullet$), 2.5 mM-dicyclohexylamine ($\circ$) or 1.25 mM-dicyclohexylamine ($\square$) or with 5 mM-dicyclohexylamine + 50 μM -spermidine ($\triangle$), 2.5 mM-dicyclohexylamine + 50 μM -spermidine ($\blacktriangle$) or 1.25 mM-dicyclohexylamine + 50 μM -spermidine ($\blacksquare$). The experiments were done three times with similar results. Data shown are from a single representative experiment.

Fig. 2. Inhibition of growth of *E. coli* (a) and *Ps. aeruginosa* (b) by dicyclohexylamine, and reversal by spermidine. Bacteria were grown as described in the Experimental section without additions (■) or with 5 mM-dicyclohexylamine (●) or 2.5 mM-dicyclohexylamine (○) or with 5 mM-dicyclohexylamine + 50 μ M-spermidine (Δ) or with 2.5 mM-dicyclohexylamine + 50 μ M-spermidine ($\blacktriangle$). Other details are as indicated in Fig. 1 legend.

Table 1. Effect of dicyclohexylamine on putrescine and spermidine contents in *Strep. uberis*, *E. coli*, *Ps. aeruginosa* and *Strep. agalactiae*

Bacteria were grown with or without additions shown in the Table as described in the Experimental section, and then the polyamines [putrescine (Pu) and spermidine (Spd)] were analysed by the method of Raina & Cohen (1966). The concentration of dicyclohexylamine was 5 mM and that of spermidine during reversal 50 μ M.

	Polyamine content (nmol/10 ⁸ cells)			
	<i>Strep. uberis</i> Pu/Spd	<i>E. coli</i> Pu/Spd	<i>Ps. aeruginosa</i> Pu/Spd	<i>Strep. agalactiae</i> Pu/Spd
Control	1.7/0.32	3.5/0.32	2.1/0.40	1.4/0.30
+ Dicyclohexylamine	3.2/0.02	3.1/0.11	2.2/0.11	1.4/0.32
+ Dicyclohexylamine + spermidine	1.7/0.34	3.7/0.38	2.4/0.42	1.7/0.55

coli and *Ps. aeruginosa*, and by 95% in *Strep. uberis*, whereas in *Strep. agalactiae* (insensitive to dicyclohexylamine) there were no changes in the intracellular concentrations of polyamines (Table 1). In *Strep. uberis* the growth in the presence of dicyclohexylamine led to the accumulation of putrescine in cells. These results suggest that spermidine is an essential compound in bacterial growth. It is also noteworthy that the accumulation of putrescine in *Strep. uberis* could have something to do with the

extreme sensitivity of the bacteria towards dicyclohexylamine.

A possible reason for the differences in the observed sensitivity towards dicyclohexylamine is shown in Fig. 4. *Strep. uberis*, *Staph. aureus*, *E. coli* and *Ps. aeruginosa*, the growth of each of which was repressed by 5 mM-dicyclohexylamine and could not be restored by putrescine, contained spermidine synthase activity that was very sensitive to dicyclohexylamine *in vitro* (over 95%

Fig. 3. Effects of dicyclohexylamine on the growth of *Micrococcus* sp. (a), *Klebsiella* sp. (b) and *Strep. agalactiae* (c)

Bacteria were grown as indicated in Fig. 1 legend without additions (■), with 10 mM-dicyclohexylamine (○) or with 10 mM-dicyclohexylamine + 50 μM-spermidine (△) or with 10 mM-dicyclohexylamine + 100 μM-putrescine (●). Other details are as indicated in Fig. 1 legend.

inhibition at 50 μM-dicyclohexylamine). On the other hand, the spermidine synthase of *Micrococcus* sp. (for which the effects of dicyclohexylamine *in vivo* could be reversed by putrescine; Fig. 3a) was only 85% inhibited at 50 μM-dicyclohexylamine, and the spermidine synthase from *Strep. agalactiae* (the growth of which was unaffected by 10 mM-dicyclohexylamine) still exhibited 10% of its activity at 1000 μM-dicyclohexylamine. Thus the extent of inhibition of the growth of the bacteria correlated well with the sensitivity of their spermine synthases to the drug *in vitro* and with its

Fig. 4. Effects of dicyclohexylamine on bacterial spermidine synthesis

Spermidine synthase activity from *Strep. uberis* (□), *Staph. aureus* (○), *E. coli* (■), *Ps. aeruginosa* (●), *Micrococcus* sp. (△) and *Strep. agalactiae* (△) were measured as described in the Experimental section. Results are expressed as percentages of the activity in the absence of any inhibitor.

ability to lower the intracellular concentrations of polyamines.

We next wanted to test whether the effect of dicyclohexylamine on the growth of the bacteria could be reversed by adding spermidine to the medium some time after the dicyclohexylamine. Fig. 5 shows the effect on *Staph. aureus* and *Strep. uberis* (the most sensitive bacteria) of the addition of spermidine after 18 h growth with dicyclohexylamine. The addition of spermidine rapidly and completely reversed the inhibition by dicyclohexylamine of the growth rates. This indicates that dicyclohexylamine has no permanent toxic effect(s) on the bacteria other than depriving them of spermidine.

Since spermidine but not spermine (or putrescine) could overcome the adverse effect of dicyclohexylamine on the growth of bacteria containing dicyclohexylamine-sensitive spermidine synthase, we tested the ability of *E. coli* and *Ps. aeruginosa* to take up spermidine and spermine from the medium. Table 2 shows that the bacteria used in our study did not take up spermine from the medium but rapidly accumulated spermidine. It is therefore reasonable to believe that the inability of spermine to counteract dicyclohexylamine stems from its inability to enter the bacterial cells.

Fig. 5. Reversal of the inhibitory effect of dicyclohexylamine on bacterial growth by subsequent addition of spermidine

Staph. aureus (▲) and *Strep. uberis* (●) were grown without additions or *Staph. aureus* (△) and *Strep. uberis* (□) with 5mM-dicyclohexylamine or *Staph. aureus* (■) and *Strep. uberis* (○) with 5mM-dicyclohexylamine alone for 18h and then 50µM-spermidine was added. Experiments were done twice with similar results. Data shown are from a single representative experiment.

Discussion

Our results, indicating that dicyclohexylamine inhibits the growth of different bacteria, are in agreement with earlier studies (Bitonti *et al.*, 1982; Pegg *et al.*, 1983; Bitonti *et al.*, 1984). The obvious reason for this phenomenon is that dicyclohexylamine is able to lower the concentration of spermidine in bacteria (Bitonti *et al.*, 1982; Pegg *et al.*, 1983; Bitonti *et al.*, 1984; Table 1), since it is a powerful inhibitor of bacterial spermidine synth-

Table 2. Uptake of [3 H]spermidine or [3 H]spermine by *E. coli* and *Ps. aeruginosa*

Experiments were performed as described in the Experimental section. Values are a means for duplicate samples. The specific radioactivities of the spermidine and spermine were 26 and 28 Ci/mol respectively.

Time (min)	Intracellular radioactivity (c.p.m./ 10^7 cells)			
	<i>E. coli</i>		<i>Ps. aeruginosa</i>	
	Spermidine	Spermine	Spermidine	Spermine
30	48 000	116	44 500	186
60	78 000	108	86 300	136

ase (Paulin *et al.*, 1983b; Pegg *et al.*, 1983; Pösö *et al.*, 1983). As also shown earlier (Bitonti *et al.*, 1982, 1984), the inhibition of the growth could be overcome by adding spermidine (Figs. 1 and 2), and also by putrescine in those bacteria that contain spermidine synthase that is less sensitive to dicyclohexylamine, suggesting that spermidine synthesis was only partially inhibited in these bacteria (Fig. 3). It is also clear from our results (Table 1) that the inhibition of spermidine synthesis *in vivo* is necessary before the growth inhibition takes place.

It is noteworthy that the inability of spermine to reverse the inhibition of growth is based, at least in *E. coli* and *Ps. aeruginosa*, on the impermeability of the bacterial membrane to spermine (Table 2). The differential uptakes of spermidine and spermine suggest that there are specific mechanisms by which polyamines cross bacterial membranes. This suggestion is even more obvious in the light of work by Bitonti *et al.* (1984), where they had wild-type *E. coli* and *Ps. aeruginosa* able to take up spermine. Spermine also reversed the inhibition of growth due to the lack of polyamines. So, it appears that among the similar bacterial strains there could be differences in the uptake system(s) for polyamines.

The biggest difference between our results and those reported by Bitonti *et al.* (1982) is that the *E. coli* used in their study was totally insensitive to 10mM-dicyclohexylamine, whereas our mastitic pathogenic strain was sensitive to the drug. This finding suggested a great variation in the sensitivity of spermidine synthases to dicyclohexylamine even among strains of the same bacterial species. Our study shows that *Strep. agalactiae* contained a spermidine synthase that still had some activity in the presence of 1 mM-dicyclohexylamine (Fig. 4), and this bacterium could also grow in the presence of 10mM-dicyclohexylamine (Fig. 3), which strongly suggests that a specific inhibi-

tion of spermidine synthase (Fig. 4) and thus of spermidine synthesis (Table 1) is needed for the inhibition of growth. Another possible explanation for the different sensitivities of the bacteria to dicyclohexylamine *in vivo* might be that they are differently permeable by the drug. At present, we do not know to what extent dicyclohexylamine is taken up by different bacteria, nor whether spermidine is able to displace dicyclohexylamine and force it out of cells, since unfortunately there is at the present time no method available for the measurement of the concentration of dicyclohexylamine in the cells. Replacing dicyclohexylamine might in theory account for the reversal by spermidine of the effects of dicyclohexylamine other than inhibition of spermidine synthase. However, the adverse effects of dicyclohexylamine could still be reversed by spermidine after 18 h exposure to the drug (Fig. 5), which suggests that the inhibition of spermidine synthesis had a specific role in preventing bacterial growth.

Strep. uberis was the only bacterium that completely stopped growing in the presence of dicyclohexylamine. This suggests that this organism has an absolute need for polyamines, thus resembling the mutant studied by Tabor (1981) and Tabor *et al.* (1981), although it is still possible that the accumulation of putrescine due to the presence of dicyclohexylamine could have some effect on the growth of *Strep. uberis*. Still another possibility having some effect on the growth could be the accumulation of decarboxylated S-adenosylmethionine in the presence of dicyclohexylamine, since in eukaryotic cells the inhibition of spermidine synthesis leads in some cases to a profound accumulation of decarboxylated adenosylmethionine (Pegg *et al.*, 1982) and this might also be the case in bacterial cells. The other bacteria did grow in the presence of dicyclohexylamine with different growth rates, thus resembling some other polyamine mutants of *E. coli* (Hafner *et al.*, 1979) that grow slowly even without polyamines.

Since we have shown earlier that ethambutol, a drug widely used against tuberculosis, inhibits powerfully and specifically the spermidine synthase from mycobacteria, but not spermidine synthase from other bacteria (Pösö *et al.*, 1983), it may be possible to develop more specific drugs against common bacterial synthases. Already the present study shows that there are large differences in the sensitivity of spermidine synthases from different bacteria to dicyclohexylamine. It may be possible to find derivatives of dicyclohexylamine or ethambutol with a great specificity for bacterial spermidine synthase. This kind of compound would be useful for the specific inhibition of bacterial growth in infections without any harmful side effects on the host.

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