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Restriction of bacterial growth by inhibition of polyamine biosynthesis by using monofluoromethylornithine, difluoromethylarginine and dicyclohexylammonium sulphate

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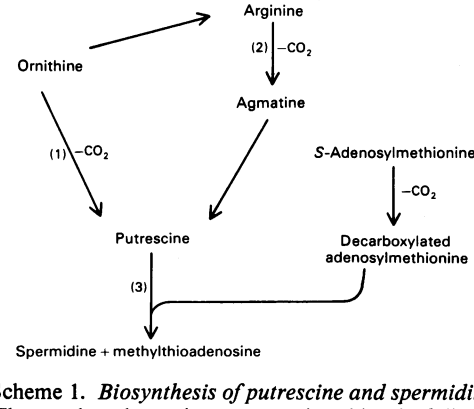
Bacterial growth was measurably slowed by a combination of drugs which inhibit polyamine-biosynthetic enzymes. Addition of DL-α-monofluoromethylornithine, which was shown to inactivate irreversibly ornithine decarboxylase extracted from *Escherichia coli* ($K_i = 0.36$ mM) and *Pseudomonas aeruginosa* ($K_i = 0.30$ mM), DL-α-difluoromethylarginine and dicyclohexylammonium sulphate to cultures of *E. coli* or *P. aeruginosa* resulted in a 40 and 70% increase in generation times (decreased growth rates) respectively, which was completely reversed by the addition of 0.1 mM-putrescine plus 0.1 mM-spermidine to the medium. Decreased intracellular polyamine concentrations correlated with increased generation times; putrescine concentration was decreased by 70% in *E. coli* and 80% in *P. aeruginosa*, while spermidine concentration was decreased by 50% in *E. coli* and 95% in *P. aeruginosa*. Subsequent investigation of the inactivation of the ornithine decarboxylase by monofluoromethylornithine indicated that it was active-site directed, as the normal substrate ornithine slowed the rate of inhibition. Specific interference with polyamine biosynthesis may be a viable approach to control of some bacterial infections.

The polyamines putrescine and spermidine are absolute requirements for the growth of the bacterium *Haemophilus influenzae* (Herbst & Snell, 1948) and mutants of the fungi *Aspergillus nidulans* (Sneath, 1955) and *Neurospora crassa* (Deters *et al.*, 1974). Mutants of *Escherichia coli* which completely lack the polyamine-biosynthetic enzymes and intracellular polyamines have growth rates significantly slower than that of wild-type strains (Tabor *et al.*, 1978; Hafner *et al.*, 1979; Jorstad *et al.*, 1980; Tabor *et al.*, 1981). Although inhibitors of polyamine biosynthesis have proved to be quite effective in limiting the proliferation of rapidly growing mammalian cells (Koch-Weser *et al.*, 1981; Sjoerdsma, 1981; Pegg & Coward, 1981; Porter *et al.*, 1981) and of parasitic protozoa (McCann *et al.*, 1981), it has not been possible to limit bacterial proliferation by using this approach (Kallio *et al.*, 1982). A part of the problem is that most bacteria can synthesize putrescine from ornithine or arginine (Scheme 1) (Pegg & Williams-Ashman, 1981). Although it has been possible to inhibit irreversibly bacterial ornithine decarboxylase (Kallio & McCann, 1981; Kallio *et al.*, 1982) and arginine decarboxylase (Kallio *et al.*, 1981) and consequently decrease intracellular putrescine concentrations, no effect on bacterial growth has been demonstrated. The present study shows that growth of *Escherichia coli* and *Pseudomonas aeruginosa* can be markedly slowed by a combination of inhibitors of polyamine



Scheme 1. *Biosynthesis of putrescine and spermidine*
The numbered reactions are catalysed by the following enzymes: (1) ornithine decarboxylase, (2) arginine decarboxylase and (3) spermidine synthase.

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biosynthesis. This combination includes DL-α-monofluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase (Kollonitsch *et al.*, 1978), DL-α-difluoromethylarginine, an irreversible inhibitor of arginine decarboxylase (Kallio *et al.*, 1981), and dicyclohexylammonium sulphate, a competitive inhibitor of spermidine synthase (Hibasami *et al.*, 1980).

Experimental

Bacterial growth

E. coli (Merrell Research Center 59) and *P. aeruginosa* (A.T.C.C. 9027) were grown as described previously (Kallio *et al.*, 1982) on minimal medium (Davis & Mingioli, 1950) at 37°C. Growth was monitored in control and drug-treated cultures by measuring the A_{550} of cell suspensions. Numbers of cells were determined by using previously prepared standard curves plotting A_{550} against number of viable cells. Generation times were calculated as described by Stanier *et al.* (1976). Cells were harvested by centrifugation (10min at 10000*g*) and washed once with phosphate-buffered saline, pH 7.2 (0.125M-NaCl, 10mM-Na_2HPO_4 and 3mM-KH_2PO_4). The cell pellet was suspended in 20mM-Tris/HCl (pH 7.5) containing 1mM-dithiothreitol and 0.1mM-EDTA, and disrupted by sonication with a Branson Cell Disruptor 350 (5 × 30s, setting 3). Cell debris was removed by centrifugation at 25000*g* for 30min, and the supernatant (approx. 16mg of protein/ml), used for the measurement of ornithine decarboxylase, was stored frozen at −20°C without appreciable loss of activity over a 2-week period.

Ornithine decarboxylase

Ornithine decarboxylase activity was measured by the release of $^{14}CO_2$ (Jänne & Williams-Ashman, 1973). Assays contained 100mM-Tris/HCl (pH 8.25 for *E. coli* and pH 7.5 for *P. aeruginosa*), 0.04mM-pyridoxal phosphate, 1mM-dithiothreitol, 10mM-L-ornithine, 2.5μCi of DL-[1-^{14}C]ornithine and 80–120μg of bacterial protein in a total volume of 1ml. The reaction was run for 30min at 37°C in a tightly stoppered flask, terminated by adding 1ml of 40% (w/v) trichloroacetic acid, and $^{14}CO_2$ was trapped on filter paper saturated with 50μl of methylbenzethonium hydroxide.

Inhibition of ornithine decarboxylase by monofluoromethylornithine

Time-dependent irreversible inhibition of ornithine decarboxylase was determined as described by Kallio *et al.* (1982). The enzyme was incubated at 20°C in 100mM-Tris/HCl (pH 8.25 for *E. coli* and pH 7.5 for *P. aeruginosa*) containing 1mM-dithiothreitol, 0.04mM-pyridoxal phosphate and various concentrations of monofluoromethylornithine. At selected times, 20μl portions of enzyme incubation were removed and transferred to the reaction flasks in which ornithine decarboxylase activity was to be determined. These flasks contained all the components of the ornithine decarboxylase reaction (including 10mM-ornithine) except DL-[1-^{14}C]-ornithine. The ornithine decarboxylase reaction flasks were kept on ice until all time points were taken, and then DL-[1-^{14}C]ornithine was added and ornithine decarboxylase activity was determined at 37°C. The transfer of the enzyme incubation to the reaction flask on ice resulted in a 45-fold dilution of the inhibitor and effectively stopped any further inhibition.

Determination of intracellular polyamines

Cells were harvested by centrifugation (10000*g*, 10min), washed once with phosphate-buffered saline, pH 7.2, and the cell pellet was extracted overnight with 1.0ml of 0.4M-$HClO_4$. Proteins were removed by filtration on Millipore membranes (0.22μm), and 20μl samples of the filtrates were analysed for polyamines with a Dionex D-300 Amino Acid Analyser with a Dionex P/N 30831 column. After separation, derivatives of polyamines were prepared with *o*-phthaldialdehyde and detected by fluorescence spectrometry. Buffers for elution and detection of polyamines were as described by Kallio *et al.* (1982).

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

Chemicals

DL-[1-^{14}C]Ornithine (58Ci/mol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.), and dicyclohexylammonium sulphate was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DL-α-Difluoromethylarginine and DL-α-monofluoromethylornithine were synthesized in our laboratories.

Results

Inhibition of ornithine decarboxylase by monofluoromethylornithine

On incubation with 0.2mM-monofluoromethylornithine at 37°C, ornithine decarboxylase extracted from *E. coli* was inhibited in a time-dependent manner, and the inhibition followed pseudo-first-order kinetics (Fig. 1). The inhibition of ornithine decarboxylase by monofluoromethylornithine was slowed by the addition of 2mM- or 5mM-L-ornithine, suggesting that the inhibitor was acting at the catalytic site of the enzyme. Dialysis of the inhibited enzyme for 24h against 10mM-Tris/HCl (pH 8.25) containing 1mM-dithiothreitol and 0.1mM-EDTA failed to restore catalytic activity. These data suggest

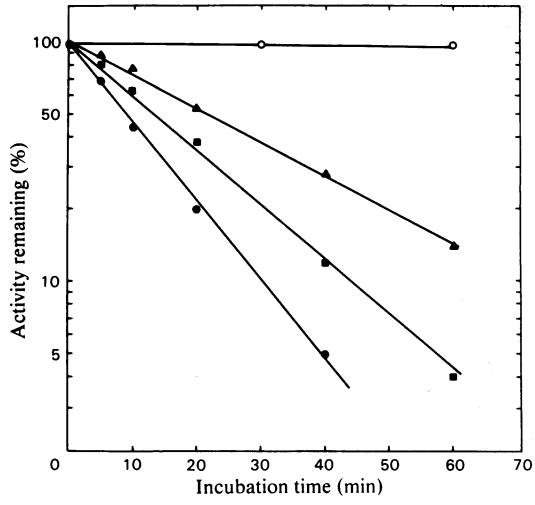

Fig. 1. *Time-dependent inhibition of E. coli ornithine decarboxylase and competition by* L-*ornithine*
Ornithine decarboxylase was prepared from *E. coli*, incubated in the absence (○) or presence (●) of 0.2 mM-monofluoromethylornithine or 0.2 mM-monofluoromethylornithine with (■) 2 mM- or (▲) 5 mM-L-ornithine, and assayed as described in the Experimental section.

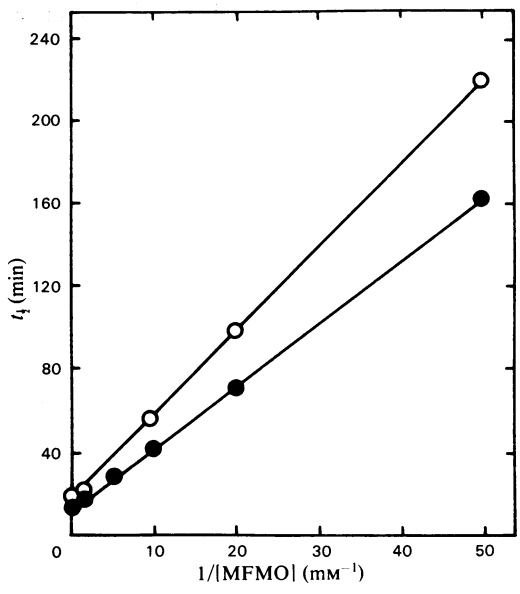

Fig. 2. *Dependence of the half-life of E. coli* (○) *and P. aeruginosa* (●) *ornithine decarboxylase activities on the concentration of monofluoromethylornithine (MFMO)*
The half-lives of the activity at different concentrations of MFMO (0.02–1.0 mM) were determined as described in the Experimental section and analysed by the procedure of Kitz & Wilson (1962) as modified by Jung & Metcalf (1975).

that the inhibition of ornithine decarboxylase by monofluoromethylornithine is irreversible and that a strong covalent bond is formed between the enzyme and the inhibitor.

Kinetic analysis of ornithine decarboxylase inhibition by monofluoromethylornithine was done by the method of Kitz & Wilson (1962) as modified by Jung & Metcalf (1975). From a plot of half-life of the enzyme activity versus concentrations of monofluoromethylornithine beween 0.02 and 1.0 mM (Fig. 2) it was possible to calculate dissociation constants (*E. coli*, $K_i = 0.36$ mM; *P. aeruginosa*, $K_i = 0.30$ mM) and the half-life of each enzyme activity at an infinite concentration of monofluoromethylornithine (*E. coli*, $t_{\frac{1}{2}} = 12$ min; *P. aeruginosa*, $t_{\frac{1}{2}} = 12$ min. Monofluoromethylornithine was also found to inhibit *E. coli* and *P. aeruginosa* ornithine decarboxylase activities (>98% inhibition) when the bacteria were grown in the presence of 2 mM-monofluoromethylornithine (Table 1), indicating that there was sufficient penetration of the cell wall by the drug.

Table 1. *Inactivation of E. coli and P. aeruginosa ornithine decarboxylase in situ by monofluoromethylornithine (MFMO)*
Bacteria were grown to stationary phase in the presence of 2 mM-MFMO, and ornithine decarboxylase was prepared and assayed as described in the Experimental section.

	Ornithine decarboxylase activity (μmol of CO_2/h per mg of protein)	
	E. coli	*P. aeruginosa*
Control	0.78	0.56
+2 mM-MFMO	0.01	0.01

Inhibition of growth of E. coli and P. aeruginosa

Incubation of *E. coli* or *P. aeruginosa* in the presence of three polyamine-biosynthetic-enzyme inhibitors (2 mM-monofluoromethylornithine, 2.5 mM-difluoromethylarginine, 5 mM- or 10 mM-dicyclohexylammonium sulphate for *P. aeruginosa* and *E. coli* respectively) resulted in a measurable increase in exponential-growth-phase generation times (decreased growth rates) of 40 and 70% respectively (Figs. 3 and 4). As a consequence, the number of cells was markedly decreased. There was a 3-fold decrease in the number of *E. coli* cells and greater than a 6-fold decrease in the number of *P. aeruginosa* cells during the exponential phase of growth. The inhibitory effects of the drug combination were completely reversed by the addition of 0.1 mM-putrescine plus 0.1 mM-spermidine to the growth medium along with the three drugs. The inhibition

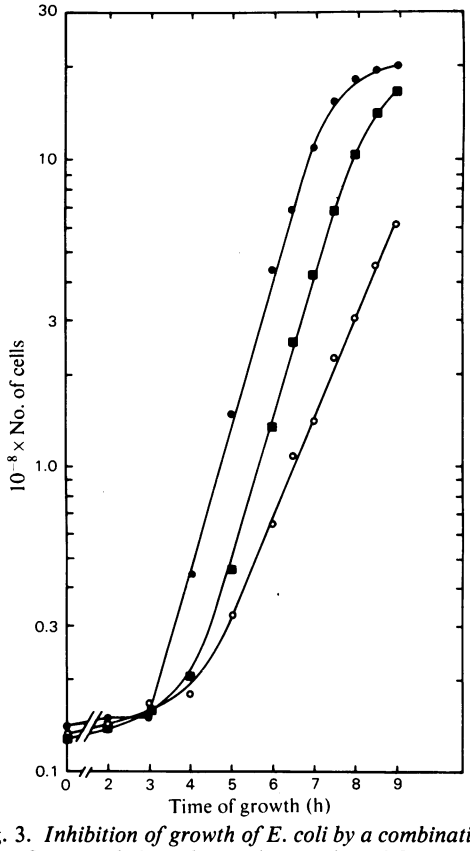

Fig. 3. *Inhibition of growth of E. coli by a combination of monofluoromethylornithine (MFMO), difluoromethylarginine (DFMA) and dicyclohexylammonium sulphate (DCHA), and reversal by polyamines*

E. coli was grown as described in the Experimental section without additions (●) or with 2 mM-MFMO, 2.5 mM-DFMA and 10 mM-DCHA without (○) or with (■) 0.1 mM-putrescine and 0.1 mM-spermidine. Generation times during the exponential phase of growth were calculated to be: control (●), 41 min; MFMO/DFMA/DCHA (○), 57 min; MFMO/DFMA/DCHA plus putrescine and spermidine (■), 43 min. The experiment was repeated five times, and results from the separate experiments were similar. Data shown are from a single representative experiment.

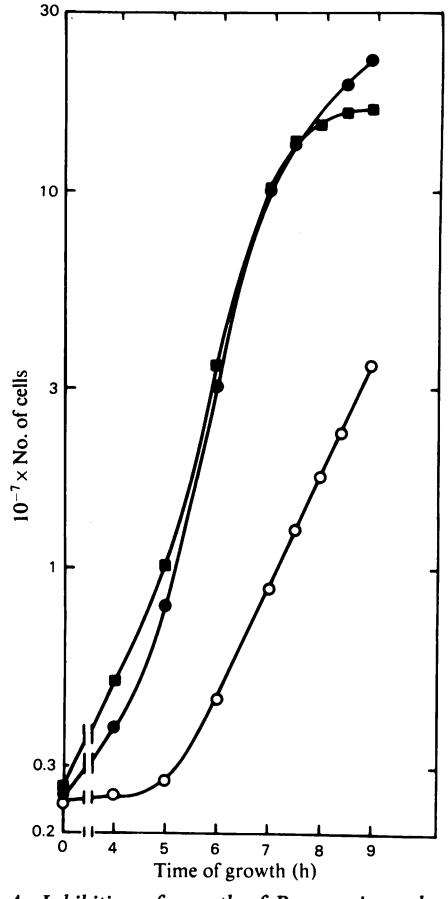

Fig. 4. *Inhibition of growth of P. aeruginosa by a combination of monofluoromethylornithine (MFMO), difluoromethylarginine (DFMA) and dicyclohexylammonium sulphate (DCHA), and reversal by polyamines*

P. aeruginosa was grown as described in the Experimental section without additions (●) or with 2 mM-MFMO, 2.5 mM-DFMA and 5 mM-DCHA without (○) or with (■) 0.1 mM-putrescine and 0.1 mM-spermidine. Generation times during the exponential phase of growth were calculated to be: control (●), 35 min; MFMO/DFMA/DCHA (○), 60 min; MFMO/DFMA/DCHA plus putrescine and spermidine (■), 37 min. The experiment was repeated three times and results from the separate experiments were similar. Data shown are from a single representative experiment.

of growth of *P. aeruginosa* was also reversed by either 5 mM-putrescine or 0.1 mM-spermidine added individually, and the inhibition of *E. coli* growth was reversed by either 0.1 mM-putrescine or 0.1 mM-spermidine.

Growth rates in the presence of the combination of monofluoromethylornithine and difluoromethylarginine (or either drug alone) were the same as controls. Dicyclohexylammonium sulphate (10 mM) had no effect on the growth of *E. coli*, but 5 mM-dicyclohexylammonium sulphate did have a partial effect on the growth of *P. aeruginosa* as compared with the three-drug combination. This effect of dicyclohexylammonium sulphate alone was completely reversible by the combination putrescine plus spermidine (each 0.1 mM).

Intracellular polyamine concentrations

Intracellular polyamine concentrations were dramatically affected by the drug treatments (Table

Table 2. *Effect of monofluoromethylornithine (MFMO), difluoromethylarginine (DFMA) and dicyclohexylammonium sulphate (DCHA) on putrescine and spermidine contents in E. coli and P. aeruginosa*

E. coli and *P. aeruginosa* were grown until control cultures entered stationary phase (usually 8–9h), at which time the cells were harvested and polyamines were determined as described in the Experimental section. Drug concentrations were: MFMO, 2mM; DFMA, 2.5mM; DCHA, 10mM for *E. coli* and 5mM for *P. aeruginosa*. When present, putrescine and spermidine were at a concentration of 0.1mM. Values in parentheses represent percentages of control. The experiment was repeated three times with similar results. Data are from a single experiment.

	Content (nmol/10^8 cells)			
	E. coli		*P. aeruginosa*	
	Putrescine	Spermidine	Putrescine	Spermidine
Control	1.70 (100)	0.26 (100)	3.11 (100)	0.64 (100)
MFMO + DFMA	0.24 (14)	0.37 (142)	2.04 (66)	0.76 (120)
DCHA	1.68 (99)	0.22 (85)	2.44 (78)	0.05 (8)
MFMO + DFMA + DCHA	0.57 (34)	0.13 (50)	0.63 (20)	0.03 (5)
MFMO + DFMA + DCHA + putrescine + spermidine	0.72 (42)	0.27 (104)	1.51 (49)	0.66 (103)

2), and these changes correlated with changes in growth. In the presence of monofluoromethylornithine and difluoromethylarginine the putrescine concentration was decreased 86% in *E. coli* and 34% in *P. aeruginosa*. Spermidine actually increased by 40% and 20% in *E. coli* and *P. aeruginosa* respectively. Dicyclohexylammonium sulphate (10mM) slightly decreased the spermidine concentration in *E. coli* (15%), whereas the spermidine concentration in *P. aeruginosa* was decreased by >90% by 5mM-dicyclohexylammonium sulphate. Incubation of *E. coli* or *P. aeruginosa* with monofluoromethylornithine, difluoromethylarginine and dicyclohexylammonium sulphate resulted in markedly decreased concentrations of both putrescine and spermidine. When polyamines were included in the medium, intracellular putrescine concentrations returned to about 50% of control values, whereas intracellular spermidine concentrations recovered to control values.

Discussion

These experiments demonstrate that specific interference with the biosynthesis of polyamines can lead to decreases in the growth rates of *E. coli* and *P. aeruginosa*. The changes in intracellular polyamine concentrations produced by monofluoromethylornithine, difluoromethylarginine and dicyclohexylammonium sulphate correlated with changes in growth in both bacteria. It is evident that the most dramatic effects on the growth of the bacteria were obtained when both putrescine and spermidine were markedly decreased, although it appeared that changes in spermidine concentrations are more closely correlated with changes in growth than were those of putrescine. There were some puzzling changes in putrescine concentrations that cannot be fully explained at this time. It is noteworthy that a larger decrease in growth was obtained with *P. aeruginosa* than with *E. coli*, and this was presumably due to the greater decrease in intracellular polyamines in *P. aeruginosa*. That the inhibitory effects of the three-drug combination were completely reversed by the addition of low concentrations of putrescine plus spermidine indicated that the drugs did, in fact, inhibit growth through inhibition of the polyamine-biosynthetic enzymes rather than by non-specific toxicity. Subsequently, a relatively high concentration of putrescine (5mM) was found to reverse completely the effects of the drug combination in *P. aeruginosa*, whereas 0.1mM-putrescine was sufficient to reverse the drug effects in *E. coli*. These effects were most probably due to putrescine itself, since there was no apparent conversion of putrescine into spermidine in either bacterium owing to the presence of dicyclohexylammonium sulfate. Spermidine (0.1mM) completely reversed the growth inhibition in both bacteria, but there was a substantial increase in the intracellular putrescine concentration when spermidine was added, possibly indicating conversion of some of the exogenous spermidine back into putrescine.

The development of the specific inhibitors for the enzymes in the bacterial polyamine-biosynthetic pathways has not been straightforward, particularly in identifying inhibitors of the bacterial ornithine decarboxylases. Monofluoromethylornithine was previously shown to be a potent enzyme-activated irreversible inhibitor of ornithine decarboxylase from rat liver (Kollonitsch *et al.*, 1978), but was not tested against an enzyme from bacteria. In the light of previous studies in our laboratories, there was no reason *a priori* to believe that monofluoromethylornithine would be an effective inhibitor of bacterial ornithine decarboxylases. It was previously demon-

strated that DL-α-difluoromethylornithine, an effective irreversible inhibitor of all eukaryotic ornithine decarboxylases studied (Metcalf *et al.*, 1978), was also active against ornithine decarboxylase extracted from *P. aeruginosa*, but was completely ineffective against the enzyme from *E. coli* (Kallio & McCann, 1981). DL-α-Monofluoromethylputrescine, an analogue of putrescine, the product of ornithine decarboxylase, was also found to inhibit eukaryotic ornithine decarboxylase (Seiler *et al.*, 1978) and ornithine decarboxylase from *E. coli*, but was a poor inhibitor of the enzyme from *P. aeruginosa* (Kallio *et al.*, 1982). It was further found that, although *P. aeruginosa* ornithine decarboxylase was sensitive to inhibition by difluoromethylornithine, growth of *P. aeruginosa* was not inhibited by the combination of difluoromethylornithine, difluoromethylarginine and dicyclohexylammonium sulphate (A. J. Bitonti, P. P. McCann & A. Sjoerdsma, unpublished work). It is obvious that inhibitors effective in eukaryotes cannot be predictably used to inhibit bacterial enzymes.

Difluoromethylarginine was shown to be a specific irreversible inhibitor of arginine decarboxylases from both *E. coli* and *P. aeruginosa* (Kallio *et al.*, 1981). When difluoromethylarginine became available it was thought that combining inhibitors of ornithine decarboxylase and arginine decarboxylase could result in a decrease in intracellular polyamines and subsequent decreased growth rate. For reasons not immediately apparent, however, in the presence of monofluoromethylornithine and difluoromethylarginine (a situation in which both decarboxylases were inhibited by >95%) intracellular spermidine concentrations were increased by 20–40% rather than decreased and growth was not restricted. The addition of dicyclohexylammonium sulphate, a known inhibitor of spermidine synthase in mammalian cells (Hibasami *et al.*, 1980), to the monofluoromethylornithine and difluoromethylarginine combination resulted in not only a decrease in intracellular spermidine concentration but a measurable slowing of bacterial growth. Concurrently, it has been determined that dicyclohexylammonium sulphate is in fact a potent competitive inhibitor of spermidine synthase from both *E. coli* and *P. aeruginosa* (A. E. Pegg, personal communication). Other inhibitors of mammalian spermidine synthase have been described (Pegg & Coward, 1981), and studies are required to determine if these compounds are also effective against bacterial spermidine synthase.

Although bacterial proliferation was not completely halted by depletion of polyamines, this result is not entirely unexpected in view of studies demonstrating that mutants of *E. coli* which, in fact, completely lack putrescine and spermidine grow at one-third the rate of wild-type organisms (Tabor *et al.*, 1978; Hafner *et al.*, 1979; Jorstad *et al.*, 1980; Tabor *et al.*, 1981). However, the general approach of inhibiting polyamines biosynthesis may be a viable approach towards the control of some bacterial infections.

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