Alteration of Membrane Permeability in *Bacillus subtilis* by Clofoctol

By FRANÇOISE YABLONSKY†

Centre d'Etudes Nucléaires – Saclay, Institut National des Sciences et Techniques Nucléaires,
91191 Gif sur Yvette, France

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When *Bacillus subtilis* was treated with a bacteriostatic concentration of clofoctol [2-(2,4-dichlorobenzyl)-4-(tetramethyl-1,1,3,3-butyl)phenol], UV-absorbing material was released. Electron micrographs showed evidence of physical alteration of the bacterial envelope. The uptake of [¹⁴C]glutamate was strongly inhibited by clofoctol, and preloaded glutamate was found to leak from the bacteria. Clofoctol caused an immediate and dramatic decrease in the amount of intracellular ATP. This was neither the consequence of the stimulation of an ATPase, nor of the inhibition of bacterial respiration. Both the proton gradient and the potential gradient across the cytoplasmic membrane collapsed and this inhibition of energy metabolism was sufficient to account for the inhibition of growth by clofoctol. At the same bacteriostatic concentration complete permeabilization of the bacteria occurred.

INTRODUCTION

Clofoctol is a new synthetic antibacterial agent which is active only on Gram-positive bacteria (Simonnet *et al.*, 1979). This compound is used in human therapy for the treatment of upper respiratory tract infections (Salet & Melekian, 1978; Vialatte, 1978).

Although Gram-negative bacteria were resistant to clofoctol, spheroplasts from *Escherichia coli* as well as protoplasts from *Bacillus subtilis* were lysed by clofoctol (F. Yablonsky, unpublished results). Thus the resistance of Gram-negative bacteria appeared to be due to the inability of clofoctol to penetrate the outer membrane of these organisms.

The mechanism of action of clofoctol is unknown at present. It does not, however, inhibit specifically DNA, RNA or protein synthesis (Simonnet *et al.*, 1979). Moreover, clofoctol binds to sensitive bacteria (10^6 to 1.5×10^7 molecules bound per cell), but not to resistant ones (Combe *et al.*, 1980).

The cell wall precursor UDP-*N*-acetylmuramyl-pentapeptide accumulated in a culture of *Bacillus cereus* T treated with clofoctol and the antibiotic also inhibited the incorporation of *N*-acetyl[¹⁴C]glucosamine into peptidoglycan (Yablonsky & Simonnet, 1982). These results suggested that clofoctol might act by inhibiting synthesis of peptidoglycan. Since clofoctol is active on protoplasts and spheroplasts, we investigated the possibility that the inhibition of wall synthesis could result from the action of clofoctol on the cytoplasmic membrane.

METHODS

Bacterial strains and growth conditions. *Bacillus subtilis* 168 Trp[−] (kindly donated by Dr S. Laurent) was grown at 37 °C in a minimum salts medium (Spizizen, 1958) containing glucose as carbon source (0.2%, w/v) and tryptophan (40 µg ml^{−1}). For the incorporation of ³²PO₄, the medium used was described by Cohen & Rickenberg (1956), except that the final concentration of potassium phosphate used was 0.5 mM.

† Present address: Laboratoires DEBAT, 153 rue de Buzenval, 92380 Garches, France

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Preparation of membrane vesicles. Membrane vesicles from *B. subtilis* were prepared according to the method of Konings *et al.* (1973). Membranes (8 mg protein ml⁻¹) were stored in 0.1 M-potassium phosphate buffer, pH 6.6, in liquid nitrogen.

For the uptake of [¹⁴C]glutamic acid, membrane vesicles were used at a final concentration of 1.5 to 2 mg ml⁻¹.

Inverted membrane vesicles from *B. subtilis* were prepared by slight modifications to the method of Tsuchiya & Rosen (1975). Bacteria were harvested at the end of the exponential phase, centrifuged at 6000 *g*, washed with cold 0.1 M-Tris/HCl buffer pH 7.5 containing 10 mM-MgCl₂, and resuspended in the same buffer containing 0.5 mg DNAase ml⁻¹ and 0.5 mM-dithiothreitol. The bacterial suspension was disrupted in an Eaton press. Surviving bacteria and coarse debris were removed by centrifuging for 10 min at 15000 *g* and the supernatant was then centrifuged in an ultracentrifuge (Beckman Spinco L/L₂) at 105000 *g* for 90 min. The pellet containing membrane particles was washed with cold 0.1 M-Tris/HCl buffer pH 7.5 and stored in the same buffer at concentrations of 12 mg protein ml⁻¹. Protein concentrations were determined by the Lowry method using bovine serum albumin as the standard.

Electron microscopy. Samples (2 to 5 ml) were fixed with formaldehyde/glutaraldehyde, postfixed with 1% (w/v) OsO₄ and further processed as described previously (Burdett & Higgins, 1978; Burdett, 1980). Sections were cut with glass knives, picked up on copper grids bearing a collodion/carbon support film and stained with uranyl acetate and lead citrate. Micrographs were taken on a Philips EM 300 electron microscope.

Leakage of cell constituents. The release of UV-absorbing material from clofoctol-treated organisms was followed after removing the bacteria by centrifugation at 6000 *g* for 10 min and measuring the absorbance of the supernatant at 260 nm.

Uptake of [¹⁴C]glutamic acid. Bacteria were incubated in growth medium, harvested at the middle of the exponential phase, washed and resuspended in fresh medium, at the same concentration of 200 µg (dry wt) of cells ml⁻¹ containing 0.025 µCi [¹⁴C]glutamic acid ml⁻¹ (0.925 kBq ml⁻¹), added at a final concentration of 0.1 mM (Kaback & Milner, 1970; Konings & Freese, 1972; Macleod *et al.*, 1973). The resuspended cultures were incubated at 37 °C with gentle shaking. Duplicate 1 ml samples were removed at intervals and the bacteria were collected on membrane filters. A sample (1 ml) was washed with 5 ml Spizizen salt medium and the radioactivity incorporated in the pool by then was determined. The other 1 ml sample was washed with 5 ml TCA (5%, w/v) to determine the incorporation of glutamate into protein.

Release of [¹⁴C]glutamic acid. Using the technique described above for the uptake of [¹⁴C]glutamic acid, bacteria were preloaded for 30 min at 37 °C in a medium containing 0.025 µCi [¹⁴C]glutamic acid ml⁻¹ (0.1 mM), harvested, washed once and resuspended in fresh medium at a concentration of 200 µg dry wt ml⁻¹. Clofoctol was then added and its effect on the release of [¹⁴C]glutamic acid examined.

Uptake of [¹⁴C]benzoic acid. *Bacillus subtilis* (*A*₆₅₀ = 0.9) was incubated with [¹⁴C]benzoic acid (50 mCi mmol⁻¹) at 0.1 µCi ml⁻¹. At various times, 1 ml samples were removed and prepared according to Bakker & Mangerich (1981).

Assay of ³²P_i incorporation into nucleotides. ³²PO₄H₃ (0.1 mCi ml⁻¹) was added to cultures of *B. subtilis* grown in Cohen & Rickenberg medium (1956). After being allowed to equilibrate for 120 min, clofoctol was then added and the concentration of nucleotides determined as described by Cashel & Gallant (1969).

Respiration of intact cells and vesicles from B. subtilis. A suspension of bacteria (2 ml) grown in Spizizen medium (*A*₆₅₀ = 1) was placed into a thermostatically controlled cell at 37 °C. Air was bubbled and the oxygen consumption was measured with a Hansatech electrode using a Servotrace oxygraph.

ATPase assay. The standard reaction mixture consisted of 33 mM-Tris/HCl buffer pH 7.5, 1.5 mM-MgCl₂, 3 mM-ATP (sodium salt) and 0.4 mg ATPase, as described by Monteil *et al.* (1974). The mixture was incubated for 30 min at 37 °C and the ATPase activity was measured by the colorimetric determination of the inorganic phosphate liberated using ammonium molybdate (Baginsky *et al.*, 1966).

Chemicals. Clofoctol was obtained from DEBAT Laboratories (Garches, France). Solutions were prepared in absolute ethanol at a concentration of 2 mg ml⁻¹ as it is very hydrophobic. [¹⁴C]glutamic acid (285 mCi mmol⁻¹), [¹⁴C]benzoic acid (50 mCi mmol⁻¹) and carrier-free ³²PO₄H₃ were products of the Commissariat à l'Energie Atomique, Saclay, France. Potassium cyanide was from Merck, dicyclohexylcarbodiimide was from Fluka and CCCP was from Sigma.

RESULTS

Effect of clofoctol on ATP

Since clofoctol decreases the growth rate of *B. subtilis* but does not specifically inhibit the synthesis of DNA, RNA or protein, it seemed possible that energy metabolism might be altered. The effect of clofoctol on the intracellular content of ATP and GTP was studied. A culture of *B. subtilis* (*A*₆₅₀ = 0.5) was treated with 4 µg clofoctol ml⁻¹ after equilibration with ³²PO₄.

Fig. 1. Effect of clofoctol on the intracellular ATP, GTP and ppGpp pools of *B. subtilis*. Bacteria were incubated with $^{32}\text{PO}_4\text{H}_3$ (0.1 mCi ml^{-1} ; 0.5 mM) ($\bullet$). After 120 min (arrow) clofoctol ($4 \mu\text{g ml}^{-1}$) was added to half the culture ($\blacktriangle$). Radioactivity in the cell-free extract after dilution with formic acid was determined in the ATP (a), GTP (b), and ppGpp (c) pools.

The addition of clofoctol to the culture decreased the nucleotide pools dramatically. The pools of ATP and GTP dropped by about 75% and 56%, respectively, during the first 30 min (Fig. 1a, b). A consistent increase in the pool of ADP was observed (data not shown). A transient accumulation of the regulatory nucleotide, ppGpp was observed during the first 30 min after exposure to the drug. The levels then returned progressively to near basal values (Fig. 1c).

ATPase activity

It seemed possible that the rapid decrease in the ATP pool caused by clofoctol might result from the inhibition of the H^+ -translocating ATP synthetase.

The effects of clofoctol on the ATPase of inverted vesicles were therefore compared with those of the known inhibitor, dicyclohexylcarbodiimide (DCCD) (Harold *et al.*, 1969). Clofoctol did not inhibit the ATPase activity, even at very high concentration ($100 \mu\text{g ml}^{-1}$), while, as expected, DCCD (1 mM) inhibited the hydrolysis of ATP by 61%.

Oxygen uptake

The effect of clofoctol on the respiration of *B. subtilis* and its membrane vesicles was studied using an oxygen electrode. Figure 2 compares the respiration of bacteria treated with clofoctol (5 and $50 \mu\text{g ml}^{-1}$) with that of bacteria treated with potassium cyanide (1 mM). The oxygen consumption was not affected by clofoctol even at a very high concentration ($50 \mu\text{g ml}^{-1}$), while

Fig. 2. Effect of clofoctol on respiratory rate of *B. subtilis*. Endogenous respiration was measured using a Hansatech electrode. Respiratory rate was measured in untreated bacteria (◆) then samples were treated (arrow) with 5 µg clofoctol ml⁻¹ (◇), 50 µg clofoctol ml⁻¹ (---), or 1 mM-KCN (—) and new rates were determined.

Fig. 3. Facilitation of proton uptake by clofoctol. Bacteria were suspended at 25 °C ($A_{650} = 10$) in KCl (50 mM)/MgCl₂ (2 mM)/glycylglycine (1 mM). Aliquots of HCl, sufficient to lower the pH to 6.2, were added at time zero, followed by addition at arrows: (a) 0.5 µg valinomycin ml⁻¹ (Val) and 2% ethanol (EtOH); (b) 0.5 µg valinomycin ml⁻¹ (Val) and 50 µg clofoctol ml⁻¹ (Clo).

it was stopped immediately with potassium cyanide. Results were similar when membrane vesicles of *B. subtilis* were used. The decrease in ATP concentration observed after clofoctol treatment did not therefore seem to be due to an interference of the drug with respiration.

Effects of clofoctol on the pH gradient

According to the chemiosmotic theory of Mitchell (1977), the cytoplasmic membrane acts as an osmotic and electric barrier, driving an adenosine triphosphatase toward the synthesis of ATP by maintaining a pH gradient. We therefore examined the effects of clofoctol on the transmembrane pH gradient using two different methods.

The first method involved measuring directly the pH of a suspension of *B. subtilis* (Fig. 3). In this experiment, the electrical restrictions on proton movements were relieved by valinomycin, which renders the membrane freely permeable to K⁺ (Harold *et al.*, 1974; Moore & Pressman, 1964). As clofoctol was very hydrophobic, it was diluted in ethanol and the same concentration of ethanol was used for the control. When clofoctol was added, stimulation of the translocation of protons across the membrane was demonstrated.

Fig. 4

Fig. 5

Fig. 4. Effect of clofoctol and CCCP on ΔpH . *Bacillus subtilis* was grown in Spizizen medium containing [^{14}C]benzoic acid ($0.1 \mu\text{Ci ml}^{-1}$) (●). At the time indicated (arrow) 10 ml samples were added to flasks containing $7.5 \mu\text{g clofoctol ml}^{-1}$ (■) or 30 mM-CCCP (▲). All the samples were filtered and washed and [^{14}C]benzoate in the cells was determined.

Fig. 5. Effect of clofoctol on the release of intracellular materials from cells of *B. subtilis*. Bacterial suspensions were treated with $4 \mu\text{g clofoctol ml}^{-1}$ (■), $6 \mu\text{g clofoctol ml}^{-1}$ (▲), or untreated (●). A_{260} of the supernatant solutions was measured after the indicated treatment times.

The second method measured the effect of clofoctol on the uptake of benzoic acid [A]. This acid permeating the membrane only in its neutral form [AH] (Bakker & Mangerich, 1981) will reach the equilibrium when $[\text{AH}_{\text{in}}] = [\text{AH}_{\text{out}}]$. Since

$$K_a = [\text{H}_{\text{in}}^+] [\text{A}_{\text{in}}^-] / [\text{AH}_{\text{in}}] = [\text{H}_{\text{out}}^+] [\text{A}_{\text{out}}^-] / [\text{AH}_{\text{out}}]$$

it follows that $[\text{H}_{\text{in}}^+] / [\text{H}_{\text{out}}^+] = [\text{A}_{\text{out}}^-] / [\text{A}_{\text{in}}^-]$. Thus in equilibrium the total species concentration $[\text{A}^T] = [\text{A}^-] + [\text{AH}]$ on both sides of the membrane is given by:

$$[\text{A}_{\text{in}}^T] / [\text{A}_{\text{out}}^T] = (1/K_a + 1/[\text{H}_{\text{in}}^+]) / (1/K_a + 1/[\text{H}_{\text{out}}^+])$$

For acids which $K_a \gg [\text{H}_{\text{in}}^+]$ and $[\text{H}_{\text{out}}^+]$, $[\text{A}_{\text{in}}^T] / [\text{A}_{\text{out}}^T] = [\text{H}_{\text{out}}^+] / [\text{H}_{\text{in}}^+]$. We used [^{14}C]benzoic acid as a permeating acid in pH studies as this acid has a fast equilibration, does not bind to membranes and proteins and does not have any metabolic effects. The uptake of [^{14}C]benzoic acid by *B. subtilis* was examined. At various times, samples were removed and the radioactivity taken up was determined.

The value of pH_{in} was calculated from the equilibrium:

$$[\text{benzoic acid}_{\text{in}}] / [\text{benzoic acid}_{\text{out}}] = [\text{H}_{\text{out}}^+] / [\text{H}_{\text{in}}^+] \quad (1)$$

Addition of clofoctol and the uncoupler CCCP at the equilibrium resulted in a loss of the accumulated benzoic acid (Fig. 4).

The internal pH calculated from equation (1) was 7.7 for the control and dropped to 7.4 when cells were treated for 10 min with clofoctol. The value of ΔpH ($\text{pH}_{\text{in}} - \text{pH}_{\text{out}}$) varied from 0.9 for the control to 0.65 for the clofoctol-treated cells. Thus clofoctol appeared to facilitate the passage of protons across the membrane, leading to the collapse of the proton gradient.

Effects of clofoctol on cell permeability

Since the decrease in the ATP pool was not due to an effect of clofoctol on ATPase or on respiration, the observed collapse of the proton gradient suggests that clofoctol might alter cell permeability in general. We used the method of Harold & Baarda (1968) to investigate the possibility that the decrease in the ATP pool observed after clofoctol treatment might reflect a general effect of the drug on cell permeability. We measured the ability of *B. subtilis* to take up the amino acid, L-glutamic acid, in the presence of clofoctol. L-Glutamic acid was concentrated against a gradient in the cells.

The uptake of glutamic acid was strongly inhibited when the bacteria ($A_{650} = 0.5$) were treated with clofoctol ($3 \mu\text{g ml}^{-1}$). In addition, *B. subtilis* preloaded with [^{14}C]glutamic acid (0.1 mM , $0.025 \mu\text{Ci ml}^{-1}$) leaked about 85% of the accumulated radioactivity during the first 5 min of incubation in fresh medium, containing clofoctol ($3.3 \mu\text{g ml}^{-1}$). In the same experiment, the effect of clofoctol on the incorporation of [^{14}C]glutamic acid into the protein fractions was also examined. The control organisms showed a linear rate of incorporation of radioactivity, while clofoctol treatment resulted in approximately 15% inhibition of this rate.

Similar results were obtained with preparations of membrane vesicles from *B. subtilis* and *E. coli* (results not shown). The amount of accumulated glutamate released from these preparations was about 40% during the first 5 min after addition of clofoctol.

The effect of clofoctol (4 and $6 \mu\text{g ml}^{-1}$) on UV leakage and on viability was measured on *B. subtilis* ($A_{650} = 0.8$). UV leakage was greater at $6 \mu\text{g ml}^{-1}$ and increased with treatment time (Fig. 5). These concentrations of clofoctol had no effect on viability during the same period (60 min).

The appearance of the bacteria was examined by electron microscopy. An effect of the drug was observed when *B. subtilis* ($A_{650} = 0.8$) was treated with $6 \mu\text{g ml}^{-1}$ clofoctol for 30 min. There was no lysis of the bacteria, but the electron micrographs revealed alterations in the bacterial envelope. Holes were seen in both the wall and the cytoplasmic membrane.

DISCUSSION

The observations reported here suggest that clofoctol alters the permeability of the envelope of *B. subtilis*. A consideration of the effects of clofoctol on *B. subtilis* suggests that this antibacterial agent affects the integrity of the cytoplasmic membrane and hence the many biochemical activities associated with it. Interaction with the membrane is suggested by the results showing leakage of cellular constituents, particularly UV-absorbing material and glutamic acid. However there was no concomitant rapid loss of viability. This suggests that, at least in the early stages, the damage to the cytoplasmic membrane caused by clofoctol may be repaired. The collapse of the intracellular pool of ATP readily accounts for the arrest of macromolecular syntheses (Simonet *et al.*, 1979), including that of the cell wall (Yablonsky & Simonet, 1982). This reduction in ATP concentration resulted from the direct leakage of ATP across the damaged cytoplasmic membrane and by the reduction of its re-synthesis by the Mg^{2+} , Ca^{2+} -dependent ATPase, as shown by an increase in ADP concentration. However, a direct effect of clofoctol on the activity of the ATPase was ruled out by the experiments with inverted vesicles. A disruption of the transmembrane proton gradient remains the most probable cause.

The chemiosmotic hypothesis states that during bacterial respiration, protons are pumped out to give an electrochemical gradient across the cell membrane. This proton-motive force has electrical and chemical components such as: $p = \Delta\psi - Z\Delta\text{pH}$, where $\Delta\psi$ is the membrane potential and $-\Delta\text{pH}$ the transmembrane pH gradient (Mitchell, 1977). The collapse by clofoctol of both ΔpH and $\Delta\psi$ resulted in additional effects similar to those of uncouplers of oxidative phosphorylation.

At present, little is known about the chemical and physical modifications of the membrane caused by clofoctol. However, electron micrographs of treated *B. subtilis* presented evidence of apparently non-random alteration of the whole envelope (both wall and cytoplasmic

membrane). These gaps were located near the septum (or a potential septum), suggesting an interference with the biosynthetic processes of the wall.

We have already shown that clofoctol inhibits *in vivo* the polymerization of UDP-N-acetylmuramyl pentapeptide leading to the accumulation of this precursor (Yablonsky & Simonnet, 1982), but could not demonstrate *in vitro* inhibition of peptidoglycan synthesis. Hence, this effect is indirect and might suggest a modification of the transfer properties of the cytoplasmic membrane, which remains the most probable target of this antibacterial agent.

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