ON THE MODE OF ACTION OF PSEUDOMONIC ACID: INHIBITION OF PROTEIN SYNTHESIS IN *STAPHYLOCOCCUS AUREUS*

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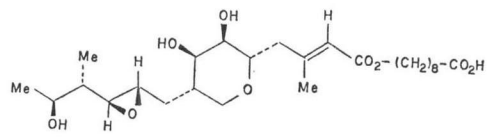
The effect of the antibiotic, pseudomonic acid, on the major metabolic processes in *Staphylococcus aureus* was studied. The primary effect of low concentrations of the antibiotic, leading to bacteriostasis, is inhibition of protein synthesis. Pseudomonic acid also severely inhibits RNA synthesis which can be prevented by chloramphenicol treatment. DNA and cell wall peptidoglycan synthesis are inhibited to a lesser extent and interference with these processes is considered to be a secondary effect. Oxidative phosphorylation as measured by ATP levels was not inhibited.

Pseudomonic acid A (hereafter referred to as pseudomonic acid), (Fig. 1), was isolated as the major component of a family of structurally related antibiotics from a strain of *Pseudomonas fluorescens*, by BANKS *et al.*[2] Its structure was elucidated by CHAIN and MELLOWS[4,5]. More recently, the absolute stereochemistry of the molecule has been determined by ALEXANDER *et al.*[1] The antibiotic has a narrow spectrum of activity confined mainly to Gram-positive bacteria (SUTHERLAND *et al.*[14]). It is relatively inactive against Enterobacteriaceae and strains of *Enterococci*. Pseudomonic acid shows no cross-resistance with a wide selection of antibiotics, suggesting, as does also its unique structure, a novel mode of action.

This paper reports the effect of pseudomonic acid on major cellular processes, in which interference with protein synthesis was identified as the primary inhibitory effect leading to bacteriostasis of *Staphylococcus aureus*.

Fig. 1. Structure of pseudomonic acid



Materials and Methods

Materials

Pseudomonic acid was isolated from a large scale fermentation of *Pseudomonas fluorescens* (NCIB 10586) and purified as previously described (CHAIN and MELLOWS[5]). The antibiotic was used throughout this investigation as its sodium salt. Nutrient broth was purchased from Oxoid Ltd., London, S.E.1. L-Phenylalanine, uridine, thymidine and ATP were from Sigma Ltd., Kingston-upon-Thames, Surrey. Polyethyleneimine (PEI)-cellulose-coated thin-layer chromatography plates were from Camlab Ltd., Cambridge. GF/C glass-fibre filters were from Whatman Ltd., Maidstone, Kent., [2-^{14}C]-thymidine (56.0 mCi/mmole) and D-[1-^{14}C]-glucosamine (3.4 mCi/mmole) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire. [^{32}P]-Phosphate (carrier-free; original specific activity 3 Ci/mmole of P) was purchased from New England Nuclear, D-6072 Dreieich, West Germany.

Methods

Culture conditions: *S. aureus* Oxford NCTC 6571 was grown in double strength nutrient

broth at 37°C on a rotary shaker. A 0.1% v/v inoculum of an overnight culture was dispersed into fresh pre-warmed medium. Experiments were started when the absorbance at 600 nm was approximately 0.2, indicating steady exponential growth.

Measurements of Protein Synthesis: The incorporation of [^{14}C]-phenylalanine into trichloroacetic acid (TCA) precipitable material, as described by UCHIDA and ZÄHNER,[15] was used. Samples (0.5 ml) were added to 10% TCA (6 ml), placed on ice for 30 minutes and finally heated at 90~95°C for 20 minutes. The mixture was filtered and the precipitate collected on Whatman GF/C glass-fibre filters, washed with 5% TCA (2×6 ml) and ethanol (2×6 ml), dried and counted in 5 ml toluene (containing 0.6% butyl-PBD and 5% naphthalene) in a Beckman LS-200B liquid scintillation spectrometer.

Measurement of RNA and DNA Synthesis: The uptake of [^{14}C]-uridine and [^{14}C]-thymidine into TCA precipitable material was followed, as described above, with the following inclusion. Culture samples (0.5 ml) were added to 2% sodium lauryl sulphate (0.5 ml), whirled and placed on ice for 30 minutes. 5% TCA (6 ml) was added to each and the samples left for a further 30 minutes before filtering, *etc.*, as above.

Measurement of Peptidoglycan (Cell Wall) Synthesis: Two methods were used.

(**1**) The uptake of D-[^{14}C]-glucosamine into TCA precipitable material, essentially as described by UCHIDA and ZÄHNER[15], was used. Culture samples (0.5 ml) were added to 10% TCA (5 ml), whirled, placed on ice for 30 minutes and finally heated to 90~95°C for 20 minutes. On cooling, each was filtered through a Whatman GF/C filter, washed successively with 5% TCA (3×5 ml) and ethanol (2×10 ml), dried and radio-counted.

(**2**) In the second method, the peptidoglycan was extracted from the cells and partially purified by a similar procedure to that of PARK and HANCOCK.[12] Culture samples (100 ml) were immediately centrifuged at 6,000 *g* for 15 minutes. The pellet was washed with water, re-centrifuged and suspended in water (2 ml). 25% TCA (0.5 ml) was added, mixed well and placed on ice for 10 minutes. After centrifugation, 4,000 *g* for 10 minutes, the residue was suspended in 75% ethanol (2.5 ml), left for 10 minutes at room temperature and re-centrifuged. The residue was re-suspended in 5% TCA (2.5 ml), heated for 6 minutes at 90°, cooled and centrifuged. The residue was suspended in 0.05 M NH_4HCO_3 (0.95 ml) containing 0.005 M NH_4OH. A solution of trypsin (0.05 ml; 1 μg/ml) was added and the mixture incubated at 37°C for 2 hours. After centrifugation, the residue was freeze-dried, re-suspended in water (1.0 ml) and 0.1 ml aliquots counted in 10 ml BRAY's scintillation fluid.

Determination of ATP Levels: The [^{32}P]-orthophosphate exchange method of CASHEL,[3] as described by OGILVIE *et al.*[11], was used. The procedure described for *E. coli* and *B. subtilis* was found to be applicable to *S. aureus*. The Rf value of ATP (0.8) was identical to that quoted by CASHEL[3] for elution [1.5 M KH_2PO_4 (pH 3.4)] on polyethyleneimine cellulose thin-layer chromatograms.

Results

Bacteriostasis and Reversibility

The primary effect of pseudomonic acid against *S. aureus* at concentrations (0.05~0.5 μg/ml) close to the minimum inhibitory concentration (MIC; 0.05 μg/ml), is bacteriostasis (HUGHES and MELLOWS, unpublished work; SUTHERLAND *et al.*[14]). Cells treated with pseudomonic acid within these concentration limits, followed by transfer to fresh medium, recover spontaneously after several hours. At higher concentrations the effect of the antibiotic becomes increasingly bactericidal.

Inhibition of the Synthesis of Macromolecules

The overall synthesis of protein, RNA and DNA was measured in the presence of increasing concentrations of pseudomonic acid on the assumption that the most sensitive process of the cell would indicate the primary action leading to bacteriostasis. The incorporation of labelled phenylalanine, uridine and thymidine was always inhibited simultaneously but protein and RNA synthesis

Fig. 2. Effect of pseudomonic acid on (a) protein, (b) RNA and (c) DNA synthesis in *S. aureus*

An exponentially growing culture (40 ml, E_{600} approx. 0.2) was labelled with either [^{14}C]-phenylalanine, [^{14}C]-uridine, or [^{14}C]-thymidine (each at 0.4 μCi/ml). After 4 minutes the culture was split into 10 ml aliquots. One subculture was used as a control, whilst the others were treated with different concentrations of pseudomonic acid (as indicated by the arrows). Samples (0.5 ml) were withdrawn and the acid-precipitable radioactivity determined.

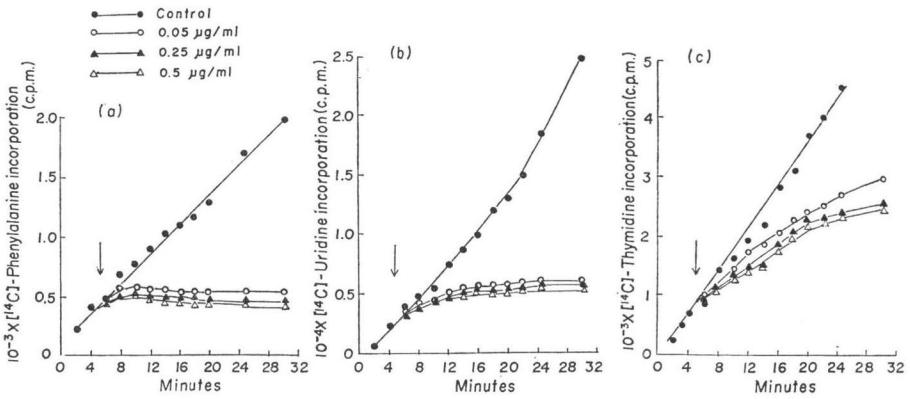


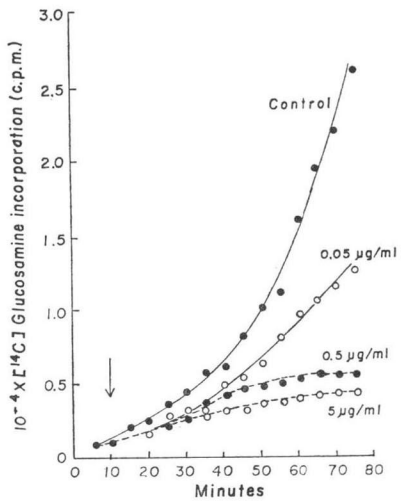
Table 1. Inhibition of protein, RNA and DNA synthesis by pseudomonic acid in *S. aureus*

To a split culture was added either [^{14}C]-phenylalanine, [^{14}C]-uridine or [^{14}C]-thymidine as described in the legend to Fig. 2. The degree of inhibition was calculated from the kinetics of incorporation in pseudomonic acid-treated cultures relative to the untreated controls, 20 minutes after the addition of the antibiotic.

Pseudomonic acid µg/ml	% Inhibition		
	Protein	RNA	DNA
0.05	88	78	55
0.10	92	80	60
0.25	96	81	63
0.50	100	86	65

Fig. 3. Effect of pseudomonic acid on peptidoglycan synthesis in *S. aureus*

D-[^{14}C]-Glucosamine (0.2 μCi/ml) was added to an exponentially growing culture (50 ml). The culture was split into four equal portions. One culture served as the control and different concentrations of pseudomonic acid were added to the remaining three cultures as indicated by the arrow. Samples (0.5 ml) were withdrawn and the acid precipitable radioactivity determined.



appeared to be more sensitive than DNA synthesis (Table 1 and Fig. 2).

Pseudomonic acid also inhibited the formation of the cell wall peptidoglycan. However, in the short term the inhibition of peptidoglycan synthesis was less pronounced than the inhibition of RNA, DNA and protein synthesis (Table 2 and Fig. 3). The more marked effect of penicillin G (Table 2), which inhibits the cross-linking process in peptidoglycan synthesis (GALE *et al.*[7]) suggested that cell wall formation is a less likely target site. This is supported by the observation

Table 2. Inhibition of peptidoglycan (cell wall) synthesis in *S. aureus*

D-[^{14}C]-Glucosamine (10 μCi) was added to an exponentially growing culture (200 ml, E_{600} approx 0.2). The culture was split into two equal portions. To one was added pseudomonic acid (0.05 μg/ml) whilst the other served as the control. Cultures were harvested at the times indicated; the incorporation of radioactivity into partially purified peptidoglycan was measured. The degree of inhibition was calculated from the kinetics of incorporation in pseudomonic acid-treated cultures relative to the untreated controls. The results are compared with the inhibitory effect of penicillin G (0.03 μg/ml, M.I.C.) obtained in a separate experiment.

Time (min.)	% Inhibition of peptidoglycan synthesis
30	24
60	46
90	75
30 (penicillin G)	81

that pseudomonic acid treated cells do not lyse as do penicillin treated cells.

Effect on Cellular ATP levels

At a concentration of 0.5 μg/ml, pseudomonic acid significantly enhanced (37%) the cellular ATP level after 30 minutes (Table 3). Under identical conditions, 2,4-dinitrophenol, a known uncoupler of oxidative phosphorylation (GALE *et al.*[7]) at 1 mg/ml (MIC) led to a 45% reduction in the ATP content of the cell. It is therefore concluded that at low concentrations pseudomonic acid does not interfere with oxidative phosphorylation and energy processes dependent on it.

A similar enhancement of the cellular ATP pool size has previously been observed in *B. subtilis* (OGILVIE *et al.*[11]) grown in the presence of granaticin, an antibiotic which specifically inhibits the aminoacylation of leucyl-tRNA (OGILVIE *et al.*[10]). This suggested that pseudomonic acid and granaticin may have a similar action and that the marked inhibition of RNA synthesis by pseudomonic acid may be a result of the stringent control mechanism brought about by the cellular deprivation of one or more amino acids (HASELTINE and BLOCK,[8] PEDERSON *et al.*[13], OGILVIE *et al.*[11]).

Table 3. Effect of pseudomonic acid on cellular ATP levels in *S. aureus*

Two aliquots (0.5 ml) of an exponentially growing culture (E_{600} approx. 0.2) were transferred to two tubes. Pseudomonic acid (0.5 μg/ml) or 2,4-dinitrophenol (1 mg/ml) was added to one tube whilst the other served as the control. [^{32}P]-Orthophosphate (0.1 ml, 50 μCi) was added to each and the cultures incubated at 37°C, with aeration from a split capillary tube. Samples (0.1 ml) were pipetted into 2 M formic acid (0.1 ml) and left on ice for 30 minutes. Aliquots (50 μl) were eluted on prewashed PEI-cellulose coated t.l.c. plates using 1.5 M KH_2PO_4. The [^{32}P]-ATP spot was located by autoradiography, removed from the chromatogram and radiocounted.

Time (minutes)	[^{32}P]-ATP c.p.m.			
	(a)		(b)	
	Pseudomonic acid treated cells	Control	2,4-Dinitrophenol treated cells	Control
0	322	330	213	262
10	684	645	527	695
20	1,174	772	457	1,118
30	1,466	1,072	746	1,347

Effect of Chloramphenicol on Pseudomonic Acid-treated *S. aureus* Cells

Certain antibiotics, *e.g.* chloramphenicol, which inhibit protein synthesis at the ribosomal level abolish the stringent control mechanism of RNA synthesis (CASHEL,[3] EDLIN and BRODA,[6] LUND

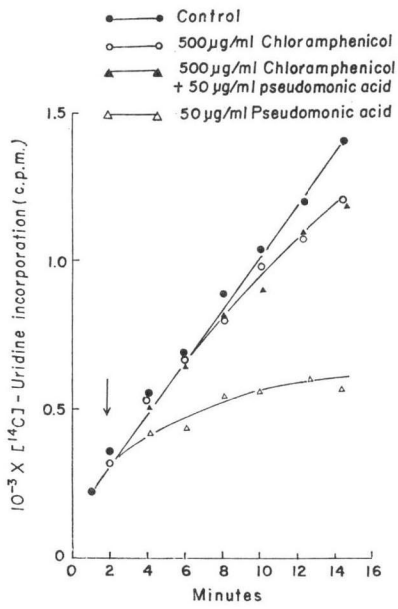
and KJELGAARD[9]). When chloramphenicol and pseudomonic acid were simultaneously added to *S. aureus* cells, the incorporation of [^{14}C]-uridine was not inhibited when compared with [^{14}C]-uridine uptake in cells treated with pseudomonic acid alone (Fig. 4). Thus chloramphenicol breaks the stringent regulation of RNA synthesis imposed by pseudomonic acid.

Conclusion

In *S. aureus* cells, pseudomonic acid markedly inhibits protein and RNA synthesis, whereas DNA and cell wall formation are affected to a lesser extent. The antibiotic does not interfere with oxidative phosphorylation since the cellular pool of ATP increased in cells treated with pseudomonic acid. The inhibition of RNA synthesis was relieved by the addition of chloramphenicol to pseudomonic acid-treated cells, suggesting that the antibiotic causes auxotrophy of one or more amino acids thus providing the signal for the stringent control of RNA synthesis. These observations provide evidence that protein synthesis is the primary target of pseudomonic acid in *S. aureus*, and further suggest that the antibiotic may cause intracellular starvation of one or more amino acids.

Fig. 4. Effect of chloramphenicol on RNA synthesis in a pseudomonic acid treated culture of *S. aureus*

An exponentially growing culture was treated with [^{14}C]-uridine (0.4 μCi/ml). The culture was split into four equal portions. Chloramphenicol, chloramphenicol plus pseudomonic acid and pseudomonic acid were added to each of three cultures as indicated by the arrow, whilst the other culture served as a control. Samples (0.5 ml) were withdrawn and the acid-precipitable radioactivity determined.



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