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DIO 9 AND CHLORHEXIDINE: INHIBITORS OF MEMBRANE-BOUND ATPase AND OF CATION TRANSPORT IN *STREPTOCOCCUS FAECALIS*

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SUMMARY

1. Dio 9, an antibiotic of unknown structure, and the synthetic bis-guanidine Chlorhexidine (1,6-di-4'-chlorophenyldiguanidohexane), inhibit the ATPase associated with isolated protoplast membranes of *Streptococcus faecalis*. They inhibit both the native, membrane-bound enzyme, and the solubilized protein. The effects of Dio 9 and Chlorhexidine differ in this respect from those of *N,N'*-dicyclohexylcarbodiimide which was previously found to inhibit only the membrane-bound ATPase.

2. When added to intact cells, Dio 9 and Chlorhexidine inhibit the net uptake of K^+ by exchange for Na^+ and H^+, and also the stimulation of glycolysis associated with K^+ uptake. However, they do not inhibit autologous exchange of $^{42}K^+$ for K^+.

3. Dio 9 and Chlorhexidine apparently do not inhibit the generation of ATP *via* glycolysis. Partial inhibition of glycolysis by these compounds may be a secondary consequence of the inhibition of ATPase.

4. The finding that Dio 9 and Chlorhexidine inhibit both the ATPase and net K^+ uptake by *S. faecalis* supports the hypothesis that the ATPase is involved in ion transport.

INTRODUCTION

The membrane-bound ATPase of *Streptococcus faecalis* has been characterized in some detail in earlier publications[1–4], but its physiological function remains unclear. Mitochondrial ATPase is thought to catalyze the terminal step in oxidative phosphorylation (review: ref. 5). It is unlikely that the ATPase associated with protoplast membranes of *S. faecalis* serves an analogous function as this organism lacks cytochromes and generates ATP entirely *via* glycolysis[6]. Indirect evidence[2,7,8] has implicated the ATPase of *S. faecalis* in ion transport. This hypothesis received some direct support from the demonstration[9] that *N,N'*-dicyclohexylcarbodiimide is a potent inhibitor of the membrane-bound ATPase of *S. faecalis* and also inhibits both ATP degradation and energy-dependent accumulation of K^+ by the intact organisms.

N,N'-Dicyclohexylcarbodiimide belongs to that heterogeneous class of compounds which inhibit the ATPase and energy-transfer processes in mitochondria[10–12].

Abbreviation: Chlorhexidine, 1,6-di-4'-chlorophenyldiguanidohexane.

We report here that two other compounds, Dio 9 and Chlorhexidine (1,6-di-4'-chlorophenyldiguanidohexane) also inhibit both the ATPase and K^+ accumulation by *S. faecalis*. Dio 9, an antibiotic of unknown structure, is a potent inhibitor of the ATPase of both mitochondria and chloroplasts[13,15], and inhibits energy transfer reactions in these organelles[13–17]. Dio 9 is active against a number of Gram-positive organisms[13] but we are not aware of any previous work on its mode of action on bacteria. Chlorhexidine, a synthetic bis-guanidine[18] inhibits the growth of many microorganisms. Bactericidal concentrations of Chlorhexidine disrupt the plasma membrane and induce leakage of cell constituents[19–22]. The effects of Chlorhexidine on mitochondria have apparently not been explored, but other bis-guanidines are known to inhibit energy transfer.

METHODS

All the procedures employed in the present study have been fully described in earlier papers listed below.

S. faecalis strain 9790 was grown on complex media containing tryptone, yeast extract, glucose and either Na_2HPO_4 (medium NaTY, ref. 7) or K_2HPO_4 (medium KTY, ref. 23). Organisms grown on medium NaTY overnight are relatively depleted of K^+ and contain large amounts of Na^+ and H^+ (ref. 7). The cells were harvested by centrifugation, washed with 2 mM $MgCl_2$ and resuspended in water. To produce cells fully loaded with K^+, the organisms were grown on medium KTY; the pH of overnight cultures was adjusted to 7 to permit exchange of any intracellular H^+ for K^+, and the cells were then collected and washed with $MgCl_2$. Cells adapted to arginine were grown on medium NaTY supplemented with arginine hydrochloride[7]. Viability was assayed by plate counts on medium KTY.

In a few experiments we employed cells in which K^+ and H^+ were totally replaced by Na^+. Such cells were prepared by incubation in sodium maleate buffer with 2μg/ml monactin[8]. This antibiotic renders *S. faecalis* permeable to cations and is later readily removed by washing the cells.

Transport experiments were conducted at room temperature. Glycolysis was monitored continuously at pH 7.5 by means of a pH-stat[7], which automatically added NaOH to neutralize the lactic acid produced by the cells; arginine metabolism at pH 6.2 was similarly followed by addition of HCl. Samples were taken by filtration through Millipore filters and washed with $MgCl_2$. Cations were extracted with trichloroacetic acid and estimated by flame photometry[24]. ATP was determined by a modification[25] of the firefly luminescence method.

Membrane-bound ATPase was prepared by osmotic lysis of protoplasts. The enzyme was dissociated from the membrane by repeated washing as described previously[2,4]. The effects of inhibitors were tested as follows: Native or solubilized enzyme, in Tris–Mg buffer (0.1 M Tris chloride, 2 mM Mg^{2+}, pH 7.5) was incubated for 10 min at room temperature with or without inhibitors. The suspension was then diluted with an equal volume of Tris chloride (0.1 M)–ATP (10 mM)–Mg^{2+} (8 mM) and incubated at 38°. P_i released was determined after 10 min (ref. 2).

Chemicals

Dio 9 and Chlorhexidine were gifts from R. J. GUILLORY and A. DAVIES, respectively. Dio 9 was dissolved in ethanol and added to cells or enzyme preparations

so as to dilute the ethanol 100-fold. Chlorhexidine was obtained as the digluconate in aqueous solution and used as such.

RESULTS

Inhibition of ATPase by Dio 9 and Chlorhexidine

Inhibition of the membrane-bound ATPase by Dio 9, Chlorhexidine and dicyclohexylcarbodiimide is illustrated in Fig. 1. The potency of the three inhibitors was comparable on a weight basis and, for dicyclohexylcarbodiimide and Chlorhexidine on a molar basis (50 % inhibition: dicyclohexylcarbodiimide, $1 \cdot 10^{-5}$ M; Chlorhexidine, $5 \cdot 10^{-6}$ M; the molecular weight of Dio 9 is not known). It should be noted that even much higher concentrations did not completely inhibit the enzyme. Another guanidine derivative, Vantocil IB (ref. 19) was approximately as effective as Chlorhexidine.

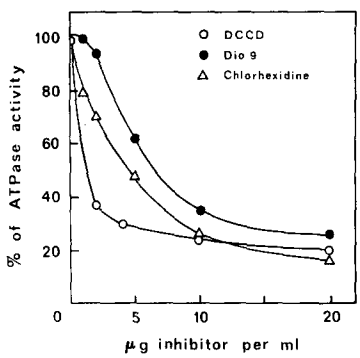


Fig. 1. Inhibition of the membrane-bound ATPase. For assay conditions see METHODS. DCCD, dicyclohexylcarbodiimide.

We found previously that dicyclohexylcarbodiimide inhibited the membrane-bound ATPase but not the solubilized enzyme[9]. By contrast, Dio 9 and Chlorhexidine inhibited the enzyme equally well in both states (Table I). Also, unlike dicyclohexylcarbodiimide[9], neither Dio 9 nor Chlorhexidine inhibited the membrane-bound NADH dehydrogenase.

Inhibition of K^+ accumulation by intact cells

Cells harvested from medium NaTY are relatively depleted of K^+ but contain large amounts of Na^+ and H^+. In the presence of an energy source such cells can accumulate K^+, with concurrent extrusion of Na^+ and H^+; cation exchange is accompanied by marked stimulation of glycolysis[7–9,24]. As shown in Figs. 2 and 3, Dio 9 and Chlorhexidine inhibited both the exchange of cations and the concurrent stimulation of glycolysis. Entirely analogous results were obtained with cells fully loaded with Na^+: again, Dio 9 and Chlorhexidine inhibited both K^+ uptake and the stimulation of glycolysis.

Arginine can replace glucose as energy source for K^+ accumulation[7,24]. Dio 9 and Chlorhexidine had no detectable effect on the rate of arginine metabolism, but reduced the rate of K^+ accumulation to one third of that of control cells.

Dio 9 and Chlorhexidine thus inhibit net uptake of K^+ by heterologous exchange for Na^+ and H^+. However, these compounds did not inhibit autologous exchange of

$^{42}K^+$ for K^+—indeed, they accelerated it somewhat (Fig. 4). It will be recalled that dicyclohexylcarbodiimide also blocks heterologous, but not autologous, cation exchange[9].

TABLE I

INHIBITION OF MEMBRANE-BOUND AND SOLUBILIZED ATPASE

The enzyme in 0.5 ml 100 mM Tris chloride (pH 7.2) and 2 mM Mg^{2+} was incubated at room temperature for 10 min with 0.1 μmole dicyclohexylcarbodiimide, 20 μg Dio 9 or 0.024 μmole Chlorhexidine. Then an equal volume of Tris chloride containing 5 μmoles ATP and 4 μmoles Mg^{2+} was added. The mixture was incubated 10 min at 38° and P_i released was determined. The absolute amounts of P_i ranged from 0.49 to 0.70 μmoles/10 min for the membrane-bound ATPase, and from 0.40 to 0.56 μmoles/10 min for the solubilized enzyme.

Enzyme	*Protein (mg)*	*Inhibitor*	*Number of experiments*	*Inhibition (%)*
Membrane–ATPase complex	0.3	Dicyclohexylcarbodiimide	7	71.8 ± 1.1
	0.3	Dio 9	5	63.6 ± 3.5
	0.3	Chlorhexidine	6	30.5 ± 3.7
Solubilized ATPase	0.04	Dicyclohexylcarbodiimide	4	0 (no significant inhibition*)
	0.04	Dio 9	4	50.3 ± 6.2
	0.04	Chlorhexidine	4	38.0 ± 3.0

* See ref. 9.

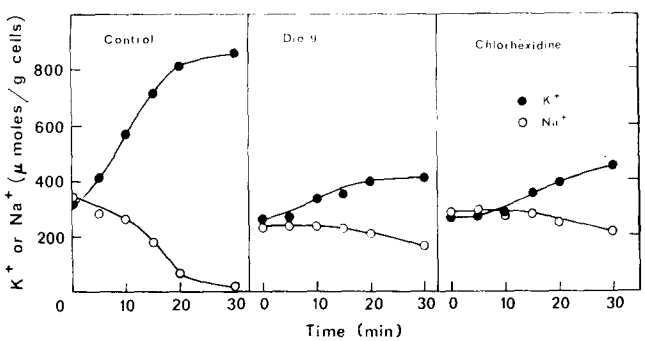
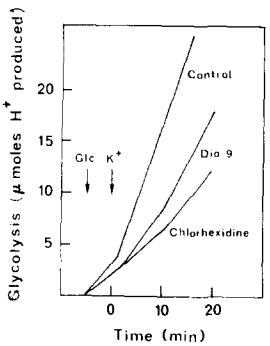


Fig. 2. Inhibition of net K^+ uptake by Dio 9 and Chlorhexidine. Cells grown on medium NaTY were resuspended in water at 1 mg/ml. Aliquots of 20 ml each were prepared; one received Dio 9 (10 μg/ml), one Chlorhexidine ($1.2 \cdot 10^{-5}$ M) while the third served as control. After 5 min glucose (4 mg/ml) was added to each, and glycolysis was allowed to proceed on the pH-stat, pH 7.5, for 5 min. At this point (designated 0 min), KCl was added to 1 mM.

Effect of Dio 9 and Chlorhexidine on integrity of the cytoplasmic membrane

A possible interpretation of the inhibition of net K^+ uptake by Dio 9 and Chlorhexidine might be that these compounds render the membrane permeable to K^+ and thus interfere with retention of K^+ by the cells. As shown in Table II, Dio 9 did not seriously impair the capacity of the cells to retain K^+, though there was some loss. Similar results had earlier been obtained with dicyclohexylcarbodiimide[9]. Dio 9 also did not induce leakage of [^{14}C]alanine from the acid-soluble pool of *S. faecalis*, nor did it promote lysis of protoplasts osmotically stabilized either by sucrose or by glycyl-

glycine. There is thus no evidence that Dio 9 has any marked effect on the integrity of the osmotic barrier.



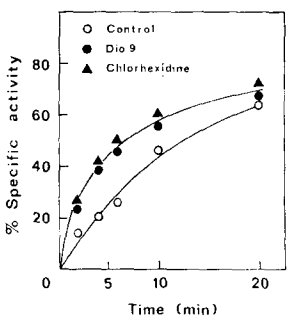


Fig. 3. Effect of Dio 9 and Chlorhexidine on the stimulation of glycolysis associated with net K^+ uptake. These cells were the same as those used for Fig. 2. Glycolysis was recorded automatically by the pH-stat and corrected for the volume of samples withdrawn.

Fig. 4. Effect of Dio 9 and Chlorhexidine on $^{42}K^+$–K^+ exchange. Cells loaded with K^+ were resuspended in water at 1 mg/ml and allowed to metabolize glucose (4 mg/ml) on the pH-stat at pH 7.5. $^{42}K^+$ (0.5 mM) of known specific activity was added at 0 min, and samples were taken at intervals for measurement of cellular K^+ and radioactivity. Dio 9 (10 μg/ml) or Chlorhexidine ($1.2 \cdot 10^{-5}$ M) were added 5 min before the $^{42}K^+$.

TABLE II

EFFECT OF DIO 9 AND CHLORHEXIDINE ON THE RETENTION OF K^+

S. faecalis was grown on medium KTY. The cells were loaded with K^+ as described under METHODS, washed and resuspended in 25 mM sodium maleate (pH 7.4) with additions as shown. Suspensions were incubated at room temperature.

Incubation conditions	*Inhibitor*	*Cellular cation (μmoles per g cells)*			
		0 min		*30 min*	
		K^+	Na^+	K^+	Na^+
Buffer only	None	865	90	675	110
	Dio 9 (10 μg/ml)			640	110
	Chlorhexidine ($1.2 \cdot 10^{-5}$ M)			435	145
Buffer *plus* glucose	None	865	90	740	35
	Dio 9 (10 μg/ml)			590	165
	Chlorhexidine ($1.2 \cdot 10^{-5}$ M)			390	220

The situation is more ambiguous with respect to Chlorhexidine, a surface-active compound known to disrupt bacterial membranes. Chlorhexidine did induce significant loss of K^+ from the cells (Table II); this may contribute to the inhibition of net K^+ uptake seen in Fig. 2, but can not account for it.

Effect of Dio 9 on ATP turnover

Since previous work has suggested that ATP is most probably the immediate energy donor for cation accumulation[7–9], the effect of Dio 9 on ATP turnover was

examined. Washed cells of *S. faecalis* have no internal energy reserves, and contain little ATP. Upon addition of glucose ATP is rapidly synthesized, reaching a steady-state level within 2 min. When the added glucose is exhausted, net breakdown of the ATP pool follows[8]. This normal pattern is shown in Fig. 5, together with the effects of Dio 9. The antibiotic had no consistent effect on the generation of ATP, nor did it

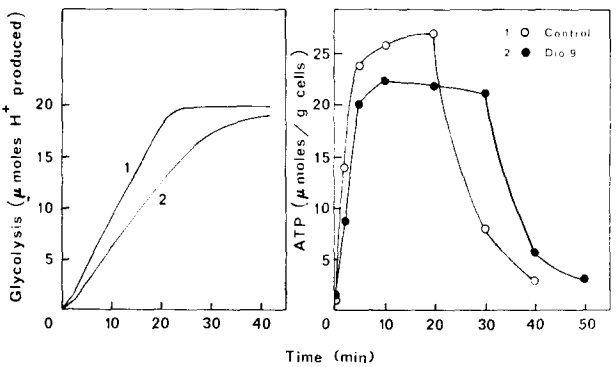


Fig. 5. Effect of Dio 9 on glycolysis and the ATP pool. Cells loaded with Na^+ were suspended in water at a density of 1 mg/ml, and placed on the pH-stat at pH 7.5. 1, to 20 ml, 10 μmoles glucose were added at 0 min; 2, 20 ml were preincubated with Dio 9, 10 μg/ml, for 5 min before addition of glucose.

prevent net degradation of the ATP pool. However, the onset of breakdown of the ATP pool was usually delayed, and the rate of glycolysis was consistently reduced by about 30%. We have chosen to illustrate results obtained with cells fully loaded with Na^+, since we know that Dio 9 inhibits net K^+ uptake by such cells. The retardation of ATP breakdown by Dio 9 was also seen in normal, K^+-loaded cells.

Chlorhexidine inhibited glycolysis to the same extent as did Dio 9, but we observed no effect on ATP turnover.

Other observations

Fresh cells of *S. faecalis* accumulate some L-[^{14}C]alanine even in the absence of glucose, but both the rate and extent of alanine uptake are stimulated by glycolysis[9]. As had previously been found for dicyclohexylcarbodiimide[9], Dio 9 and Chlorhexidine blocked the glucose-dependent component of alanine uptake but did not affect the portion which was independent of metabolism. However, Dio 9 and Chlorhexidine did not materially inhibit uptake of $^{32}P_i$.

Dio 9 stopped growth of *S. faecalis* in complex media (NaTY and KTY) at about 5 μg/ml. After addition of the inhibitor the cells continued to incorporate [^{14}C]leucine into protein at about half the control rate for nearly one generation. Incorporation of [^{14}C]uracil into the total cellular RNA was somewhat more strongly inhibited and soon ceased altogether. Chlorhexidine stopped growth at $2 \cdot 10^{-6}$ M, followed by slow lysis of the cells; macromolecule synthesis was not examined.

DISCUSSION

The hypothesis that the membrane-bound ATPase of *S. faecalis* is functionally related to cation transport rests entirely on indirect evidence. Its strongest support is

the finding that dicyclohexylcarbodiimide, a potent inhibitor of the membrane-bound ATPase, inhibits both ATP turnover and K^+ accumulation by intact cells[9]. In this report we describe two additional compounds, apparently unrelated in structure to dicyclohexylcarbodiimide, which elicit similar metabolic effects. Both Dio 9 and Chlorhexidine strongly inhibit the ATPase of isolated protoplast membranes, and prevent energy-dependent net uptake of K^+ by exchange for Na^+ and H^+ in intact cells. Curiously, and again like dicyclohexylcarbodiimide[9], Dio 9 and Chlorhexidine did not inhibit autologous exchange of $^{42}K^+$ for K^+. Neither Dio 9 nor Chlorhexidine appeared to inhibit the generation of ATP. It seems reasonable to propose that Dio 9 and Chlorhexidine inhibit the ATPase even in intact cells and that the block in K^+ uptake is a consequence thereof. This interpretation, which calls for a reduced rate of ATP turnover in the presence of the inhibitors, readily explains the partial inhibition of glycolysis by both agents. Unfortunately, we have been unable to demonstrate directly that Dio 9 or Chlorhexidine inhibit ATP degradation *in vivo*. The effect expected, of the order of 30%, may be too small to be detected by measurement of the total ATP pool; more sensitive procedures may be required to reveal the predicted inhibition of turnover.

Even though their effects on intact cells are similar, the site of action of Dio 9 and Chlorhexidine is probably not identical with that of dicyclohexylcarbodiimide. The latter inhibits membrane-bound ATPase but not the solubilized enzyme[9]. It is quite clear that dicyclohexylcarbodiimide reacts with an unidentified component of the membrane and that it inhibits the ATPase indirectly, perhaps by a transmitted effect on the conformation of the enzyme[9–12]. By contrast, Dio 9 and Chlorhexidine inhibit both the membrane-bound and the solubilized ATPase of *S. faecalis* membranes. SCHATZ, PENEFSKY AND RACKER[15] have previously noted that Dio 9 inhibits the solubilized ATPase of yeast mitochondria. Probably, therefore, Dio 9 and Chlorhexidine react directly with the ATPase protein. It may be significant in this context that Dio 9 and Chlorhexidine, unlike dicyclohexylcarbodiimide, did not inhibit the membrane-bound NADH dehydrogenase of *S. faecalis*.

On the surface, the ATPase of mitochondrial membranes appears functionally very different from the ATPase associated with the plasma membrane of fermentative cells such as erythrocytes or *S. faecalis*. The former is thought to catalyze the terminal step in oxidative phosphorylation (review: ref. 5); the latter apparently mediates the utilization of glycolytically generated ATP for membrane transport. Yet it has been known for some time that oligomycin, the classical inhibitor of ATPase and oxidative phosphorylation in mitochondria, inhibits also ATPase and cation transport in erythrocytes[26]. The present observations with Dio 9 and Chlorhexidine, as well as our earlier work with dicyclohexylcarbodiimide[9], add to the growing evidence that a fundamental unity underlies processes apparently as diverse as oxidative phosphorylation in mitochondria and cation transport by *S. faecalis*. Similarities at the molecular level between the ATPase of mitochondria and of *S. faecalis*[2,4] point in the same direction.

Dio 9, Chlorhexidine and also dicyclohexylcarbodiimide all stop the growth of *S. faecalis* at concentrations comparable to those which inhibit the ATPase and energy-dependent transport of K^+ and of alanine. The cessation of growth may thus be a consequence of the inhibition of energy-linked transport processes. The precise role of the ATPase in membrane functions remains to be determined.

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