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Mechanism of Action of EM 49, Membrane-Active Peptide Antibiotic

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EM 49 (recently renamed octapeptin) is a membrane-active peptide antibiotic that has been reported to affect the structure of bacterial membranes (K. S. Rosenthal, P. E. Swanson, and D. R. Storm, Biochemistry 15:5783-5792, 1976). In this study, it is shown that the effects of EM 49 on bacterial metabolism are similar to those of uncouplers of oxidative phosphorylation. EM 49 stimulated bacterial respiration within a narrow concentration range corresponding to minimum inhibitory concentrations and inhibited respiration at concentrations comparable to minimum biocidal concentrations. In addition, the peptide increased membrane proton permeability and lowered the adenosine 5'-triphosphate pool size. Parallel studies done with the related antibiotic polymyxin B demonstrated that the two peptides differed considerably in their effects on bacterial respiration. In contrast to EM 49, polymyxin B did not stimulate respiration at any concentration. It is proposed that the primary action of EM 49 is to disrupt the selective ion permeability of the cytoplasmic membrane, thereby relaxing the membrane potential.

A number of membrane-active antibiotics have served as useful tools for studying the structure and function of biological membranes (13, 21, 24). Recently, a new peptide antibiotic, EM 49 (recently renamed octapeptin), has been isolated (17) that disrupts membrane structure (19, 22). EM 49 resembles the polymyxins structurally; however, the antimicrobial spectra of these peptides are significantly different. Both antibiotics are cyclic peptides containing a high percentage of 2,4-diaminobutyric acid with a fatty acid attached through an amide linkage. However, the polymyxins are decapeptides containing a C:8 or C:9 fatty acid, and EM 49 is an octapeptide containing a C:10 or C:11 β-hydroxy fatty acid.

In this report, it is shown that the actions of EM 49 on bacterial metabolism are analogous to those of uncouplers of oxidative phosphorylation (29). The permeability of bacterial membranes with respect to protons is rapidly increased by EM 49, and bacterial respiration is enhanced at concentrations of the antibiotic corresponding to minimum inhibitory concentrations. At higher concentrations of the peptide, comparable to minimum biocidal concentrations, respiration is inhibited. Previous studies with immobilized EM 49 and polymyxin B have established that these antibiotics can affect bacterial respiration and growth by interacting with the outside surface of membranes (12). Therefore, the mechanism for EM 49 action must be distinct from uncouplers, such as carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) or ionophores (e.g., valinomycin), which are capable of diffusing back and forth across membranes (4, 21).

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MATERIALS AND METHODS

Materials. EM 49 as well as *Escherichia coli* strains SC 9251, SC 9252, and SC 9253 were supplied by the Squibb Institute for Medical Research. *Bacillus subtilis* GSY 201 was obtained from George Ordal. CCCP, dicyclohexylcarbodiimide (DCCD), dessicated firefly tails, and polymyxin B sulfate were purchased from Sigma Chemical Co. Synthetic medium contained 50 mM potassium phosphate, 0.12 mM $MgCl_2$, 1 mM $(NH_4)_2SO_4$, 20 mM sorbitol, and 1 g of casein hydrolysate per liter at pH 7.0. Minimal medium was that described by Davis and Mingioli (2), with sorbitol substituted for glucose. Low-ionic-strength medium was synthetic medium diluted to 40% with distilled water.

Minimum growth-inhibitory concentration and minimum biocidal concentration determinations for aerobic growth. Antimicrobial activities were determined in shaker culture. Antibiotics at varying concentrations were added to 10 ml of low-ionic-strength medium, 0.1 ml of a late log-phase inoculum was added, and the culture was incubated for 18 h at 37°C. Since the growth and respiration effects were sensitive to ionic strength, media of comparable ionic strength were used for both of these studies. Minimum growth-inhibitory concen-

tration is defined as the minimum concentration of antibiotic that completely prevented growth of the bacteria, as monitored by light scattering with a Klett spectrophotometer. Minimum biocidal concentration is defined as the minimum concentration of antibiotic at which there were no viable cells after 18 h at 37°C. Viable cell count was determined by serial dilution and plating on nutrient agar.

Antibiotic activity under anaerobic conditions. Media were prepared anaerobically by first boiling and then aspirating the solution for 30 min with nitrogen. Culture tubes were filled and sealed in an anaerobic hood. An N_2-CO_2 mixture was utilized in the culture tubes. The inoculum was prepared by transferring *E. coli* SC 9251 three times under anaerobic conditions. Antibiotic and 0.1 ml of the anaerobic *E. coli* inoculum were added, and growth was monitored by light scattering at several time intervals up to 72 h.

Preparation of spheroplasts. Spheroplasts were prepared by the lysozyme-ethylenediaminetetraacetate method (9). Before use, spheroplasts were washed twice in minimal medium containing 20% sucrose and then suspended in this same solution at a density of 80 Klett units. The extent of spheroplast formation was monitored by phase-contrast microscopy and osmotic sensitivity upon dilution into distilled water. The efficiency of spheroplast formation was estimated to be greater than 98%.

Proton influx after antibiotic treatment. Proton flux was monitored by the method of Feingold (3), with several modifications. *E. coli* was grown to late log phase in nutrient medium. The cells were harvested at 3,000 × *g* at 25°C for 15 min and washed twice with 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.0, to increase the intracellular pH. The cells were then washed twice in 50 mM KCl and suspended in the same solution at a cell density of approximately 3×10^{10} cells per ml. The cell suspensions were stirred, and the pH was monitored continuously. Before each experiment, the pH of a fresh cell suspension was brought to approximately pH 6.0 with 0.1 M HCl and allowed to remain at this pH for 2 min. With the cell suspension at pH 6.0, polymyxin B, EM 49, DCCD, or CCCP was added at various concentrations. These reagents alone did not change the pH of a 50 mM KCl solution.

Measurement of oxygen uptake. *E. coli* and *B. subtilis* were grown to early log phase (80 to 110 Klett units) in synthetic medium and diluted to 35 Klett units with distilled water. Spheroplasts, prepared as described below, were suspended in minimal medium containing 20% sucrose at a density of 85 Klett units. Five milliliters of the bacterial or spheroplast suspension was saturated with O_2 by gently bubbling air through the solution. Oxygen uptake was monitored at 37°C in a stirred cell with a Clark O_2 electrode (Yellow Springs Instrument Co.). The rate of oxygen uptake was monitored for several minutes until a linear rate was established, and then a solution of antibiotic in the same medium was added and the rate of oxygen utilization was continually monitored. Inhibition of the respiration rate is expressed as the change in slope relative to the initial slope in the absence of added antibiotic. Unless otherwise indicated, the extent of respiration inhibition is reported for 0.2 min after addition of the peptide. The dilutions affected by addition of antibiotic were negligible, and addition of buffer or growth medium containing no antibiotic was without effect upon the rate of oxygen uptake.

Measurement of ATP pool sizes. Adenosine 5'-triphosphate (ATP) levels were measured by the firefly luciferase assay (10). These assays were performed in standard scintillation vials. Measurement of ATP-induced luminescence was started 20 s after initiation of the assay and was monitored for 30 s with a Beckman LS scintillation counter. ATP standards were prepared fresh with each set of determinations, and the ATP assay cocktail was stable for 3 h. All determinations were done in triplicate, with a standard error of 5% or less. For each experiment, *B. subtilis* cultures were sequentially inoculated every 20 min with a 1% inoculum. Cells were then allowed to grow for 4 to 6 h, to a density of 2.5×10^8 cells per ml, before treatment with EM 49 or polymyxin. Viable cell counts were determined before and after each experiment. Samples of 0.1 ml were removed at various time intervals after addition of EM 49 or polymyxin B and were diluted into 0.4 ml of dimethyl sulfoxide (25°C) followed by addition of 2.5 ml of ATP assay buffer (4°C) 2 min later. ATP assay buffer contained 5 mM $NaAsO_4$, 20 mM glycylglycine, pH 8.0, and 4 mM $MgSO_4$. The samples were stored at 4°C until they were assayed. In one set of experiments, extracellular ATP was determined after treatment with 20 μg of EM 49 per ml. These samples were filtered through a 0.22-μm filter to remove bacteria. For experiments examining synergism between EM 49 and DCCD or CCCP, the peptide was introduced 6 min after the other reagent. This time period was sufficient for the primary reagent to elicit its effect.

Assay for *B. subtilis* chemotaxis. The microscope assay of Ordal was used to study the actions of EM 49 and polymyxin B on the motility of *B. subtilis* (18).

RESULTS

Effects of EM 49 on bacterial growth and respiration. Treatment of *E. coli* or *B. subtilis* with low concentrations of EM 49 resulted in rapid changes in their rates of respiration. Three strains of *E. coli* were examined, SC 9251 and two polymyxin-resistant strains derived from this strain, SC 9252 and SC 9253. In addition, the effects of EM 49 on the respiration of *B. subtilis* GSY 201 were also examined. EM 49 was biostatic at concentrations of less than 2 μg/ml and biocidal at higher concentrations (Table 1). The peptide stimulated bacterial respiration at low concentrations, corresponding to minimum inhibitory concentrations, and inhibited respiration at higher levels, comparable to minimum biocidal concentrations.

TABLE 1. *Effects of EM 49 and polymyxin B on bacterial respiration*

Antibiotic	Strain	Concn (μg/ml)[a]			
		Minimum inhibitory[b]	Optimum for respiration stimulation[c]	Minimum biocidal	Minimum for respiration inhibition[d]
EM 49	*E. coli* SC 9251	1.7 ± 0.1	2.0 ± 1.0	4.5 ± 0.2	4.6 ± 1.0
	E. coli SC 9252	0.8 ± 0.2	0.5 ± 0.1	1.3 ± 0.3	1.5 ± 0.2
	E. coli SC 9253	0.3 ± 0.1	0.6 ± 0.1	1.0 ± 0.2	1.2 ± 0.2
	B. subtilis GSY 201	0.2 ± 0.1	0.2 ± 0.1	1.2 ± 0.1	0.7 ± 0.2
Polymyxin B	*E. coli* SC 9251	1.2 ± 0.1		3.0 ± 1.0	0.3 ± 0.1
	E. coli SC 9252	>100		>200	>200
	E. coli SC 9253	>200		>200	>200
	B. subtilis GSY 201	0.6 ± 0.1		0.9 ± 0.1	0.3 ± 0.1

[a] Average of three or more determinations.

[b] Minimal inhibitory concentration of the peptide that completely inhibited bacterial growth.

[c] Optimal concentration of the peptide for stimulation of the rate of respiration. The rate of respiration was stimulated by EM 49 over a limited concentration range (Fig. 2) but not by polymyxin B at any concentration.

[d] Minimal concentration of the peptide to inhibit the rate of respiration by 10% after 0.2 min of treatment.

Interaction of the peptide with bacteria was quite rapid. Changes in the rate of respiration were detected within 10 to 15 s after addition of EM 49 (Fig. 1). The effects of EM 49 on bacterial respiration varied qualitatively and quantitatively with peptide concentration. For example, at concentrations less than the minimum biocidal concentrations, EM 49 increased the rate of respiration. At concentrations equivalent to or greater than the minimum biocidal concentrations, the antibiotic inhibited respiration (Fig. 2). The maximum stimulation of the respiration rate was 15 to 20%, with 100% inhibition occurring at higher concentrations of the peptide. The kinetics for inhibition of *B. subtilis* respiration by EM 49 were first order. However, with *E. coli*, plots of the log of the extent of respiration inhibition versus time were not linear and could be fit by two or more straight lines. In addition, inhibition of respiration of *B. subtilis* compared with that of *E. coli* was generally more rapid and occurred at lower concentrations of EM 49. These differences between the susceptibilities of *E. coli* and *B. subtilis* to EM 49 may be due to the *E. coli* outer membrane system, which can function as a barrier for some antibiotics (14).

The data described above suggested that the primary mechanism for EM 49 antibiotic activity might involve perturbation of bacterial respiration. To test this hypothesis, the minimum inhibitory concentration of EM 49 for *E. coli* SC 9251 was determined under anaerobic conditions. The minimum inhibitory concentration was identical under anaerobic and aerobic conditions, 1.7 μg/ml. This suggests that the effects of EM 49 on respiration cannot be the primary killing mechanism. The peptide does, however, alter membrane permeability with respect to several ions, including protons and potassium (discussed below). These changes in membrane permeability could explain the effects of the peptide on bacterial respiration and

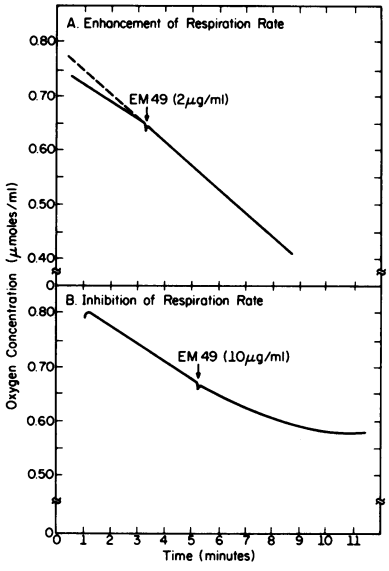

FIG. 1. *Effect of EM 49 on respiration of E. coli SC 9251. E. coli SC 9251, grown to early log phase in synthetic medium, was diluted to 35 Klett units with distilled water. The initial respiration rate of the bacterial suspension was established (2 to 3 min), and then EM 49 was added. The bacteria were treated with 2 (A) or 10 (B) μg of EM 49 per ml. The change in respiration rate 0.2 min after treatment with 2 μg of EM 49 per ml was an enhancement of 20%. Treatment of E. coli with 10 μg of EM 49 per ml inhibited respiration within 10 s, and complete inhibition occurred after 5 min.*

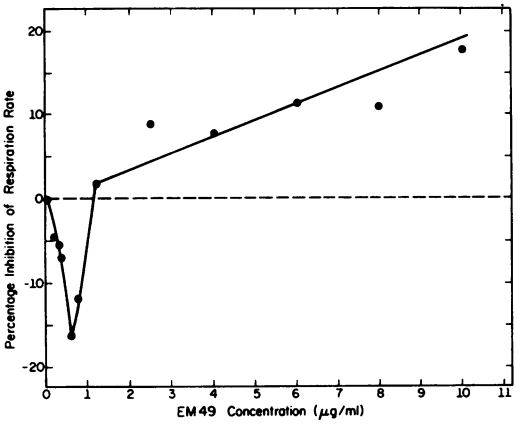

FIG. 2. *EM 49 concentration dependence for the initial change in respiration rate of E. coli SC 9253. E. coli SC 9253 was treated with varying concentrations of EM 49, and the rates of oxygen consumption were monitored as described in the text. The change in respiration rate 0.2 min after treatment with EM 49 is reported. In the concentration range of 0.25 to 1.25 μg of EM 49 per ml, the rate of O_2 consumption was enhanced; at higher concentrations, the peptide inhibited respiration.*

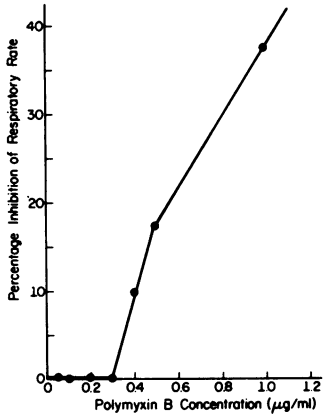

FIG. 3. *Polymyxin B concentration dependence for inhibition of E. coli SC 9251 respiration. Inhibition of the respiration rate was determined as described in the text, and the reported values are for 0.2 min after treatment with polymyxin B. No stimulation of respiration was observed at any concentration of polymyxin B.*

the equivalent biostatic concentrations under anaerobic and aerobic conditions. Even under anaerobic conditions, an intact membrane permeability barrier is required, and the membrane potential is maintained by the Ca^{2+}, Mg^{2+}-adenosine triphosphatase (ATPase) (20).

Effects of polymyxin B on bacterial growth and respiration. Polymyxin B and EM 49 affected bacterial respiration and growth quite differently. For example, *E. coli* SC 9252 and SC 9253 were polymyxin resistant but very sensitive to EM 49 (Table 1). EM 49 stimulated respiration at low concentrations; polymyxin B did not. Polymyxin B exhibited a concentration-dependent inhibition of bacterial respiration that correlated, to a certain extent, with minimum inhibitory and biocidal concentrations (Table 1).

Treatment of *E. coli* SC 9251 or *B. subtilis* GSY 201 with concentrations of polymyxin B greater than 0.3 μg/ml inhibited respiration within 10 to 15 s (Table 1). Although the growth of *E. coli* SC 9251 was equally susceptible to EM 49 and polymyxin B (minimum inhibitory concentrations were 1.7 and 1.2 μg/ml, respectively), EM 49 stimulated respiration at these concentrations. The extent of respiration inhibition caused by polymyxin B after 0.2 min of treatment increased with peptide concentration (Fig. 3).

The respiration of the two polymyxin B-resistant strains, *E. coli* SC 9252 and SC 9253, was unaffected by polymyxin B up to 200 μg/ml (Table 1). Again, the correlation between the concentration dependence for antibiotic activity and effects upon bacterial respiration suggested either a causal relationship or a common mode of inhibition.

Effects of EM 49 and polymyxin B on spheroplast respiration. The cytoplasmic membrane has been implicated as a target for both polymyxin B (23, 28) and EM 49 (22), although both peptides can inhibit growth and respiration of gram-negative bacteria solely by interaction with outer membranes (12). To directly study the interactions between these peptides and cytoplasmic membranes, the influence of polymyxin B and EM 49 on spheroplast respiration was examined. In addition, the role of the outer membrane as a determinant for polymyxin resistance was evaluated by comparing spheroplasts made from *E. coli* SC 9251, SC 9252, and SC 9253. The latter two strains are resistant to polymyxin B.

Treatment of spheroplasts with EM 49 affected their rates of oxygen uptake in a way analogous to its effect whole cells. The rate of respiration for *E. coli* SC 9251 and SC 9253 spheroplasts was stimulated 5 to 10% at concentrations of EM 49 in the range of 1 to 2 μg/ml (Fig. 4A). The effects of EM 49 on SC 9252 spheroplast respiration were not examined in this concentration range. At higher concentrations of the peptide, the rates of respiration were inhibited to an extent that depended upon EM 49 concentration. As with whole cells, the changes in O_2 uptake were rapid. The data in

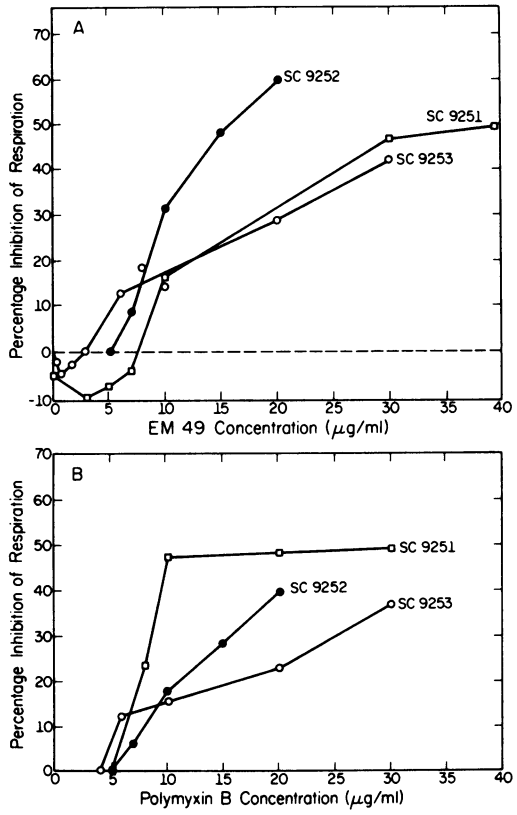

FIG. 4. *Concentration dependence for the effects of EM 49 (A) and polymyxin B (B) on spheroplast respiration. Spheroplasts were prepared from E. coli SC 9251 (□), SC 9252 (●), and SC 9253 (○) by the method of Kaback (9). Before use, the spheroplasts were washed twice with minimal medium containing 20% sucrose and suspended in the same medium at a density of 80 Klett units. The rates of respiration were determined as described in the text, and the percentages of inhibition or stimulation of respiration after 0.2 min of treatment with the peptides are reported.*

Fig. 4 represent the change in respiration rate after 0.2 min of treatment.

Polymyxin B had no effect on the growth or respiration of *E. coli* SC 9252 or SC 9253 at concentrations of the peptide below 100 μg/ml (Table 1). In contrast, the respiration of spheroplasts from all three strains was quite susceptible to polymyxin B (Fig. 4B). At concentrations greater than 5 μg/ml, respiration was rapidly inhibited, and the extent of inhibition increased with polymyxin concentration. The concentration dependence for inhibition of spheroplast respiration cannot be directly compared to that for whole cells, since 20% sucrose was present for stabilization of the spheroplasts. High concentrations of sucrose have been observed to influence the effects of EM 49 and polymyxin B on bacterial respiration (K. S. Rosenthal and D. R. Storm, unpublished data). Although the respiration of all three spheroplast preparations was inhibited by polymyxin B at concentrations as low as 5 μg/ml, SC 9251 spheroplasts were still more susceptible to polymyxin B than were SC 9252 or SC 9253 spheroplasts. These data indicate that the resistance of *E. coli* SC 9252 and SC 9253 respiration to polymyxin B was due, at least in part, to their outer membranes.

Increased proton permeability. It has been previously reported that polymyxin B (28) affects the selective permeability of bacterial membranes and stimulates the release of cytoplasmic components into the media. Because of the proposed role of the membrane proton potential for oxidative phosphorylation (30), the influence of EM 49 on membrane proton permeability was examined. An artificial proton potential was established by preincubating *E. coli* SC 9251 in a pH 8.0 tris(hydroxymethyl) aminomethane-hydrochloride buffer to increase the intracellular pH, followed by washings and resuspension in unbuffered 50 mM KCl at pH 6.0. Addition of EM 49 at 15 μg/ml resulted in an increase of 0.12 pH units (Fig. 5). At the density of cells used in these experiments (3×10^{10} cells per ml), the minimum concentration of EM 49 at which measurable proton flux could be detected was 10 μg/ml. The peptide concentration dependence for this phenomenon cannot be directly compared to that for inhibition of growth or respiration since the density

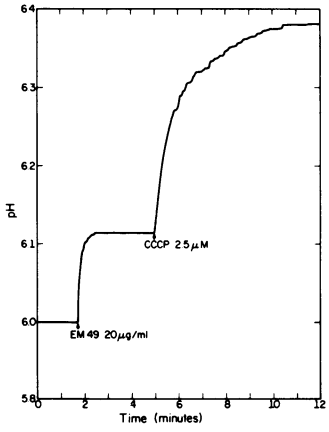

FIG. 5. *Proton efflux upon EM 49 treatment. The pH of a cell suspension, prepared as described in the text, was monitored before and after addition of 20 μg of EM 49 per ml. CCCP, dissolved in ethanol, was added to a concentration of 2.5 μM. Ethanol by itself had no effect on the pH of the cell suspension.*

of cells used in the latter experiments was several orders of magnitude lower. The weak organic acid uncoupler CCCP caused a change of 0.4 pH units under identical conditions. If the pH change mediated by CCCP represents complete relaxation of the proton gradient, then EM 49 was only partially effective in this respect. When the membrane potential is relaxed by an uncoupler, the cells attempt to maintain the proton gradient through ATP hydrolysis catalyzed by the Ca^{2+},Mg^{2+}-ATPase (18, 27). If it is assumed that the Ca^{2+},Mg^{2+}-ATPase counteracts the effects of EM 49, then inhibition of this enzyme should enhance the proton flux stimulated by EM 49. DCCD is a potent inhibitor of the Ca^{2+},Mg^{2+}-ATPase (5). Pretreatment of cell suspensions with 12.5 mM DCCD resulted in a proton flux of 0.08 pH units (Fig. 6A). Upon addition of EM 49 to DCCD-pretreated cells, there was an additional increase of 0.3 pH units. A similar effect was obtained when the order of these two reagents was reversed, and CCCP had no additional effect (Fig. 6B). Thus, synergism between DCCD and EM 49 gave a proton flux comparable to CCCP. Addition of CCCP to cells pretreated with DCCD gave the expected results; i.e., a net change of 0.4 pH units. These observations support the proposal that the increase in proton permeability stimulated by EM 49 is partially counteracted by the Ca^{2+},Mg^{2+}-ATPase. It would be predicted on the basis of the observations cited above that EM 49 treatment should depress intracellular levels of ATP. The antibiotic caused a rapid decrease in *B. subtilis* ATP concentrations (Fig. 7). The ATP concentrations of untreated controls increased somewhat during the course of these experiments; however, this was due to continued bacterial growth. At 15 μg/ml, EM 49 depressed ATP levels 85% compared with untreated controls. Because of the sensitivity of this assay for ATP, it was necessary to use relatively concentrated suspensions of bacteria (2.5×10^8 cells per ml). Therefore, the EM 49 concentration dependence for effects on ATP pool size cannot be directly compared to the concentration dependence for effects on bacterial respiration or growth inhibition. The latter determinations were made with bacterial suspensions of 10^5 to 10^6 bacteria per ml. A measurable drop in ATP concentrations occurred within 1 to 2 min after addition of EM 49. In one set of experiments, extracellular ATP was determined after removal of bacteria by filtration. No extracellular ATP was detected with EM 49-treated *B. subtilis*. When bacterial cells were pretreated with DCCD and then EM 49, the rate of ATP decline was three times less than that with EM 49 alone. EM 49 had analogous effects on the *E. coli* ATP pool size. This result is consistent with the proposal that relaxation of the membrane potential by EM 49 stimulates ATP hydrolysis by the Ca^{2+},Mg^{2+}-ATPase. EM 49 treatment of *B. subtilis* also stimulated the release of K^+. However, the significance of this effect is difficult to evaluate since a relatively minor percentage of the total cellular K^+ was released. For example, approximately 2% of

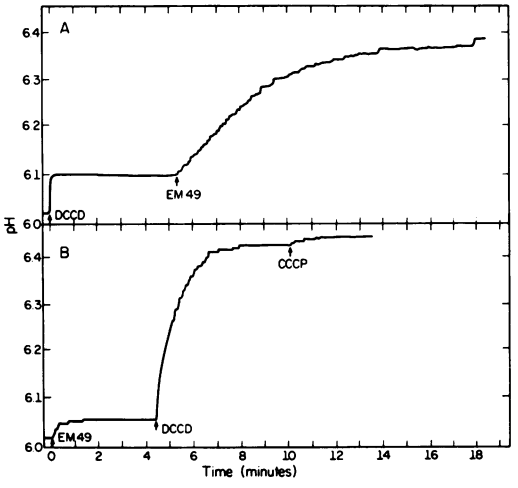

FIG. 6. *Synergistic effects of EM 49 and DCCD on proton efflux. The pH of a cell suspension, prepared as described in the text, was treated first with: (A) DCCD (12.5 mM) followed by EM 49 (10 μg/ml); (B) EM 49 (10 μg/ml) and then 3 min later with DCCD (12.5 mM). CCCP was added after 10 min in experiment (B).*

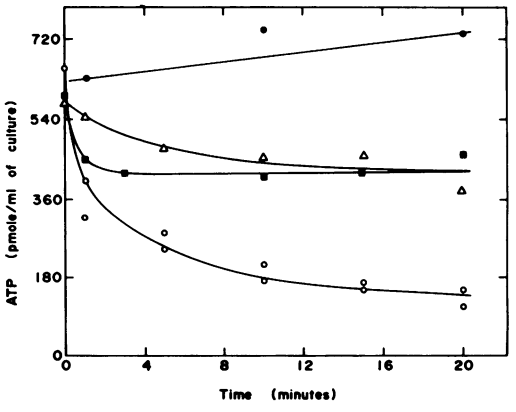

FIG. 7. *Decrease in ATP pool size of B. subtilis by EM 49 or CCCP treatment. B. subtilis GSY 201 was untreated (●) or treated with 10 (△), or 15 (○) μg of EM 49 per ml or with CCCP at 0.25 and 2.5 μM (■) after 5 h of growth. Aliquots of the cultures were removed and assayed as described in the text.*

the intracellular K^+ was released after 20 min of treatment with EM 49 at 20 μg/ml.

Effect of EM 49 on the motility of *B. subtilis*. Recent studies have demonstrated the importance of the bacterial membrane potential for *B. subtilis* chemotaxis (18, 26). Uncouplers, such as CCCP, cause a transient period of tumbling, which is also elicited by chemotactic repellents. EM 49, but not polymyxin, caused *B. subtilis* to tumble. This response occurred only at EM 49 concentrations in the range of 0.2 to 0.9 μg/ml, which compared favorably with the concentrations of EM 49 that stimulated respiration. Higher concentrations of EM 49, or polymyxin greater than 0.5 μg/ml, completely inhibited bacterial motility.

DISCUSSION

Bacterial oxidative phosphorylation occurs in the cytoplasmic membrane and is dependent upon the integrity of the membrane for maintenance of a transmembrane proton gradient. Alteration of membrane permeability can affect both respiration and ATP production. For example, an uncoupler such as CCCP, dinitrophenol, or trifluoromethoxycarbonylcyanidephenylhydrazone relaxes the membrane potential (16), increases the rate of respiration (4), inhibits ATP production (30), stimulates ATP hydrolysis by the Ca^{2+}, Mg^{2+}-ATPase (18, 27), and influences bacterial chemotaxis (18). EM 49 affected bacterial metabolism in a similar manner. However, unlike the weak organic acids mentioned above, EM 49 uncoupled oxidative phosphorylation only within a narrow concentration range of the peptide. At higher concentrations, EM 49 inhibited respiration and motility and caused more extensive permeability changes. The effects at EM 49 on bacterial respiration were quite rapid and correlated with the biological activities of the peptide. The primary effect of EM 49 appears to be disruption of the membrane permeability barrier. Under comparable conditions, the earliest detectable changes in proton permeability, rates of oxygen consumption, and ATP concentration were observed at 5, 10, and 60 s, respectively. However, the sensitivity of the assays used to follow these various parameters varied considerably, which imposes serious limitations on comparative kinetic studies.

Although the mechanism for alteration of membrane permeability by EM 49 or polymyxin has not been defined, certain aspects of this process are now more clearly understood. These antibiotics bind to and disrupt the structure of phospholipid and lipopolysaccharide aggregates (4, 15, 22). The permeability of phospholipid liposomes for polar molecules has been shown to be increased by polymyxin B (7, 8). Inhibition of bacterial growth, binding of the antibiotics to membranes and lipids, and perturbation of membrane structure are all specifically inhibited by Ca^{2+} and Mg^{2+} ions (6, 11, 22, 25). These observations suggest that polymyxin and EM 49 may competitively displace magnesium or calcium from negatively charged phosphate groups on membrane lipids. In addition, the fatty acids attached to polymyxins or EM 49 are crucial for antibiotic activity, indicating that the fatty acid tails may insert into the hydrophobic domain of the membrane (25). The combination of these interactions may be sufficient to alter packing of the membrane lipid bilayer, thereby increasing the rates of diffusion of polar or charged molecules across the membrane. At concentrations of EM 49 exceeding the minimum inhibitory concentration, there are general membrane permeability changes with the efflux of cytoplasmic components (22). However, at lower concentrations of EM 49, corresponding to minimal inhibitory concentrations, which result in stimulation of respiration, these general permeability changes were not detected. It is proposed that low concentrations of EM 49 cause structural changes within the lipid bilayer sufficient to allow leakage of protons, resulting in relaxation of the membrane proton gradient. Inhibition of respiration at higher concentrations of EM 49 or polymyxin B may reflect extensive membrane damage, resulting in the efflux of metabolites used for reducing potential in electron transport or disorganization of the electron transport chain.

Although EM 49 and polymyxin B have similar structures, their biological activities and effects on respiration were quite distinct. The most striking contrast between the two peptides was the stimulation of O_2 uptake at low concentrations of EM 49 that was not observed at any concentration of polymyxin B. Presumably, these differences in antibiotic activity and effects on O_2 uptake are attributable to the structural differences between the two antibiotics. In this respect, it is notable that polymyxin B is a decapeptide with a covalently attached C:8 or C:9 fatty acid and that EM 49 is an octapeptide containing a β-hydroxy C:10 or C:11 fatty acid. Both peptides are polycations containing five to six diaminobutyric acid residues; however, phenylalanine and threonine residues in polymyxin B are replaced by leucine residues in EM 49 (19). Which of these structural differences is responsible for the differences in activity between EM 49 and polymyxin B is not known. However, the fact that rela-

tively minor structural alterations produced significant changes in the activities of these peptides with respect to bacterial respiration suggests that their interactions with membranes may involve some degree of specificity.

It is quite interesting that the respiration of the two polymyxin-resistant strains, *E. coli* SC 9252 and SC 9253, was insusceptible to polymyxin B, whereas their corresponding spheroplasts were very susceptible to the peptide. This indicates that the polymyxin resistance of these two strains was partially due to their outer membranes. The role of the outer membrane as a barrier, particularly for specific antibiotics, is well established (14), and these data support the implied barrier function of the gram-negative outer membrane system. Compositional analysis of membranes from *E. coli* SC 9251, SC 9252, and SC 9253 have shown several compositional changes, none of which correlated clearly with polymyxin resistance (22). In summary, these peptides are useful tools for studying the structure and function of bacterial membranes and provide interesting model systems for examining lipid-peptide interactions.

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