Suppression of Tricarboxylic Acid Cycle in *Escherichia coli* Exposed to Sub-MICs of Aminoglycosides

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The metabolic activity of *Escherichia coli* ATCC 25922 challenged with sub-MICs of aminoglycosides was analyzed with a batch calorimeter. High-performance and gas-liquid chromatographic techniques were utilized to evaluate the concentrations of metabolic reactants, intermediates, and end products. The data reported indicate that aminoglycosides inhibit or delay bacterial catabolism of carboxylic acids, with the following relative degrees of activity: amikacin > gentamicin > sisomicin > netilmicin > kanamycin. The decrease in total biomass production was proportional to the degree of tricarboxylic acid cycle inhibition.

Since the discovery of streptomycin in 1944, the mechanism of action of aminoglycoside antibiotics has attracted considerable interest. Studies have revealed that a complex set of conditions may influence their uptake and pleiotropic effects (14, 19, 20).

The uptake of these antibiotics is demonstrated to be dependent on different mechanisms: an initial energy-independent phase associated with ionic binding to the bacterial cell surface, followed by slow (EDP1) and fast (EDP2) energy-dependent steps. Binding of aminoglycosides to ribosomes is required for initiation of the latter phase (8, 10, 11, 19, 20, 33).

Electron transport through the respiratory chain has been shown to provide the energy required for the energy-dependent uptake (1, 2, 10–12, 28). Although the transmembranous electrical potential (ΔΨ) is indicated as the driving force for aminoglycoside entry, quinone-deficient mutants with a normal ΔΨ have a significantly lower uptake of gentamicin (9). These studies support the view that electron transport may have some specific role other than its requirement to produce cellular ΔΨ.

In this investigation, we utilized the calorimetric technique to analyze the interplay between aminoglycosides and respiratory chain activity.

Extensive data have been gathered on microbial growth and metabolism by using calorimetry (4, 6, 7, 22–24, 29). This technique has also demonstrated its usefulness in investigations of the activities of various antibacterial and antifungal drugs (3, 13, 21, 25, 26, 31, 32). Most of these studies, however, did not correlate different power contributions with different catabolic pathways in the bacterial cell. If no relationship between these two is established, measurement of power production represents merely a non-specific parameter of bacterial viability and thus conveys no information on the antibiotic effect at the biochemical level.

In this investigation, each component of the thermograms produced by *Escherichia coli* growing in Mueller-Hinton broth was correlated to the sequential activation of the catabolic pathways operating in the bacteria (27).

Our findings indicate that aminoglycosides inhibit carboxylic acid catabolism and electron flow through the respiratory chain.

MATERIALS AND METHODS

*Bacterial strain. E. coli* ATCC 25922 (Bactrol disks; Difco Laboratories, Detroit, Mich.) was utilized. Several other strains isolated from clinical specimens were also tested.

**MIC determination.** The MICs of gentamicin, sisomicin, amikacin, kanamycin, and netilmicin were evaluated by the macrotube dilution procedure (34). Fresh dilutions of each antibiotic were made in Mueller-Hinton broth to achieve final concentrations of 0.06 to 32 μg/ml. Bacterial counts were adjusted to 10⁵ CFU/ml by turbidity determinations.

**Microcalorimetric analysis.** *E. coli* ATCC 25922, grown overnight at 37°C in Mueller-Hinton broth, was diluted to 10⁵ CFU/ml and then exposed to graded concentrations of gentamicin, sisomicin, amikacin, kanamycin, and netilmicin. The levels of these antibiotics ranged from 1/32 the MIC to 1/2 the MIC. The bacterial suspension (1 ml) was then placed into each vessel for calorimetric recording. The thermic behavior of the strain under anaerobic conditions was evaluated by preparing the bacterial suspension in an anaerobic incubator or by covering the final aerobic suspension with a layer of mineral oil. The heat effect was evaluated by a batch-type microcalorimeter (2277 BioActivity monitor; Thermostraf-Micromat, Sweden), with a thermostat setting of 37°C. The voltage signal was amplified at a sensitivity of 1 mW. The heat produced was measured by integrating the recorded power-time (PT) curves. Growth curves were constructed employing an MS-2 research system apparatus (Abbott Laboratories, North Chicago, Ill.).

**Analysis of metabolic products by HPLC.** The biochemical analysis of the metabolic compounds produced during bacterial growth employed high-performance liquid chromatography (HPLC) with a modular system (Gynkotek, Munich, Federal Republic of Germany) connected to an integrator (model 4290; Varian, Walnut Creek, Calif.). The analyses were performed without pretreatment extraction (18) as follows: the growth medium was collected at different times of the thermal curves; the cells were removed by centrifugation in a microfuge for 5 min at 9,000 × g; 20 μl of the sample was injected into an HPX-87H cation exchange column (300 by 7.8 mm), Aminex) for organic acids (Bio-Rad Laboratories, Richmond, Calif.). The instrument was first calibrated by injecting a standard mixture containing 3 mM α-ketoglutaric acid, 6 mM pyruvic acid, 6 mM succinic acid, 30 mM L(+)-lactic acid, 50 mM acetic acid, and 0.14 mM fumaric acid. To obtain a more accurate quantification of

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individual components, an internal standard was utilized (isovaleric acid).

Analysis of metabolic products by gas-liquid chromatography. The extraction of nonvolatile organic acids was carried out according to a previously reported method (15). One milliliter of each sample was collected at different times of the thermal curves and pipetted into a screw-cap culture tube fitted with a Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.)-lined cap. The culture was acidified to pH 2.0 with 50% (vol/vol) aqueous H2SO4. After addition of 2 ml of methanol and 0.4 ml of 50% (vol/vol) H2SO4, the tube was placed in a 55°C water bath for 30 min; then 1 ml of distilled water and 0.5 ml of chloroform were added. To mix the compounds, the tube was gently inverted about 20 times, and 5 µl of the chloroformic phase was injected into a gas-chromatograph model 3700 (Varian) equipped with a flame ionization detector. A glass column (180 cm by 4 mm, internal diameter) packed with Chromosorb 101 (Johns-Manville Products Corp., Denver, Colo.) was used. Analysis conditions included the following: injector at 220°C; detector at 240°C; oven isothermal at 190°C; and nitrogen, hydrogen, and air flows, respectively, at 30, 30, and 300 ml/min.

RESULTS

Microcalorimetric analysis. Before the results are presented, a description of calorimetric analysis is needed. The determination of heat production must not be considered as a nonspecific measurement of the temperature of a biological system. Heat is a defined thermodynamic parameter whose quantification in standardized conditions allows a direct determination of the changes in enthalpy (ΔH) of the reactions occurring in the vessel (6). This biophysical determination is successfully applied to the study of simple chemical or biochemical processes. Nevertheless, pioneering work in the 1960s indicated that this kind of analysis could also be successfully applied to cellular systems (for a review, see reference 6). All the heat produced by these systems has been demonstrated to be dependent on catabolic reactions (6). In particular, when the energy source and the end products of catabolism are simple and well known molecules, such as glucose, organic acids, alcohol, and CO2, it is possible to correlate the calorimetrically recorded heat produced, e.g., by a growing bacterium, with the theoretical heat calculated from the enthalpy of formation on the reactants and end products, which are known and tabulated.

In principle, comparative biochemical and thermochemical analyses allow one to ascertain which catabolic pathways are responsible for each component of the recorded thermogram. If this correlation can be established (and this was the goal of our research), calorimetry can be utilized as a simple, direct, and continuous method to monitor the catabolic pathways operating in the cell. In this case, the technique could be very useful in studying bacterial metabolism in different environmental conditions as well as in the presence of biologically active molecules such as antibiotics.

The thermograms obtained by the growth of E. coli ATCC 25922 in Mueller-Hinton broth under aerobic and anaerobic conditions are shown in Fig. 1. The reproducibility of the PT curves was determined by utilizing the three different channels on the chromatograph; the three PT curves run at the same time produced identical profiles. Slight differences were observed in measurements performed on different days. Growth curves determined in parallel experiments are also included.

The PT curves obtained by the growth of E. coli strains isolated from clinical specimens demonstrated no significant differences in the general shape and in the total area from those produced by E. coli ATCC 25922. A slight delay in the appearance of the second component was seen in most strains (data not shown).

The PT curves of aerobically growing bacteria can be divided into two primary thermic events: the first is made up of three peaks, and the second is made up of one major peak. The parallel growth curve is characterized by a diauxic mode of growth, which is strictly related to the different metabolic and thermic phases.

The first peak appeared about 1 h after the beginning of the recording and reached its maximum (50 µW/ml) at hour 2. At the end of this transient peak, the partial O2 pressure (pO2) in the medium, measured polarographically in parallel experiments with a Clark electrode, was equal to zero. Peaks 2 and 3 were very close together and reached their maxima about after 4 and 5 h, respectively. These two peaks reached about the same height, corresponding to 180 to 200 µW/ml. They were strictly related to a first exponential growth phase, which started after a quiescent period lasting about 1 h. The end of this phase corresponded to the exhaustion of the available glucose and a parallel increase of lactic and carboxylic acids in the medium (Table 1). The interval between these initial events and the last component of the PT curve detectable only in the aerobic culture was correlated to a parallel decrease in the growth rate. This lag phase ended after about 2 h, when both the growth curve and the PT curve started to rise again. They stopped about 5 h later, nearly 12 h after the beginning of the recording. The maximum output of power was reached in this phase and amounted to 450 to 550 µW/ml. The rapid decrease of the PT curve suggested some substrate limitation occurring within the vessel. The gas analysis performed at this time indicated a complete exhaustion of O2 in the atmosphere of the reaction vessel (data not shown).

The bacterial growth in anaerobic environment demonstrated some important differences in power production and in the growth curves: growth was characterized by the absence of the last and highest peak of the PT curve and of the second exponential phase of growth. This peak disappeared when the anaerobiosis was obtained either by preduction in an anaerobic incubator or by covering the air-water interface with mineral oil.

Analysis of metabolic products and thermochemical correlation of microbial growth. To evaluate the metabolic path-
The reaction.

The heat produced corresponds to the balance between catabolic and anabolic reactions according to the formula:

\[ Q = \Delta H_{\text{met}} = Q_{\text{cat}} + Q_{\text{an}} \]

with

\[ Q_{\text{cat}} = \sum m_i \Delta H_{\text{cat},i} \]

and

\[ Q_{\text{an}} = \sum n_j \Delta H_{\text{an},j} \]

In the first relationship, \( \Delta H_{\text{met}} \) is the enthalpy change due to metabolism, \( Q_{\text{cat}} \) is the heat production due to catabolism, and \( Q_{\text{an}} \) is the heat production due to anabolism. In the last two relationships, \( m_i \) and \( n_j \) are the number of moles of reaction for components \( i \) and \( j \) of catabolism and anabolism, respectively, which occurred during the growth; \( \Delta H_{\text{cat},i} \) and \( \Delta H_{\text{an},j} \) are the corresponding enthalpies of reaction per mole.

In bacterial cells such as \( E. \ coli \) grown in well-defined chemical media (4–6), but also in eucaryotic cellular systems (16, 17), \( \Delta H_{\text{an}} \) is negligible and we can therefore assume that \( \Delta H_{\text{met}} = Q_{\text{cat}} \) for calculations.

\( \Delta H_{\text{cat}} \) can be calculated from the enthalpies of formation given in tabulated thermodynamic data.

In Table 1, the results of experimental and theoretical \( Q \) calculations run on the last aerobic component of the PT curve are reported.

The experimental value of \( Q \) was 9.2 ± 0.8 J/ml (the standard error of the mean was calculated from 10 different experiments run in triplicate). The theoretical \( Q \) calculated from the moles of organic acids consumed (35), assuming at first approximation a complete oxidation of each acid to \( \text{CO}_2 \) and \( \text{H}_2\text{O} \), gives a value of \( Q = 10.3 \pm 1.4 \) J/ml. The theoretical \( Q \) derived from consumption of available oxygen (22.0 \( \mu \text{mol} / \text{vessel} \) [the standard error was determined from eight duplicate measurements]), assuming that all \( \text{O}_2 \) was utilized for organic acid combustion, was 9.94 ± 1.3 J/ml. The \( \Delta H \) value used for calculation was \(-452 \text{kJ/mol} \) of \( \text{O}_2 \).

Since we did not measure significant differences in pH at the end of the experiments (pH 7), no corrections were made for the heat of proton ionization.

The data reported show a fitting correlation between the experimental \( Q \) values, as determined calorimetrically, and the theoretical \( Q \) values, as calculated by \( \text{O}_2 \) consumption and organic acid combustion. The \( Q \) values calculated from these two latter parameters are practically identical.

This result indicated that the heat effect investigated can be totally ascribed to the tricarboxylic acid (TCA)-dependent combustion of organic acids to \( \text{CO}_2 \) and \( \text{H}_2\text{O} \).

**Microcalorimetric analysis in the presence of aminoglycoside antibiotics.** The thermograms produced by the growth of \( E. \ coli \) ATCC 25922 in Mueller-Hinton broth in the presence of sub-MICs of gentamicin, sisomicin, amikacin, kanamycin, and netilmicin are shown in Fig. 2. The MICs, evaluated by the macrotube dilution procedure, were as follows (micrograms per milliliter): 0.25 for sisomicin; 0.5 for gentamicin and netilmicin; 2 for amikacin; and 4 for kanamycin. Addition of aminoglycoside antibiotics substantially modified the shape of thermograms. At the MIC, no power production was observed. The presence of sub-MICs of aminoglycosides selectively suppressed or delayed the metabolic phase corresponding to the last component of the PT curve. A concomitant and proportional decrease in total biomass formation was observed.

Inhibition by gentamicin (Fig. 2A) was complete at 1/2 the MIC (curve a) and at 1/4 the MIC (curve b); a marked delay was present at 1/8 the MIC (curve c), and it appeared also at 1/16 the MIC (curve d); no significant differences from the control culture were shown at 1/32 the MIC. Sisomicin (Fig. 2B) demonstrated a complete inhibition of the final calorimetric peak at 1/2 the MIC (curve a); at 1/4 the MIC, it caused a considerable delay in the appearance of this thermic component (46 instead of 13 h) (curve b); a smaller but significant delay was evident also in the presence of concentrations 1/8 and 1/16 the MICs (curves c and d). Amikacin produced the highest inhibitory activity. When the antibiotic was added to the medium (Fig. 2C), the fourth thermic event completely disappeared at 1/2 and 1/4 the MICs (curves a and b); it caused a delay in the appearance of the last calorimetric peak, decreasing proportionally from 1/8 to 1/32 the MIC (curves c to e), and an inhibitory activity was still present at 1/64 the MIC (curve f). Netilmicin (Fig. 2D) produced a less efficient inhibition of calorimetric peak 4; 1/4 the MIC caused only a delay of this power component (35 instead of 13 h; curve b), and a shorter delay was induced by concentrations of 1/8 and 1/16 the MICs (curves c and d). Kanamycin (Fig. 2E) was the only aminoglycoside tested that demonstrated no disappearance of peak 4 at 1/2 the MIC.
At this concentration, however, it demonstrated a partial activity in the first group of thermic events as well; all antibiotic concentrations, however, produced a progressive delay of peak 4.

The first anaerobic peaks of the PT curve were generally not affected by sub-MICs of all antibiotics tested.

**DISCUSSION**

Utilizing the calorimetric technique, we were able to evaluate the modulation of the bacterial respiration induced by aminoglycosides. This was possible since, for the first time, the different heat components of the PT curves were
successfully correlated with the different metabolic activities operating sequentially in the cell. These activities were identified in a first transient aerobic phase of growth, when bacteria utilize all the oxygen dissolved in the media; this phase is followed by a second phase of growth in an anaerobic environment, when glycolysis becomes the catabolic pathway operating in the cell to supply the energy for anabolic syntheses. A third metabolic step then ensues, the aerobic catabolism of carboxylic acids accumulated in the media during anaerobic growth. This phase starts at the air-liquid interface and continues only at this level. Growth at this interface, moreover, is responsible for the characteristic thick ring of bacteria adherent to the glass tube after an overnight culture; this ring was totally absent in anaerobic cultures (data not shown).

From the data reported in this study, it appears that aminoglycosides can have a significant antibacterial effect at sub-MICs. Growth inhibition is dose dependent and is proportional to the inhibition of respiratory activity involved in the catabolism of carboxylic acids.

Some minor differences are evident, however, among the different aminoglycosides tested. Amikacin proved to be the most active: even at 1/64 the MIC, a slight effect was evident. The relative degrees of antibacterial activity were: amikacin > gentamicin > sisomicin > netilmicin > kanamycin. In terms of molar ratios, the most active drug was gentamicin.

To explain our results, some speculations on the mechanism of action of aminoglycosides at a molecular level can be proposed. Inhibition of power output observed at sub-MICs could simply be one aspect of the inhibition of growth and metabolic activity, i.e., the aminoglycoside-induced bacteriostasis. However, in our opinion, these results can be also explained by the hypothesis of Bryan and Kwan on the mechanism of aminoglycoside transport (8). These authors postulated a direct role of quinones (or a series of transporters linked to quinones) in aminoglycoside transport. Requirement of electron transport has been proposed to produce a negative polarity of the transporter due to reduction. This would facilitate binding of a polycationic antibiotic. The bound aminoglycoside would be driven across the mem-

FIG. 2. PT curves recorded during the growth of ATCC 25922 in Mueller-Hinton broth in the presence of sub-MICs of various antibiotics. Curves: C, no antibiotic; a, 1/2 the MIC; b, 1/4 the MIC; c, 1/8 the MIC; d, 1/16 the MIC; e, 1/32 the MIC; f, 1/64 the MIC. PT curves are from single experiments representative of the three performed in triplicate. Less than 5% variation was observed among the different curves run with the same antibiotic.
brane by a threshold value of $\Delta V$ and transferred to ribosomes, the final cellular targets.

In our opinion, this process would necessarily limit the quantity of available quinones (or other transporters) for electron flow. Since carboxylic acid catabolism is linked to bacterial respiration, a block of electron flow would produce a concomitant impairment of the TCA cycle.

This bacteriostatic inhibition is evident only at sub-MIC levels of drugs, because at the MIC or above, the quantity of antibiotic entering the bacterial cell during the first aerobic phase is sufficient to induce the bactericidal, irreversible uptake of the aminoglycoside.

The bacteriostatic effect finally produces a nearly 80% inhibition of biomass increase as compared with the biomass of a matched control culture run in the absence of an antibiotic. Although the importance, if any, of this inhibition from a clinical point of view remains open to discussion, it is noteworthy that, in the remaining 20% of the bacterial population, the observed decrease in the adhesive capacity of E. coli mediated by type 1 fimbriae (manuscript in preparation) parallels the decreased TCA cycle activity. Although this result could be dependent on an altered protein synthesis, as proposed by other authors (30), it could also be due to a lower degree of anabolic synthesis in a cell deprived of ATP, in which the main catabolic pathway for its production, the TCA cycle, has been blocked by the aminoglycoside.

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