KINETICS OF CHLOROTETRACYCLINE UPTAKE IN STAPHYLOCOCCUS AUREUS BY A FLUORESCENCE TECHNIQUE

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Received July 30, 1973

SUMMARY: The antibiotic chlorotetracycline (CTC) is used as a fluorescent chelate probe to investigate the kinetics of its uptake into Staphylococcus aureus. CTC binds to divalent cations in an aqueous solution with enhanced fluorescence. This fluorescence is polarity dependent, being higher in apolar solutions. Upon addition of CTC to dispersions of S. aureus, a time dependent fluorescence enhancement is detected demonstrating that the CTC-divalent cation complex migrates into the apolar regions of the membrane. This uptake, which follows saturation kinetics, is energy dependent. A K_m of 162 μM was obtained for CTC concentration ranges of 0.2-100 μgm/ml.

Tetracycline antibiotics inhibit protein synthesis in cell free ribosomal preparations of both mammalian and bacterial cells (1,2,3,4). The antimicrobial activity is attributed partly to their ability to be accumulated in bacteria by an energy dependent transport process (5,6,7) which is absent from mammalian cells (8). The rate of [^{3}H]-tetracycline uptake by the Gram-negative bacteria Escherichia coli has been shown to be non-saturating at tetracycline concentrations from 0.1-400 μgm per milliliter (9). On the other hand, tetracycline transport in the Gram-positive bacteria S. aureus H has been reported to be mediated by two processes. At low antibiotic concentrations the uptake follows saturation kinetics while at high concentrations the antibiotic becomes rate limiting (10). Hutchings suggests that several modes exist for tetracycline uptake. The system exhibiting saturation kinetics is presumably responsible for transport of the antibiotic at pharmacological levels.

In this paper we describe the use of CTC fluorescence to study its active accumulation by bacteria. Caswell and Hutchison (11, 12,13) have previously used tetracycline fluorescence to probe biological membranes. The fluorescence

is enhanced when CTC chelates divalent cations such as calcium or magnesium. This fluorescence is further enhanced when the complex is placed in an apolar environment such as when it binds to microsomes, mitochondria or erythrocyte ghosts (11,12,13,14). Active transport of CTC into bacterial cells can be monitored by following its time dependent fluorescence enhancement.

Materials and Methods

Chlorotetracycline HCl (CTC) was obtained from Nutritional Biochemical Corporation and used without further purification. CTC is unstable in alkaline media. Consequently, fresh solutions were prepared daily prior to use.

Cultures of S. aureus were maintained on BBL Brain Heart Infusion Agar slants. The cells were grown with agitation at 37°C in Difco Brain Heart Infusion Broth. They were collected by centrifugation at 10,000xg at late log phase and washed three times with fifty volume portions of 10mM Tris-HCl buffer, pH 7.0. Pelleted cells were stored for no more than one week at 2°C. Bacterial solutions were prepared from these cells.

Fluorescence scans were made on an Hitachi-Perkin Elmer MPF-2A spectrophotofluorimeter and appear as uncorrected scans. Exitation wavelengths were 400 nm with slit widths being set at 16 for both monochromaters. The time dependence fluorescence studies were run on a thermostated Turner 430 spectrofluorometer with both monochromaters set at 60 nm. Bacterial magnesium concentrations were determined on a Perkin-Elmer Atomic Absorption Model 303 spectrophotometer.

Results and Discussion

The effects of magnesium, magnesium plus an apolar environment and a solution of respiring S. aureus cells on the fluorescence of CTC is shown in Fig. 1. These cells were allowed to incubate at 25°C for two hours before their fluorescence spectra was measured. The cell dispersions show a fluorescence enhancement of 9 times over the free CTC in the same buffer. The

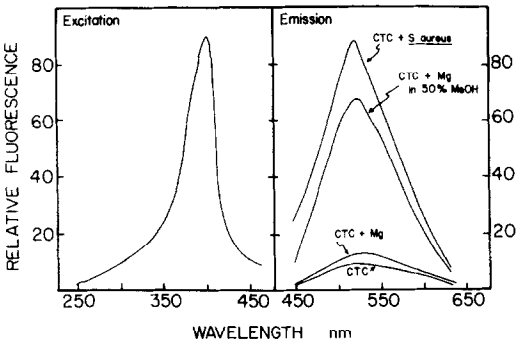

Fig. 1. Excitation and emission spectra of CTC and the effects of added magnesium, methanol and respiring bacteria. The emission spectra all contain 100 μgm/ml CTC in 10 mM Tris-HCl, pH 7.0 and 0.2% D-glucose. Where indicated, the following additions were made: 0.285 μgm/ml Mg^{2+}, 0.285 μgm/ml Mg^{2+} in a 50% methanol solution and finally a suspension of S. aureus, 1.25 mg/ml dry weight. The excitation spectra is of the same S. aureus solution.

magnesium concentration of the bacterial solution was determined to be 0.285 μgm/ml. An aqueous CTC solution of equivalent magnesium concentration was prepared. This results in a slight fluorescent enhancement over the CTC solution. If, however, the same solution were prepared in the presence of 50% methanol, a substantial enhancement is seen over the aqueous solution. These results suggest that in the presence of the bacteria the fluorescence of the CTC is greatly enhanced as the CTC-magnesium complex moves into the apolar environment of the cell (11,12).

The above results suggest that the kinetics of the active transport of CTC into the S. aureus may be followed by monitoring the fluorescence enhancement of the CTC with time. Results of such experiments are shown in Fig. 2. In the presence of glucose the fluorescence of the bacterial solution increases rapidly for the first twenty minutes and then rises only slowly. If the cells are first killed by heating, this time dependent enhancement is absent although an initial enhancement proportional to the concentration of respiring cells is observed. This indicates no movement of the CTC-magnesium complex into the apolar regions of the cell membrane. The initially observed enhancement presumably reflects adsorption to the bacterial surface. In the absence of glucose

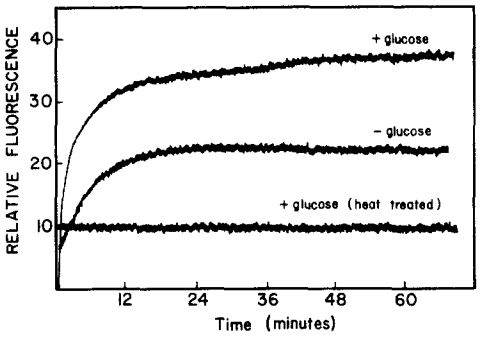

Fig. 2. The time dependent fluorescence enhancement of CTC by S. aureus. Cells are incubated for two hours in 10 mM Tris-HCl, pH 7.0, at a concentration of 1.25 mg/ml. The solutions contain 0.2% glucose, 0.2% glucose with the cells being treated at 100°C for five minutes at the beginning of the incubation period, or no glucose. At zero time CTC was added to a final solution concentration of 100 μgm/ml.

as an energy supply, the rapid time dependent fluorescence enhancement is still present but the maximum fluorescence levels reached under these conditions are only about half that found in the presence of glucose. Furthermore, there is no additional fluorescence enhancement after the first twenty minutes. These results are consistent with the explanation that although the transport of CTC required an energy source, the staphylococcal cells are able to use metabolic intermediates to energize the system (10).

To further characterize the fluorescence enhancement as an active transport process, the effect of various metabolic inhibitors was investigated. Representative data are shown in Table I for cells incubated in the presence and absence of glucose. The results clearly indicate that azide and fluoride inhibit the transport of CTC into the cells in both the presence and absence of an external energy source, with azide being a better inhibitor than fluoride under the conditions cited. Freezing the cells had less effect on the transport than did either of the metabolic inhibitors.

The effect of varying the concentration of CTC on the initial rate of fluorescence enhancement was next investigated. Figure 3A is representative of data which was obtained using a concentration range of 10-100 μgm/ml CTC.

TABLE I

Incubation Solution	Relative Fluorescence
0.2% Glucose	1.00
0.2% Glucose + 10^{-2}M NaN_3	0.10
0.2% Glucose + 10^{-2}M NaF	0.49
0.2% Glucose + heating	0.00
0.2% Glucose + freezing	0.67
No Glucose	0.35
No Glucose + 10^{-2}M NaN_3	0.02
No Glucose + 10^{-2}M NaF	0.16
No Glucose + heating	0.00
No Glucose + freezing	0.27

S. aureus were suspended in solutions of 10 mM Tris buffer, pH 7.0, at a concentration of 2.50 mg/ml and containing additions listed in the table. After incubation of these solutions for two hours they were diluted with equal volumes of 200 µgm/ml CTC. After an additional two hours the fluorescence of the samples was measured at excitation wavelength 400 nm and emission wavelength 520 nm.

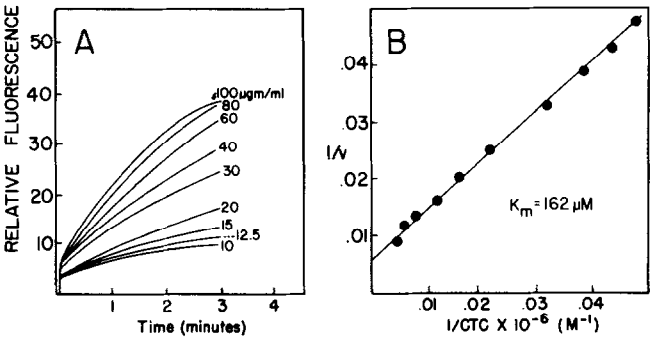

Fig. 3. Initial rates of CTC fluorescence enhancement at CTC concentrations of 10-100 µgm/ml. Cells were suspended in Tris buffer containing 0.2% glucose for two hours incubation period as before. At zero time CTC was added to the solutions at the concentrations indicated and the resulting fluorescence enhancement recorded. In Fig. 3B the initial rates are plotted as a double reciprocal plot.

The results are suggestive of saturation kinetics. When initial rates are plotted as double reciprocal plots, typical Michaelis-Menten kinetics are observed (Fig. 3B). The K_m thus obtained was 162 μM and was highly reproducible. Similar K_m's were obtained using any concentration range from 0.2-100 μgm/ml.

The fluorescent technique of monitoring the kinetics of tetracycline uptake has advantages over other kinetic determination techniques in that the initial rates are directly measured and recorded. Thus it was shown that chlorotetracycline is actively transported into the cells by a saturating system of sites at levels previously thought to be nonsaturating. Further work on the kinetics of uptake of other fluorescent tetracyclines is presently being pursued on various other bacterial strains.

Acknowledgment

This work was supported in part by Washington State University Research Committee funds.

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