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CHARACTERIZATION OF THE ACTIVE TRANSPORT OF CHLOROTETRACYCLINE IN STAPHYLOCOCCUS AUREUS BY A FLORESCENCE TECHNIQUE*

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The antibiotic chlorotetracycline (CTC) is used as a fluorescent chelate probe to investigate its active transport in respiring Staphylococcus aureus cells. CTC chelation to magnesium or calcium leads to fluorescence enhancement. This enhancement is further increased when the polarity of its environment is decreased, as occurs when the complex moves from an aqueous environment into a membrane. Upon addition of CTC to a dispersion of S. aureus cells, a time dependent fluorescence enhancement is detected which is a monitor of the transport of the CTC-divalent cation complex into the membrane. This uptake has been shown to be energy dependent and exhibits saturation kinetics with an apparent K_m of 107 ± 20 μM by the same technique. The initial rates of antibiotic uptake are shown to have a pH optimum between 5.5 and 6.5. The effects of exogenously added EDTA and paramagnetic Mn^{2+} indicate that the CTC-divalent cation complex is transported to the inside of the membrane. Exogenously added magnesium inhibits the accumulation process. This implies that the membrane CTC binding site involves a divalent cation sequestered away from the surface of the membrane, and only free CTC is bound to that site. The uptake of CTC is also temperature dependent with a maximal rate at 40°. Arrhenius plots of the initial fluorescence enhancement rates are found to be biphasic with a 27° transition temperature. The break in the plots presumably reflects an order-disorder transition involving the fatty acids of the cell membrane. Thus, transport of the CTC involves movement through the fatty acid region of the membrane. This movement is facilitated by the more fluid state of the membrane above the transition temperature.

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INTRODUCTION

Both eukaryotic and prokaryotic ribosomal fractions have been shown to be sensitive to tetracycline antibiotics. The antibiotics appear to interfere with the binding of aminoacyl-tRNA to the ribosome-mRNA complex (1–4). On the other hand, the antimicrobial activity of these antibiotics appears to lie in their ability to be actively accumulated in gram-positive and in gram-negative bacteria by an energy dependent transport process (5–9) which is absent from mammalian cells (10). This transport process has received less attention than the elucidation of the mode of action of tetracycline antibiotics but is, nonetheless, of great interest since it is the process which confers specificity to the antibiotic. The transport system is of further interest because it involves active accumulation of a toxic substance not structurally related to any known nutrient.

Several recent attempts to demonstrate saturation kinetics of the transport system of the tetracyclines using radioactively labeled compounds have been unsuccessful (7, 8). These results have led investigators to postulate models which may involve two phases of uptake; the adsorption of the tetracyclines to the bacterial cell surface and the transport of the antibiotic once a significantly high concentration of the antibiotic has been reached (11, 12). It is possible, however, that kinetic measurements of the transport will not yield linear plots because of difficulty in obtaining initial transport rates using the common technique of monitoring radioactively labeled substrates; that is, the initial saturation of the transport system may occur in a time period too short to measure accurately. To help fully understand the mechanism of tetracycline transport in intact Staphylococcus aureus, our laboratory has recently developed a fluorescence technique designed to rapidly monitor the initial uptake of chlorotetracycline in respiring bacterial cells (13). The uniqueness of this fluorescence technique lies in the fact that it is the first example of a transport substrate whose kinetics and environment can be measured directly while it is intimately bound to the transport system and being actively transported. The results presented in this paper provide new insights into the tetracycline transport system and suggest modified models for mechanisms of its transport through the S. aureus membrane. In addition, the data support recent proposals that transport systems are responsive to the physical characteristics of membrane lipids.

In examining the active transport of tetracycline, we have made use of the fluorescent properties as has been reported previously. Caswell and Hutchison (14–16) have used the antibiotic chlorotetracycline as a fluorescent probe of biological membranes. They have shown that the fluorescence is enhanced when the CTC chelates diamagnetic divalent cations such as calcium or magnesium. This CTC-divalent cation complex fluorescence is polarity dependent, being greater in apolar environments such as within microsomal, mitochondrial, or erythrocyte membranes (14, 15, 17, 18). These studies have used the tetracyclines to investigate magnesium and calcium interaction with membranes. We are using the fluorescence to study the tetracycline interaction with its own specific transport system. Information about both transport and divalent metal ion roles can thus be obtained.

MATERIALS AND METHODS

Chemicals

Chlorotetracycline hydrochloride and tetracycline hydrochloride were obtained

from Nutritional Biochemical Corporation. These tetracyclines were used without further purification. Because the tetracycline antibiotics are unstable to aqueous media, fresh solutions were prepared daily prior to use.

Bacteria

Cultures of a wild type strain of S. aureus were the gift of the Department of Bacteriology and Public Health, Washington State University. Tetracycline resistant strains 12715 and 14154 were purchased from the American Type Culture Collection as was the wild type strain 13801 (strain H). These cells were grown under conditions previously described (13). Cells were collected by centrifugation at 10,000 × g at late log phase and washed three times with fifty volumes of 10 mM tris-HC1 buffer, pH 7.0. The cells were used immediately after collection. Tetracycline resistant strains were grown on Difco Bacto-Agar in the presence of Baltimore Biological Laboratories Sensi-Disc Microbial Susceptibility Test Discs.

Fluorimetry

Kinetic measurements of the fluorescence enhancement of respiring bacterial solutions using a Turner Model 430 spetrophotofluorimeter have been previously described (13). All fluorescence measurements were made at an excitation wavelength of 400 nm and an emission wavelength of 520 nm. Bacterial suspensions were prepared by diluting pelleted bacteria with a 10 mM tris-HC1, pH 7.0, and 0.2% D-glucose solution to a final concentration of 2.5 mg/ml as determined by dry weight analysis. At time zero, equal volumes of a tetracycline solution and the bacterial dispersion prepared in an identical buffer are added together in a 1 cm fluorescence cuvette. The cuvette is then placed in a thermostatted fluorimeter and the kinetics of the fluorescence enhancement are followed on a strip chart recorder. K_m's and standard deviations reported herein were determined using a Wang 700C calculator and double reciprocal plot program 1047A/652 of the Wang 700 Series Program Library.

In the initial fluorescence rate studies reported in Table I, bacterial dispersions were prepared as above and incubated for two hours. Inhibitor, if used, was added to achieve concentrations listed in Table I prior to the incubation period. An equal volume of CTC in 10 mM tris-HC1, pH 7.0, was then added to bring the final CTC concentration to 100 μg/ml. The initial fluorescence rates were measured and converted to relative rates.

RESULTS

The time course of fluorescence enhancement of chlorotetracycline is shown in Fig. 1 for a 1.25 mg/ml suspension of S. aureus cells. In the presence of an energy source, the fluorescence of the bacterial suspension increases rapidly for the first ten minutes after the addition of chlorotetracycline and then continues to rise only slowly after that. In the absence of glucose, the kinetics of uptake are similar but with two major differences. The maximum fluorescence levels reached are only about half that of the S. aureus supplied with an energy source. In addition, there is no further enhancement of the fluorescence after the initial uptake. If the S. aureus cells are heat-treated at 100° for five minutes immediately before the chlorotetracycline is added, there is no time-dependent enhancement although a time zero fluorescence enhancement proportional to the concentration of the cells is observed.

Table I.

Preincubation Solution	Relative Initial Fluorescence Rates
0.2% Glucose	1.00
0.2% Glucose + 10^{-2} M NaN_3	0.28
0.2% Glucose + 10^{-2} M NaCN	0.10
0.2% Glucose + 10^{-2} M NaF	0.37
0.2% Glucose + 10^{-2} M N-ethyl maleimide	0.59
0.2% Glucose + heating	0.00
0.2% Glucose + freezing	0.75
0.2% Glucose + 10^{-3} M EDTA	0.79
No Glucose	0.46
No Glucose + 10^{-2} M NaN_3	0.20
No Glucose + 10^{-2} M NaCN	0.06
No Glucose + 10^{-2} M NaF	0.36
No Glucose + 10^{-2} M N-ethyl maleimide	0.24
No Glucose + heating	0.00
No Glucose + freezing	0.44
No Glucose + 10^{-3} M EDTA	0.49

These results suggest that the fluorescence enhancement of CTC is monitoring the active transport of a chlorotetracycline-divalent cation complex to apolar regions of the membrane. The initial time zero fluorescence levels presumably are indicative of CTC-divalent cation complex binding at the cell surface. This conclusion can be drawn from the fact that the heat-treated cells do not actively transport CTC (5). The initial time zero fluorescence levels of S. aureus cells in the presence and absence of glucose both appear to have initial fluorescence values like the heat-treated cells. Any additional time-dependent enhancement requires an energy source and viable cells. Without energy, viable staphylococcal cells are still able to transport a limited amount of the antibiotic, presumably by utilizing existing metabolic intermediates (7). In the presence of glucose, the rapid time-dependent enhancement of fluorescence occurs for the first ten minutes and then continues to rise only slowly. It should be noted that at some point after the initial kinetic fluorescence enhancement, fluorescence levels monitored may represent contributions from many species. Some of these species may be ribosome-bound CTC or CTC at another cytoplasmic binding site. Because of the many factors contributing to the observed fluorescence, only initial rates of fluorescence enhancement are discussed. These initial rates can be determined before other contributions to the observed fluorescence complicate the kinetic data. All evidence supports that the initial fluoresence enhancement directly reflects the active transport of CTC.

To demonstrate that the fluorescence technique employed is a true monitor of the active transport system of chlorotetracycline, the effects of various metabolic inhibitors were examined. The data in Table I are indicative of suspensions of S. aureus cells preincubated in the presence and absence of glucose, azide, fluoride, cyanide, and the sulfhydryl reagent N-ethyl maleimide. Because of its large absorption maximum at 380 nm, dinitrophenol could not be used. The results indicate that the uptake of CTC as reported by the rates of fluorescence enhancement is reduced by the presence of these inhibitors. The initial fluorescence levels of the heat-treated cells, however, are not affected by the presence of glucose. This is an expected result if these fluorescence levels are merely a measure of the CTC divalent cation complex binding to the surface of the cells.

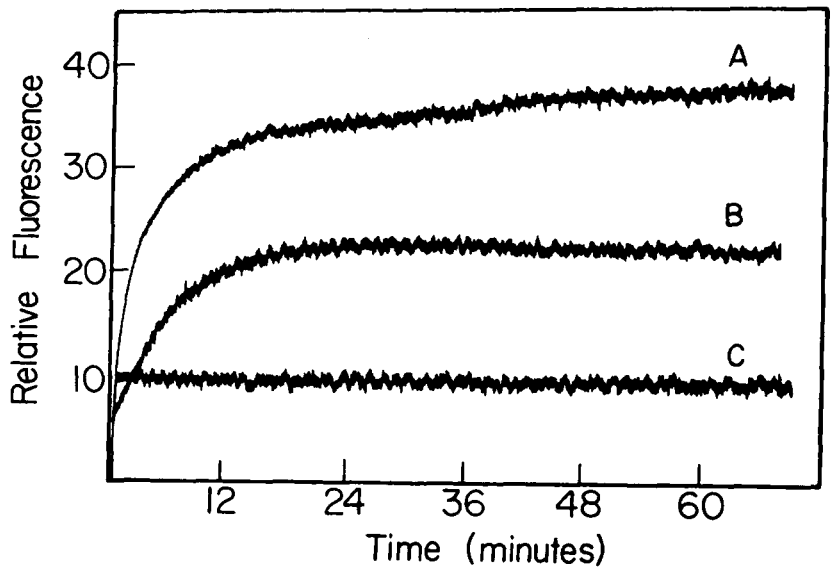

Fig. 1. The time-dependent fluorescence enhancement of CTC by S. aureus. Cells are incubated for two hours in 10 mM tris-HCl, pH 7.0, at a concentration of 1.25 mg/ml (Curve B). At time zero CTC was added to a final solution concentration of 100 μg/ml. Curves A and C were run under identical conditions with the addition of 0.2% glucose (Curve A) or heating the S. aureus cells at 100° for five minutes (Curve C) before the addition of CTC.

Location of the CTC divalent cation complex within the respiring S. aureus cells was examined by using an external divalent cation chelating agent and a paramagnetic ion to quench fluorescence of the antibiotic. If the CTC divalent cation complex is located on the external surface of the cell, the addition of EDTA will quench the fluorescence of the complex by removing the cation from the CTC complex. An example of this is seen in Fig. 2 where exogenous EDTA is added to heat-treated cells. The results indicate that the CTC-divalent cation complex is indeed bound to the cell surface and is available to the EDTA. If, on the other hand, the EDTA is added to respiring cells after there has been a significant fluorescence enhancement, there is no quenching of the fluorescence, indicating that the complex is sequestered away from sites available to EDTA. This is also shown in Fig. 2. Furthermore, S. aureus cells pretreated with EDTA before the addition of chlorotetracycline do not show a retardation of fluorescence enhancement, implying that the CTC binds to a magnesium or calcium site not available to exogenously added EDTA. The addition of a large excess of a paramagnetic divalent cation such as manganese to respiring cells does cause quenching of fluorescence. The paramagnetic divalent cations when bound to tetracyclines quench the fluorescence because of enhanced intersystem crossing from the excited singlet state to the triplet state (19). Thus, as Mn^{2+} competes with bound magnesium or calcium for the chlorotetracycline, quenching of fluorescence occurs.

Measurements of the pH optimum for initial rates of fluorescence enhancement were carried out at 25°. Figure 3 indicates an optimal pH of 5.5 to 6.5 with the rates falling off rapidly on either side of this. Chlorotetracycline fluorescence is known to be pH sensitive in its unchelated state but as a divalent cation complex it shows no appreciable change in fluorescence enhancement over a pH range of about 4.5–9.5 (17; Dockter and Magnuson, unpublished results). This optimum is the same as that reported by Hutchings using (^{3}H)-tetracycline to measure accumulated antibiotic (7).

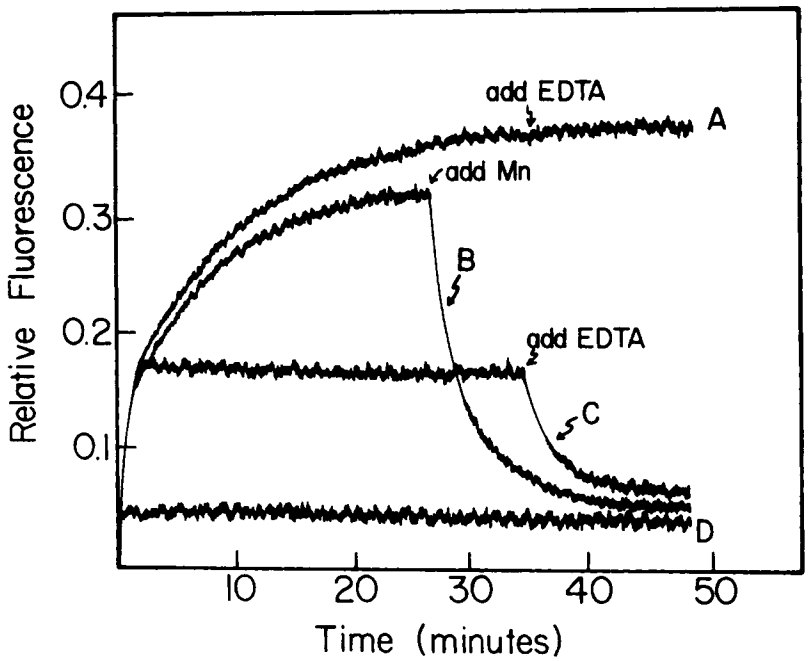

Fig. 2. Fluorescence of CTC, observed at 520 nm, in a suspension of S. aureus. Conditions are as in Fig. 1. A) Increase in fluorescence as a function of time. The addition of EDTA to a concentration of 0.5 mM has no effect. B) Fluorescence of a solution of S. aureus to which Mn^{2+} is added to a final concentration of 0.5 mM. C) Heat-treated cells to which EDTA is added. D) Fluorescence level of CTC in the absence of respiring S. aureus cells.

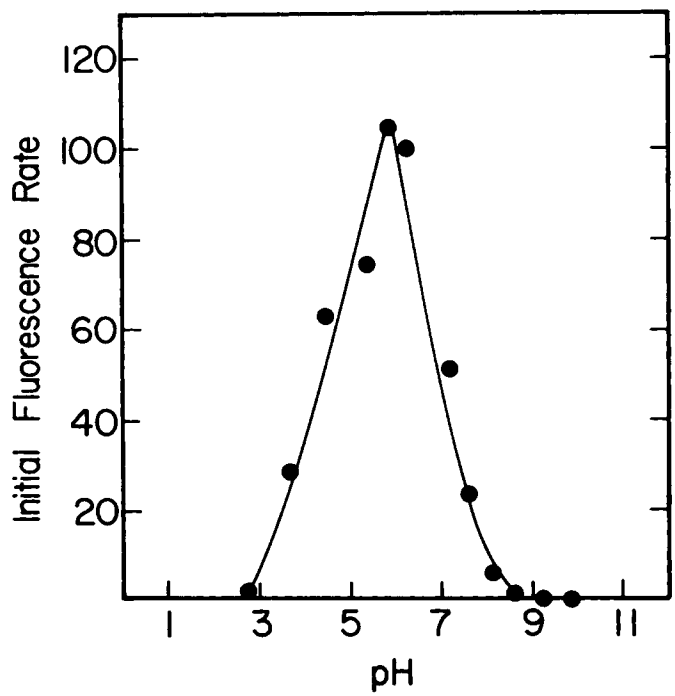

Fig. 3. The initial fluorescence enhancement rates as a function of pH at 25°. Experimental conditions are the same as described previously.

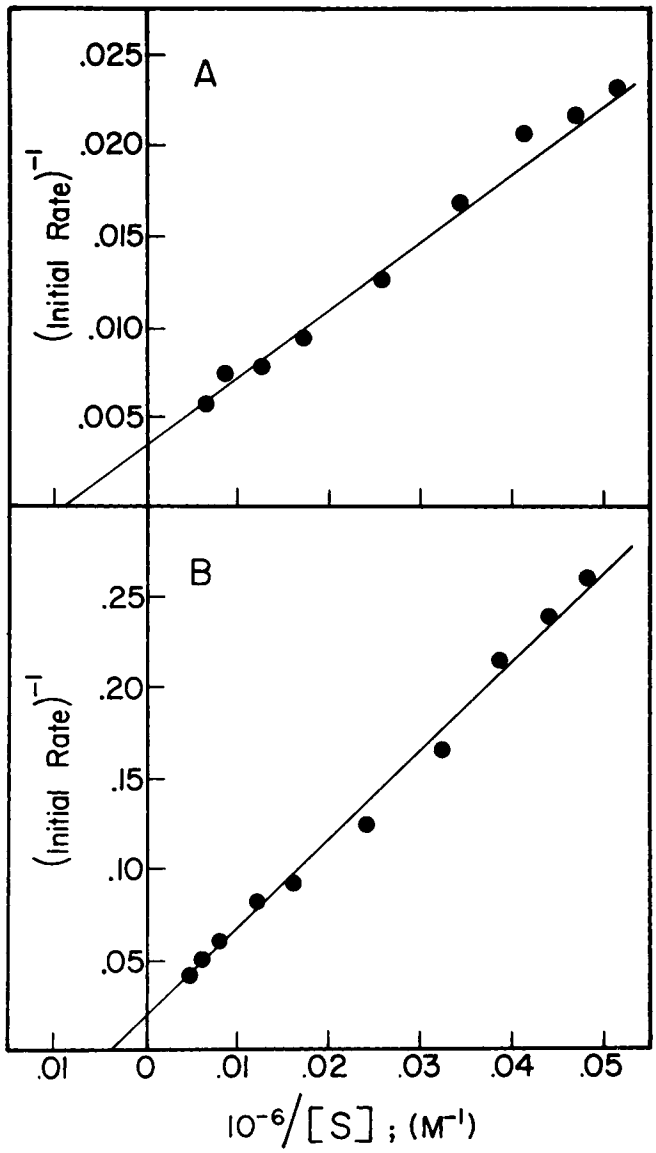

Fig. 4. Double reciprocal plots of initial fluorescence enhancement rates versus chlorotetracycline (A) and tetracycline (B) antibiotics. The K_m's obtained are 107 ± 20 μM and 254 ± 32 μM, respectively, for 25° and pH 7.0.

Initial rates of fluorescence enhancement have been shown to be dependent on the concentration of chlorotetracycline added to the media (13). The results are suggestive of Michaelis-Menten saturation kinetics for the CTC transport system. Plots of the reciprocal of the initial fluorescence enhancement rate versus reciprocal of concentration have been made for both chlorotetracycline and tetracycline using this fluorescence technique (Fig. 4). The apparent K_m's obtained by this method are found to be 107 ± 20 μM for CTC and 254 ± 32 μM for tetracycline at 25° and pH 7.0.

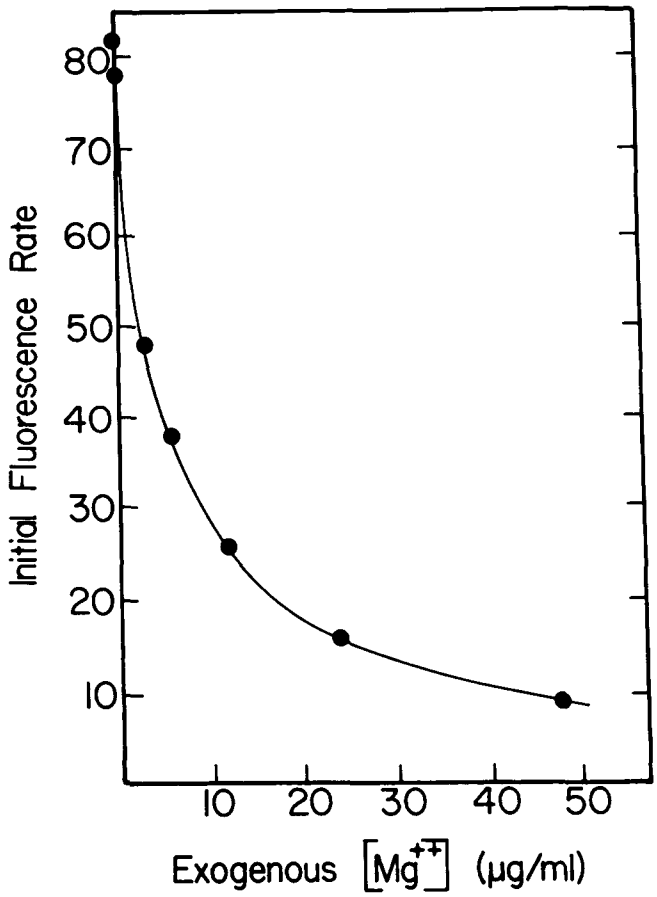

Fig. 5. The effects of exogenously added magnesium on the initial fluorescence enhancement rate of CTC. Respiring S. aureus suspensions at 25°, pH 7.0, described previously have CTC added to them to a final concentration of 50 µg/ml in the presence of the indicated amount of magnesium. Initial fluorescence enhancements are then recorded as a function of time.

The effect of exogenous magnesium on the fluorescence enhancement caused by transport of CTC is shown in Fig. 5. As the results indicate, exogenous magnesium inhibits the initial rate of fluorescence enhancement. Since CTC chelates divalent cations, exogenously added cations can compete for the CTC with membrane-bound divalent cation binding sites and effectively lower the concentration of uncomplexed CTC. Using the dissociation constant of 267 µM reported by Caswell and Hutchison (14) for the CTC-magnesium complex in an aqueous solution, it can be shown that the initial fluorescence rates shown in Fig. 5 are directly proportional to the free CTC concentration. Thus, the CTC-divalent cation complex formed in solution will not bind to the active transport site. Furthermore, at concentrations of exogenous magnesium from 0-10 µg/ml, competitive inhibition of the transport system is observed (data not shown). This further suggests that uncomplexed CTC must bind initially to a magnesium ion already at the transport site.

The initial rates of fluorescence enhancement as the CTC moves into the membrane are temperature-dependent. The initial rate of increase in fluorescence of CTC is shown in Fig. 6 as a function of incubation temperature. The initial transport rate has a temperature

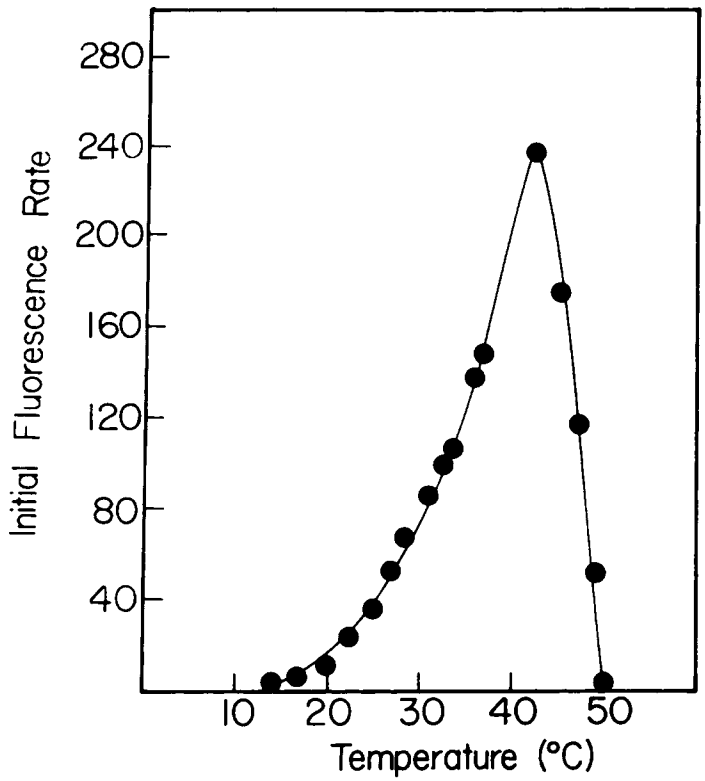

Fig. 6. Dependence of initial fluorescence enhancement rate after addition of 50 μg/ml CTC to a suspension of S. aureus cells is shown as a function of temperature. Cell suspensions are buffered with 10 mM tris-HCl at pH 7.0, as described previously.

maximum of about 35–45°. Below a temperature of 10°, there is apparently no transport of CTC into the membrane as the rate observed is zero. Above 45°, the initial fluorescence enhancement rate falls off sharply and above 50° there is no fluorescence enhancement. This 50° maximum is an irreversible point and apparently signals the destruction of the transport system. Arrhenius plots for the dependence of the initial fluorescence enhancement rate with temperature are shown in Fig. 7 for the temperature range of 10–40°. These plots are biphasic with a defined slope above and below a 27° transition point. These results suggest the transport system is influenced by a phase transition at this temperature which results in the lowering of the activation energy of the transport system. When K_m's are determined by double reciprocal plots at 20° and 37°, temperatures above and below the transition points, there is no appreciable difference found, with the K_m's both being within the uncertainty limits of each other and those measured previously at 25°.

A final test used to demonstrate that fluorescence enhancement reports transport of the antibiotic was to investigate fluorescence enhancement in tetracycline resistant mutants. It is generally believed that resistant bacteria lack the transport system (9). Two mutant strains of S. aureus reported to be tetracycline resistant were grown under identical conditions and measured for their relative fluorescence enhancement rates. Their resistance was confirmed by growth test on agar plates containing antibiotic susceptibility test discs. A wild-type strain ATCC #13801 was used as a control. The results in Fig. 8 show that

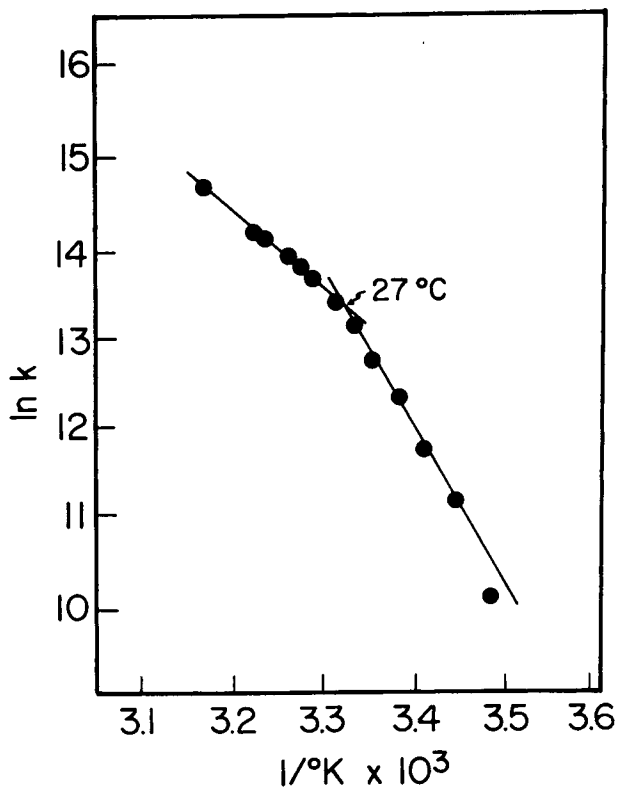

Fig. 7. The data shown in Fig. 6 are replotted as an Arrhenius plot, natural log of the molar initial fluorescence rate (k) versus the reciprocal of the absolute temperature. The plot is biphasic in nature with a transition temperature indicated at 27°.

with tetracycline resistant strain ATCC #12715, no fluorescence enhancement is observed with time when compared to the wild-type strain ATCC #13801. Thus, this mutant may be constitutive for some resistance factor or simply lacking a functional transport system. The kinetics of the resistant strain ATCC #14152, including K_m's, are similar to the wild-type cells under normal growth conditions, but the time-dependent fluorescence enhancement may be reduced by growing the cells in a subinhibitory level of chlorotetracycline. This CTC resistant strain thus appears to be of the inducible type.

DISCUSSION

The monitoring of the active transport of an antibiotic by a fluorescence technique has been described. This method of studying bacterial active transport is not unique. Recently Reeves et al. (20) have used a fluorescent galactoside to study the transport system and oxidation of D-lactate in bacterial vesicles. This probe, however, is not actively transported although it does compete with sugar for binding. The tetracyclines, on the other hand, are actively transported and their fluorescence characteristics allow us to use them as an intrinsic probe for studying the kinetics of transport. In addition CTC fluorescence has allowed us to examine interactions between the antibiotic and the transport protein binding site. Lipids are definitely involved in the transport process. Obviously, further investigations on lipid composition will enable us to elaborate further on their role in transport.

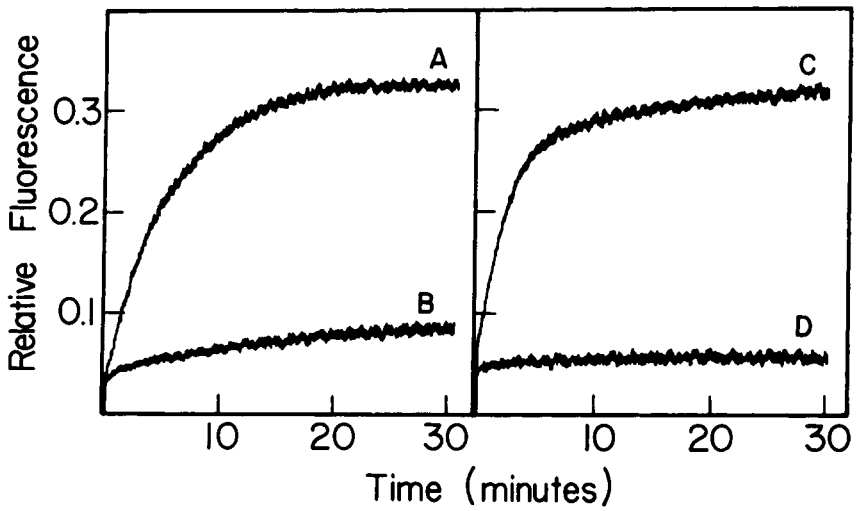

Fig. 8. Fluorescence enhancement of CTC by three strains of S. aureus cells. Conditions are the same as in Fig. 1. Curve A: CTC sensitive strain ATCC #13801. Curve B: CTC resistant strain ATCC #12715. Curve C: CTC resistant strain ATCC #14154. Curve D: CTC resistant strain ATCC #14154 cells grown in the presence of 1.0 μg/ml CTC as an inducer.

A number of laboratories have proposed mechanisms for the transport of tetracyclines in bacterial cells (7, 9, 11, 12). All of these models have at least in part been based on the fact that the tetracycline transport system was not saturable as seen by kinetics of the radioactively labeled antibiotic. Hutchings (7) postulates that S. aureus has two uptake mechanisms. At medium concentrations between 0.1 and 0.5 μg/ml saturation kinetics were observed. This conclusion was based on times to reach half of the concentration needed to inhibit protein synthesis. Above 0.5 μg/ml saturation kinetics could not be demonstrated by this technique. Franklin (9) also suggested that the technique may not be valid, since the physiological state of the cells may vary considerably in these experiments. By monitoring initial fluorescence enhancement rates, we have been able to demonstrate saturation kinetics over concentration ranges of 0.2 to 100 μg/ml with both tetracycline and chlorotetracycline.

The results here indicate that there may be two types of binding sites available to the tetracyclines. The first, as originally suggested by DeZeeuw (11), is most likely due to nonspecific adsorption of the antibiotic to the membrane surface. This may contribute to an initial time zero fluorescence level with the S. aureus cells. It should be pointed out that an initial level for viable cells was not determined precisely since uptake or fluorescence enhancement occurs immediately. With heat-treated cells a constant level of fluorescence is observed. This may reflect CTC binding sites created by heat denaturation of surface components. This level with heat-treated cells is quenched by EDTA, but no quenching occurs with viable cells. This suggests that the CTC-divalent cation complex bound at the transport site is sequestered away from the membrane surface. Furthermore, the uptake of CTC is inhibited by exogenously added magnesium at concentrations higher than those previously examined for S. aureus (7). Similar results have been reported for E. coli membrane preparations (8). Franklin (21) has proposed that addition of magnesium to the media leads to competition between the free magnesium and the membrane-bound magnesium for tetracycline. Results of our technique suggest that the unchelated antibiotic

apparently binds at some transport site not available to EDTA in the membrane. It binds to magnesium or calcium and is then transported. The CTC-divalent cation complex formed in solution cannot be bound at the transport site.

In this paper we compare all kinetic data using initial velocities. Total fluorescence is not usually discussed because, as noted previously, contributions to the observed fluorescence may come from many CTC species. It should be noted that manganese quenches all fluorescence enhancement very quickly, implying that the CTC must be in some site easily accessible to divalent metal ions. We hope to more clearly define the contributors to overall fluorescence with later work on membrane fractions. Sudies with the tetracycline resistant strains ATCC #12715 and #14154 indicate that the resistance mechanism of both of these cell types is their inability to transport the tetracycline in the energy-dependent step. It also supports our hypothesis that the fluorescence enhancement directly reflects the transport system. This resistance appears to possibly be constitutive in the 12715 S. aureus cells. On the other hand, the 14154 cells appear to be inducible when grown at subinhibitory concentrations of the antibiotic. These results correlate well with previously published results (22–25) in which many of the tetracycline resistant S. aureus strains have been shown to be inducible and lacking the ability to accumulate the antibiotic. Previously, the resistance mechanism was thought to be only inducible (26). We are presently investigating further properties of the resistance mechanism and its role in membrane function.

Arrhenius plots of the temperature dependence of the tetracycline transport are found to be biphasic as measured with a transport bound probe. The transition point appears at 27°. Abrupt changes in the activation energy of the transport, as demonstrated here, have been shown to reflect reversible temperature-dependent phospholipid phase transitions in the membranes of E. coli (27–31) and Acholeplasma laidlawii (32, 33). Below the transition points, the lipids of the membranes are considered to be in a rather rigid, semi-crystalline state. At temperatures above the transition point the membrane lipids become more fluid and give rise to increased lipid permeability. In our present study, increased membrane fluidity may result in increased movement of the CTC to some lipid association transport site or it may reflect the enhanced rate of movement of the CTC transport complex through the membrane.

Recent spin label studies of Shimshick and McConnell (29) in phospholipid dispersions suggest that the lipid bilayers do not have sharp phase transitions but rather a gradual onset of phase separations. Above a critical temperature (t_h) all of the lipids are in a free fluid state. Below the t_h the lipids separate into fluid and nonfluid domains until a second critical temperature (t_l) is reached. Here all of the lipids are in the nonfluid state. Tsukogoshi and Fox (34) have pointed out that membrane processes might conceivably be effected by the change in state of the lipids at t_h, t_l or both t_h and t_l. Linden et al. (30) have reported finding two characteristic temperatures in fatty acid auxotrophs of Escherichia coli in both spin label studies and in Arrhenius plots of rate studies of sugar transport. We have attempted to identify a lower characteristic temperature for the CTC transport. Although a second change in the slope of the Arrhenius plots could not be detected at lower temperatures, a discontinuity in the plots did occur at 10° where the observed rate goes to zero. This finding suggests that the 27° transition corresponds to t_h and the 10° discontinuity to the t_l. Both the upper temperature of the phase transition and the lower temperature discontinuity can be shifted to lower temperatures as the degree of unsaturation of the fatty acids in the S. aureus is increased (unpublished results). A similar effect was reported for both the t_h and t_l in the E. coli system (30).

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