Journal of Supramolecular Structure 4:515 (475)–520 (480) (1976)

LIGHT-INDUCED TETRACYCLINE ACCUMULATION BY RHODOPSEUDOMONAS SPHAEROIDES

Jürgen Weckesser and James A. Magnuson

Program in Biochemistry and Biophysics, Department of Chemistry, Washington State University, Pullman, Washington

Light has been used as a primary energy source in studies of tetracycline transport by Rhodopseudomonas sphaeroides. Accumulation of the antibiotic occurs in light, while efflux occurs in dark. Both fluorescence enhancement and radioisotopic tracing have been used to monitor transport. K_m's obtained from both techniques are similar. Light-induced accumulation of tetracyclines is inhibited by a variety of inhibitors, including antimycin A, N-ethylmaleimide, carbonylcyanide m-chlorophenylhydrazone, and 2,4-dinitrophenol. A rapid efflux is observed after loading when cells are placed in the dark or treated with inhibitors.

INTRODUCTION

Tetracycline antibiotics are believed to be accumulated through an active process by both Gram-negative and Gram-positive bacteria. Franklin (1) has recently reviewed much of the work. Most transport studies have been conducted by following influx or efflux of a radioisotopically labeled tetracycline. This laboratory has demonstrated that fluorescence of the antibiotics themselves can be used to follow accumulation in Staphylococcus aureus (2–4). We have also been successful in applying this to other bacteria, both Gram-positive and Gram-negative (unpublished work). The study reported herein is a preliminary report of an investigation employing light as a primary energy source for active transport of tetracyclines. The phototrophic bacterium R. sphaeroides was chosen for these studies. As with most tetracycline-sensitive bacteria, we observed a large, time-dependent fluorescence enhancement for the tetracycline. This enhancement parallels uptake and has been used to examine the transport process.

MATERIALS AND METHODS

Rhodopseudomonas sphaeroides 28/3A was obtained from the strain collection of

Jürgen Weckesser is at the Institut für Biologie II, Lehrstuhl für Mikrobiologie der Universität, D-78 Freiburg i. Br., Germany.

the Institut für Biologie II, Lehrstuhl für Mikrobiologie der Universität, Freiburg i. Br., Germany. Cells were grown anaerobically at 30°C in light (500 foot-candles) in the malate-yeast extract medium R8ÄH of Drews (5). Bacteria at late log phase were collected by centrifugation (8,000 × g, 5 min). They were washed twice with MOPS buffer (20 mM morpholinopropane sulfonic acid, 15 mM KCl, pH 7.1 as adjusted with NaOH). The bacteria were resuspended in the same buffer to the desired concentration.

[7-^{3}H] Tetracycline hydrochloride from Amersham/Searle was diluted with unlabeled tetracycline hydrochloride from ICN Pharmaceuticals as described previously (4). Solutions of labeled (sp act 1,000 cpm/μg) or unlabeled tetracyclines were always prepared just prior to use.

Light-dependent accumulation of [7-^{3}H] tetracycline was observed both in culture medium and in MOPS buffer (see above). For uptake studies in culture medium, 20 ml of growing bacteria (OD_{660nm} = 1.0) were added to 20 ml of R8ÄH medium, which had been deoxygenated with a stream of nitrogen and contained 100 μg/ml of [7-^{3}H]-tetracycline. Light energy, 600 foot-candles, was supplied to this dispersion by a common 60-W incandescent bulb. This is a suitable light source for purple bacteria (6). At indicated time intervals, 2-ml samples were withdrawn, filtered through 1.2 μm Millipore filters, and washed three times with 2 ml of 4°C MOPS buffer. The filters were counted in 10 ml of Bray's solution (7). Bacterial growth which occurred during the experiment was accounted for by correcting for cell numbers determined from an optical density measurement.

The fluorescence experiments were carried out in MOPS buffer as discussed previously (3). Equal volumes of bacteria and tetracycline were mixed in a fluorescence cuvette and the fluorescence emission at 520 nm was observed upon excitation at 400 nm. For continuous monitoring of the light-dependent uptake a 50- or 100-ml incubation bottle was illuminated with 600 foot-candles of light outside the spectrofluorimeter sample compartment. A pump was used to circulate the bacteria dispersion between the fluorimeter and incubation bottle. Light energy was removed by either stopping the pump or removing the light source from the incubation bottle. Inhibitors were added to final concentrations as indicated either upon mixing of bacteria in the single mixing experiments or upon addition to the incubation mixture when the flow system was employed. Antimycin A was added as a 10 mM ethanol solution. Carbonylcyanide m-chlorophenylhydrazone (CCCP), 1 mM, and 0.1 M 2,4-dinitrophenol or 0.1 M N-ethylmaleimide (NEM) were added as aqueous solutions.

RESULTS

The uptake of [7-^{3}H] tetracycline from culture medium was found to be light dependent. As shown in Fig. 1, R. sphaeroides accumulates tetracycline in the light. In the dark, a rapid efflux occurs. After efflux, light can again be used to induce accumulation. When cells are not exposed to light after mixing with antibiotic, relatively little accumulation is observed. The cells become leaky after exposure to light for 60 min in the presence of the antibiotic. This presumably is due to the antibacterial effect of the antibiotic.

When suspensions of R. sphaeroides in MOPS buffer are incubated with tetracycline, the time-dependent fluorescence enhancement is also determined by the light source. Using the circulating flow system, enhancement of fluorescence was observed upon ex-

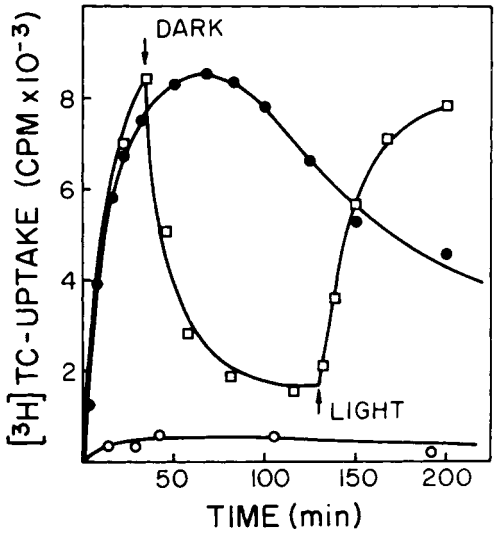

Fig. 1. Light dependence of [7-^{3}H] tetracycline, [^{3}H] TC, uptake by R. sphaeroides in culture medium. The cells were kept in the dark, ○, or continuously exposed to light, ●. A 30-min exposure to light, □, is followed by a dark period which is followed by exposure to light.

posure to light. Diminution of fluorescence was observed when the light was removed or the pump stopped, leaving the bacteria in the cuvette exposed only to 400 nm light from the fluorimeter. To establish that fluorescence enhancement monitors accumulation, the fluorescence was monitored in a dispersion of bacteria. In a parallel experiment with bacteria from the same culture carried out under identical conditions, [7-^{3}H] tetracycline uptake was monitored by sampling at appropriate times. Figure 2 demonstrates clearly that fluorescence enhancement parallels total accumulation as detected by tracer techniques.

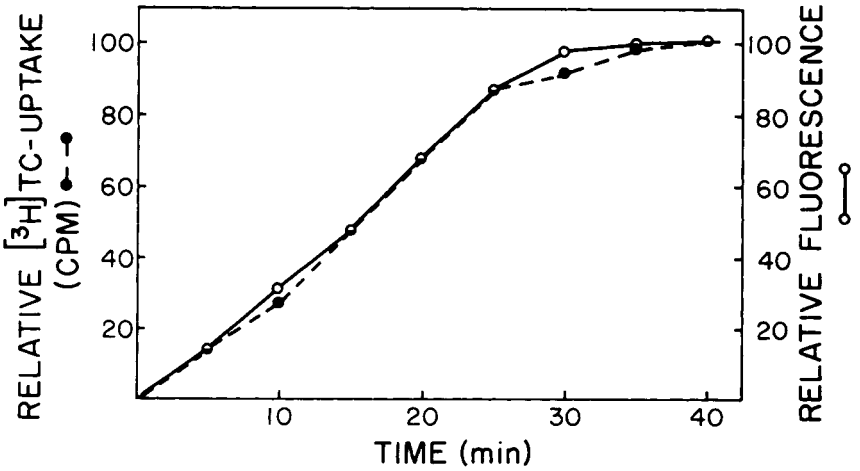

Fig. 2. Time-dependence in light of fluorescence enhancement, ○, and [7-^{3}H] tetracycline, [^{3}H] TC, accumulation, ●. The values of each are normalized to show correspondence.

To establish that the fluorescence enhancement and tracer accumulation resulted from carrier-mediated transport, K_m's were determined from rates of uptake observed in both methods. Figure 3 presents data in a double reciprocal plot for fluorescence enhancement. The K_m determined from Fig. 3 is 400 μM for cells in the presence of 0.25 mM Mg^{2+}. A K_m of 300 μM was found for osmotically shocked cells in MOPS buffer with no Mg^{2+}. In the tracer studies (Fig. 4), a K_m of 540 μM for intact cells in MOPS with 0.5 mM Mg^{2+} was found. Rates were obtained from samples withdrawn at 1, 4, and 10 min. Uptake was linear over this time period. These three values are identical within experimental error.

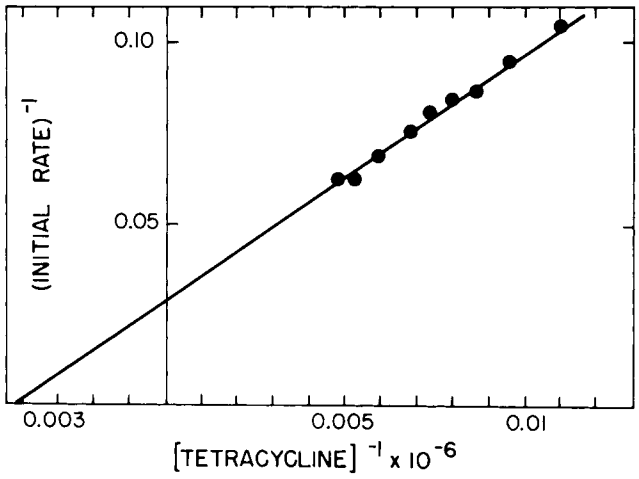

Fig. 3. Lineweaver-Burk plot from initial fluorescence enhancement rates. Bacteria were in MOPS buffer containing 0.25 mM Mg^{2+}.

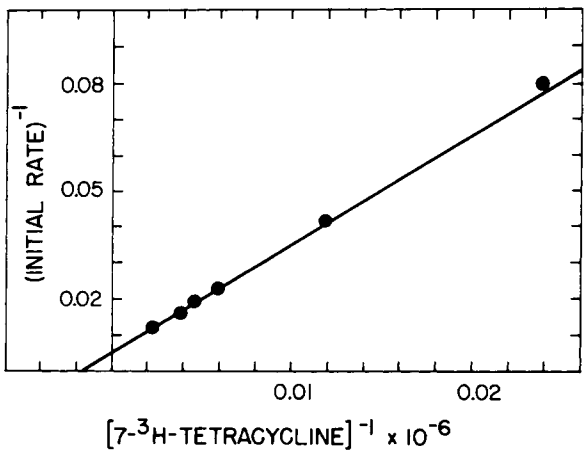

Fig. 4. Lineweaver-Burk plot using [7-^{3}H] tetracycline accumulation rates at various antibiotic concentrations. Bacteria were in MOPS buffer containing 0.5 mM Mg^{2+}.

When light is removed or inhibitors are added, efflux of the accumulated antibiotic occurs. Efflux occurs at all times during accumulation. At steady state in light, cells exchange accumulated antibiotic with that in the medium. Figure 5 shows one type of experiment. After accumulating [7-^{3}H] tetracycline, cells were diluted into cold tetracycline of the same concentration. Because of exchange, the amount of label in the cells decreases, although the absolute amount remains constant if cells are diluted into labeled tetracycline of the same concentration. In a similar manner, it can be shown that exchange will occur at various accumulation times. Steady state is reached, therefore, when efflux balances influx.

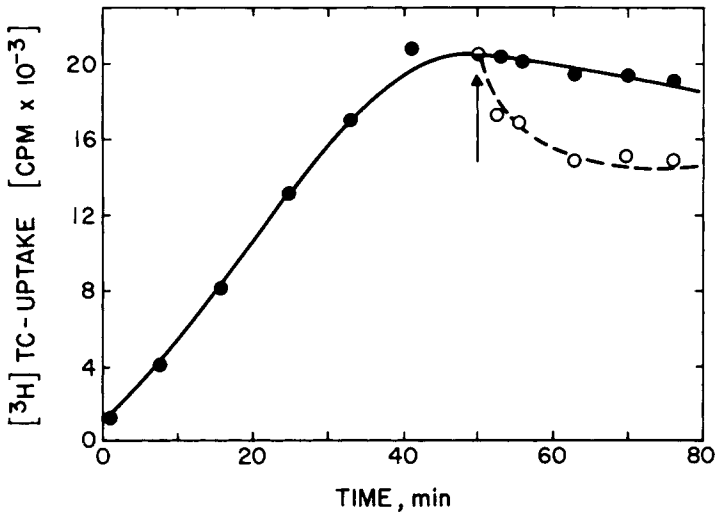

Fig. 5. Exchange of [7-^{3}H] tetracycline, [^{3}H]TC, with unlabeled tetracycline. Bacteria were diluted at the arrow into unlabeled tetracycline, ○. Steady-state level remains constant in cells in original dispersion before dilution, ●.

DISCUSSION

Light is an excellent energy source for studies of tetracycline accumulation by the phototrophic R. sphaeroides. Light can be introduced continuously in controlled amounts during the time course of uptake. It is also easily removed. With S. aureus or E. coli glucose will serve as an energy source, but experimental difficulties obviously arise when one wants to remove the energy quickly.

Our results clearly show that fluorescence follows the carrier-mediated transport of tetracycline antibiotics. This has been shown with both correlation of fluorescence enhancement and tracer accumulation. The K_m for transport determined in both techniques is the same. Brewer (8) recently has concluded that fluorescence does not follow accumulation in E. coli W-6. Although reasons for this are not clear at this time, he has worked with a system in which he may have induced changes in quantum yield.

In many experiments with Gram-negative bacteria, a carrier-mediated transport mechanism has not been convincingly demonstrated. Franklin (1) has reviewed the situation in detail. Besides R. sphaeroides, this laboratory has been able to demonstrate

saturation kinetics with a variety of Gram-negative organisms including E. coli (Lindley and Magnuson, unpublished results).

Because the light source can be manipulated easily, efflux is amenable to study. The efflux rates are very rapid, but fluorescence allows one to monitor this process continuously. The relation of efflux to influx and the energy requirement are now being examined. Lindley and Magnuson (unpublished results) have demonstrated a complex permeability dependence on Mg^{2+} with Gram-negative bacteria. This is also presently under investigation.

ACKNOWLEDGMENTS

This work was supported in part by Washington State University Research Committee Funds. J. Weckesser thanks the Deutsche Forschungsgemeinschaft for a fellowship.

REFERENCES

1. Franklin, T. J., CRC Crit. Rev. Microbiol. 2:253 (1973).
2. Dockter, M. E., and Magnuson, J. A., Biochem. Biophys. Res. Commun. 54:790 (1973).
3. Dockter, M. E., and Magnuson, J. A., J. Supramol. Struct. 2:32 (1974).
4. Dockter, M. E., and Magnuson, J. A., Arch. Biochem. Biophys. 168:81 (1975).
5. Drews, G., Zbl. Bakt. I. Abt. Orig. Suppl. 1:170 (1965).
6. Biebl, H., and Drews, G., Zbl. Bakt. II 123:425 (1969).
7. Bray, G. A., Anal. Biochem. 1:279 (1960).
8. Brewer, G. J., Biochemistry 13:5038 (1974).