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IN VITRO [^{3}H]-ERYTHROMYCIN BINDING TO *STAPHYLOCOCCUS AUREUS*

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Abstract—Characteristics of erythromycin binding to *Staphylococcus aureus* were determined by using kinetics and equilibrium binding experiments. Both methods yielded identical values of the dissociation constant, i.e. 0.1 μM. This value was in accord with that found with a bacterial extract of ribosomes which are the organelles where erythromycin exerts its action. This good agreement shows that the dissociation constant of erythromycin determined with intact bacteria is a good reflect of specific bacterial receptors of macrolides, i.e. ribosomes. In addition, mechanism of uptake of the antibiotic by *Staphylococcus aureus* was investigated. Passive diffusion process was shown to be mainly responsible for this phenomenon.

The specific receptors of sensitive microorganisms [1–6] have now been well identified for the macrolide group. Only a few investigations report the precise evaluation of binding parameters of these antimicrobial agents to bacteria and their sites of action. This paper presents such a study with the major representative of 14 membered macrolides, i.e. erythromycin, and a susceptible strain of *Staphylococcus aureus*. We were interested in checking whether the binding parameters of the drug obtained with the intact bacteria were really reflecting those of the receptor, i.e. the ribosomes where macrolides exert the inhibition of protein synthesis. In addition, an investigation concerning the mechanism of uptake of antibiotics by the bacteria was carried out.

MATERIAL AND METHODS

Drugs. [^{3}H]-erythromycin (25 Ci/mmole) was supplied by the Laboratory of Structural Organic Chemistry (Université des Sciences et Techniques du Languedoc, Montpellier). Radiochemical purity was better than 98% as determined by thin layer chromatography on silica gel in carbon tetrachloride–ethanol–dimethylformamide (7:2:1). Unlabelled erythromycin was supplied by Abbott Laboratories.

Bacterial strain and culture media. The strain was selected in the laboratory collection of the Service de Bacteriologie (Hôpital Henri Mondor). *S. aureus* 209P (CNCM 53156) were grown in overnight at 37° on Petri dishes with Mueller–Hinton agar. Then a mixture of 0.4 ml Brain Heart Infusion Broth (BHI) (Difco) and 19.6 ml water was seeded with two *S. aureus* colonies and incubated for 18 hr at 37°. At this time, the stationary phase was attained and remained constant for 3 hr, since after that period, the optical density measurements (624 nm) and the cell counting showed a decrease in viable bacteria.

Preparation of bacterial ribosomes. *S. aureus* 209P ribosomes were prepared as described by Mao [3]. Bacterial cells crushed with alumina were centrifuged at 30,000 *g* for 1 hr at 4°. Then, the supernatant was subjected to another centrifugation at 105,000 *g* for 2 hr in order to collect the ribosomes in the pellet. The pellet was resuspended in Tris buffer at pH 7.4 (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 25 mM Tris). The protein concentration was measured by the Lowry method [7]. The RNA concentration was determined by the orcinol method [8].

Binding assays. All studies were routinely carried out in polypropylene tubes containing an aliquot of the bacterial or ribosomal suspension incubated with [^{3}H]-erythromycin in the presence or absence of unlabelled erythromycin depending upon the type of experiments, i.e. kinetics or equilibrium studies of [^{3}H]-erythromycin. After incubation, bound and free ligands were then separated by fast ultrafiltration through Millipore filters 0.5 μm, EHWP 02500. Each filter was rapidly washed at 4° with an additional volume of twice 10 ml of Tris buffer. The radioactivity on the filter disc was counted in 5 ml of Picofluor® 30 in scintillation spectrometer Packard Tri Carb 460 CD.

Association and dissociation kinetics of [^{3}H]-erythromycin binding to S. aureus. The bacterial suspension prepared in BHI broth was incubated with [^{3}H]-erythromycin. The final concentration of [^{3}H]-erythromycin was 10^{-8} M and the mixture contained 10^7 cfu/ml. Then, the association kinetics of [^{3}H]-erythromycin binding was measured at 37° for 90 min in the presence and absence of potassium cyanide (KCN 10^{-3} M) and 2–4 dinitrophenol (DNP 10^{-4} M). The association kinetics of [^{3}H]-erythromycin alone was also measured at various temperatures: 4, 25, 37 and 42°. The dissociation kinetics was carried out by adding unlabelled erythromycin (10^{-4} M) to a reaction mixture previously incubated at 37° for 60 min and containing [^{3}H]-erythromycin (10^{-8} M) and 10^7 cfu/ml. In all these experiments 500 μl ali-

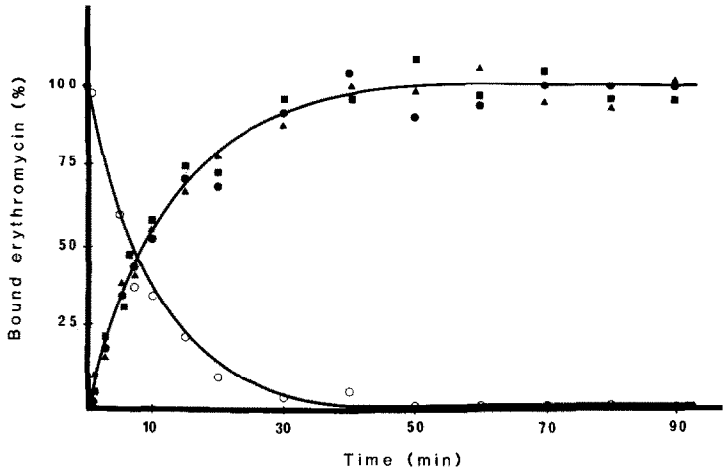

Fig. 1. Kinetic studies of [^{3}H]-erythromcyin binding to *Staphylococcus aureus*. Association kinetics was carried out by incubating [^{3}H]-erythromycin (10^{-8} M) with bacteria (4.5 10^7 cells/ml) at 37° (●—●) and in the presence of KCN (10^3 M) (■—■) and 2.4 dinitrophenol (10^{-4} M) (▲—▲). Dissociation kinetics (○—○) was measured after the addition of unlabelled erythromycin (10^{-4} M) to a reaction mixture containing [^{3}H]-erythromycin (10^{-8} M) and bacteria (4.5 10^7 cells/ml) in which equilibrium was attained after 50 min incubation.

quots of the reaction mixtures were filtered and the radioactivity counted as indicated above.

Equilibrium studies of [^{3}H]-erythromycin binding to S. aureus. The binding of [^{3}H]-erythromycin was measured after 1 hr incubation at 37° of a portion of bacterial suspension in BHI broth (10^7 cfu/ml) with Tris buffer containing increasing amounts of [^{3}H]-erythromycin (final concentrations = 10^{-9}–8.10^{-7} M) in the presence and absence of 10^{-4} M unlabelled erythromycin. 500 µl of each of these reaction mixtures were filtered and the radioactivity was measured as indicated above. Specific binding was defined as total bound radioactivity minus the radioactivity not displaced by 10^{-4} M erythromycin.

Equilibrium studies of [^{3}H]-erythromycin binding to ribosomes of S. aureus. The binding of [^{3}H]-erythromycin was studied at 37° after 1 hr incubation of the reaction mixture. Preliminary experiments showed that equilibrium was attained at that time. The reaction mixture consisted of ribosomes (final protein concentration: 0.250 mg/ml) and of increasing amount of [^{3}H]-erythromycin (10^{-9}–8.10^{-7} M) in the presence and the absence of 10^{-4} M unlabelled erythromycin. The procedures to evaluate the binding were the same as described above.

Calculations of kinetic and binding constants. The kinetic dissociation constant (k_{-1}) and association constant (k_{+1}) were calculated according to the equation of Engel *et al.* [9]. The ratio k_{-1}/k_{+1} yields the equilibrium dissociation constant (K_D). K_D was also estimated with the density of binding sites (B_{max}) from equilibrium binding studies by means of the non-linear squares method using a Gauss–Newton algorithm [10].

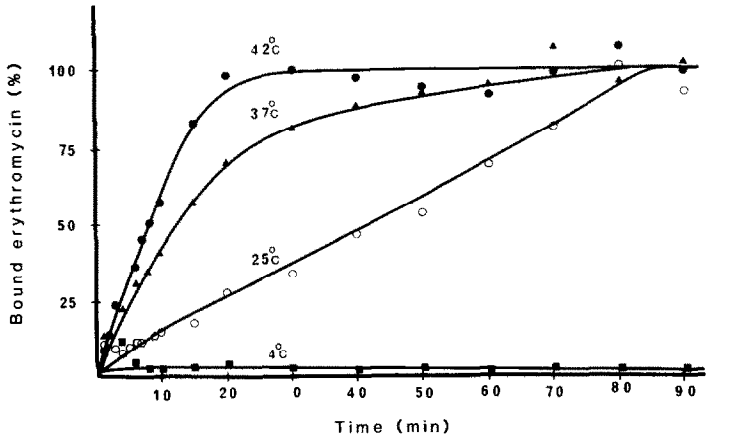

Fig. 2. Kinetics of [^{3}H]erythromycin binding to *Staphylococcus aureus* at different temperatures. Association kinetics was measured by incubating bacteria (10^7 bacteria/ml) and [^{3}H]-erythromycin (10^{-8} M). The temperatures of incubation were 42° (●—●), 37° (▲—▲), 25° (○—○) and 4° (■—■).

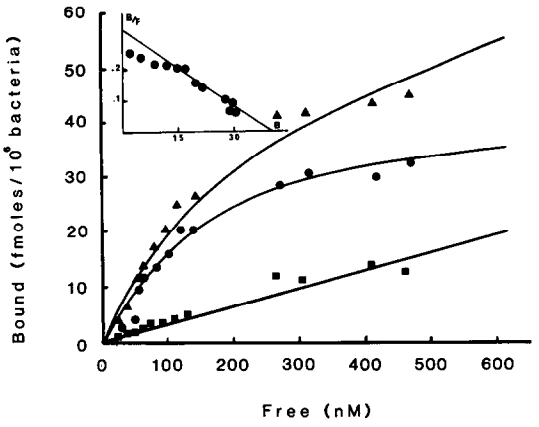

Fig. 3. Equilibrium binding of [^{3}H]-erythromycin to *Staphylococcus aureus*. Specific binding (●—●) was calculated by difference between total (▲—▲) and non specific (■—■) bindings in presence of 10^{-4} M erythromycin. Binding studies were performed at 37° after a period of 60 min incubation. The reaction mixture contained 10^7 cfu/ml. Scatchard plot for specific [^{3}H]-erythromycin binding is shown in insert.

RESULTS

Kinetics of [^{3}H]-erythromycin binding to S. aureus

The association kinetics of [^{3}H]-erythromycin to *S. aureus* shows that equilibrium is reached at 50 min (Fig. 1). The presence of potassium cyanide and 2–4 dinitrophenol did not alter this association kinetics. As shown in this figure, the reversibility of [^{3}H]-erythromycin binding is demonstrated by the dissociation curve. The association and dissociation constant rates were $k_{+1} = 6.5.10^5$/M/min and $k_{-1} = 0.073$/min respectively. The value of the equilibrium dissociation constant, i.e. $K_D = k_{-1}/k_{+1}$ was 0.11 μM. The kinetics of [^{3}H]-erythromycin binding varies with temperature. As illustrated in Fig. 2, the uptake of the drug decreases as the temperature lowers. No uptake was noted at 4°.

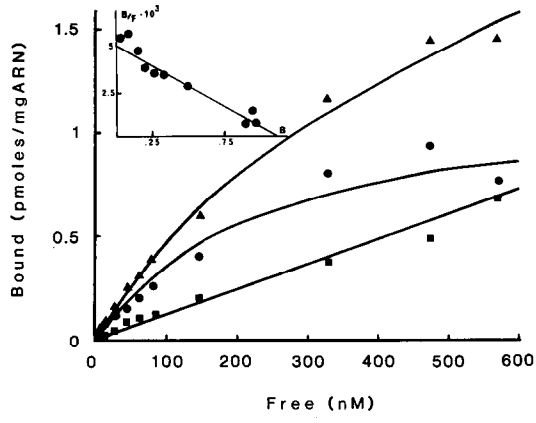

Fig. 4. Equilibrium binding of [^{3}H]-erythromycin to a ribosomal preparation of *Staphylococcus aureus*. Specific binding (●—●) was calculated by difference between total (▲—▲) and non specific (■—■) bindings in presence of 10^{-4} M erythromycin. Binding studies were performed at 37° after a period of 60 min incubation. Scatchard plot for specific binding of [^{3}H]-erythromycin is shown in insert.

Equilibrium studies of [^{3}H]-erythromycin binding to S. aureus

Figure 3 shows the equilibrium binding experiments of [^{3}H]-erythromycin to *S. aureus*. The specific binding was saturable with one class of binding sites as shown by the linear Scatchard plot (Fig. 3, insert). The calculation of the parameters by the non-linear method yielded an equilibrium dissociation constant $K_D = 0.15$ μM and a density of binding sites $B_{max} = 34.5$ fmoles/10^6 cfu.

Equilibrium studies of [^{3}H]-erythromycin binding to ribosomes of S. aureus

Likewise, the binding to ribosomes showed one class of saturable binding sites (Fig. 4). The density of binding sites was $B_{max} = 1.20$ pmoles/mg RNA. The dissociation constant was $K_D = 0.22$ μM very close to that obtained with the bacterial cells.

DISCUSSION

Our results showing that the association kinetics of [^{3}H]-erythromycin is not altered by uncoupling agents of oxidative phosphorylation such as KCN and DNP seem to indicate that no active process is involved in the uptake of the antibiotic by *S. aureus*. This observation is in accordance with that of Mao *et al.* [11]. In addition, Perrin *et al.* found that passive diffusion process accounted for the penetration of pristinamycin into *S. aureus* [12]. The decrease in the binding which was noted with the fall of temperature is likely to be due to a decreased brownian movement of the drug molecules, which may constitute a significant impediment to the permeation of erythromycin into the bacteria. The dissociation kinetics clearly shows that the interaction between erythromycin and *S. aureus* is reversible.

These association and dissociation kinetics experiments provided rate constants whose ratio (k_{-1}/k_{+1}) yielded a dissociation constant (K_D) identical to that determined in the equilibrium binding experiments with *S. aureus*. In addition, it should be emphasized that these values were very close to the K_D value found in the equilibrium binding experiments with ribosomes of *S. aureus*. The agreement between these values gives a good evidence that the dissociation constant measured on whole bacteria is that of the site of action or the binding site of the macrolides, within the bacterial cell, i.e. the ribosomes [13]. The density of binding sites (B_{max}) measured on *S. aureus* and the ribosomes cannot be compared since a proper comparison requires the knowledge of the yield of the ribosomes extraction from a given number of bacterial cells. In our experiments, the yield could not be evaluated precisely.

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REFERENCES

1. S. Siegrist, S. Velitchkovitch, F. Le Goffic and N. Moreau, *J. Antibiot.* **35**, 866 (1985).
2. S. Siegrist, J. Lagouardat, N. Moreau and F. Le Goffic, *Eur. J. Biochem.* **115**, 323 (1981).
3. J. C. H. Mao, *J. Bacteriol.* **94**, 80 (1967).
4. K. Tanaka, H. Teraoka, T. Nagira and M. Tamaki, *Biochim. biophys. Acta* **123**, 435 (1966).
5. H. Teraoka and K. H. Nierhaus, *J. mol. Biol.* **126**, 185 (1978).
6. S. B. Taubman, N. R. Jones, F. E. Young and J. W. Corcoan, *Biochim. biophys. Acta* **123**, 438 (1966).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farral and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
8. M. W. Nirenberg, in *Methods in Enzymology* (Ed. S. P. Colowick), Vol. 6, p. 17. Academic Press, New York (1964).
9. G. Engel, D. Hoyer, R. Berthold and H. Wagner, *Naunyn-Schmiedeberg's Arch. Pharmac.* **317**, 277 (1981).
10. R. Zini, P. D'Athis, J. Barré and J. P. Tillement, *Biochem. Pharmac.* **28**, 2661 (1979).
11. J. C. H. Mao and M. Putterman, *J. Bacteriol.* **95**, 1111 (1968).
12. P. Perrin, M. L. Capmau and F. Le Goffic, Abst. 99/C7. Reunion Interdisciplinaire de Chimiotherapie Antiinfectieuse, Paris, 8 December 1983.
13. J. C. H. Mao and M. Putterman, *J. mol. Biol.* **44**, 347 (1969).