STUDIES ON PRISTINAMYCIN SYNERGISM IN *STAPHYLOCOCCUS AUREUS*

PATRICIA LACROIX, MARC AUMERCIER,
MARIE-LOUISE CAPMAU and FRANÇOIS LE GOFFIC

Centre National de la Recherche Scientifique,
Centre d'Etudes et de Recherches de Chimie Organique Appliquée,
(Laboratoire Associé à l'Ecole Nationale Supérieure de Chimie de Paris),
2 à 8, rue Henry Dunant 94320 Thiais, France

(Received for publication February 1, 1986)

Binding experiments were performed with both components of the pristinamycin complex (pristinamycin I_A (PI_A) and pristinamycin II_A (PII_A)) using ribosomes from sensitive and resistant *Staphylococcus aureus*. Fluorescence polarization was used to measure PI_A binding. The results obtained show a direct correlation between inhibition, synergy and the enhancement of the affinity of PI_A for its receptor in the presence of PII_A. The uptake of PI_A by intact cells seems to be directly correlated with affinity between PI_A and ribosomes, a phenomenon which is probably shared with the macrolide antibiotics.

Pristinamycins[1] comprise a complex of two structurally distinct molecules that act on the 50S subunit of bacterial ribosomes. Individually these compounds have a bacteriostatic activity against Gram-positive bacteria. As a mixture, they exhibit strong synergistic and bactericidal effects[2~4].

Previous studies with *Escherichia coli* cell free extracts have shown that the pristinamycins bind to the bacterial ribosome. The interaction of PI_A with its receptor site is enhanced by the presence of PII_A[5~11] and the latter antibiotic inhibits protein synthesis *in vitro*. Studies with staphylococcal ribosomes are difficult because the ribosomes are not easy to prepare in active form and have different physico-chemical properties with respect to those of *E. coli*[12].

We have previously demonstrated that both molecules PI_A and PII_A enter the bacteria by the means of passive diffusion and suggested that their strong binding to bacterial ribosomes could help their accumulation within the cell[13,14]. These results prompted us to develop further studies on cell-free extracts from *Staphylococcus aureus*. We report here the analysis of the binding of pristinamycins to *S. aureus* ribosomes and compare our results with those previously obtained on *E. coli* ribosomes. We also discuss binding experiments with ribosomes from PI_A sensitive and resistant *S. aureus* strains which explain the relationship between the accumulation and the binding of these antibiotics.

Materials and Methods

Chemicals

All chemicals were of analytical grade whenever available. Reagents sources were; polyethyleneglycol (PEG) 6000 from Touzart et Matignon, pristinamycins from Rhône-Poulenc. [^{3}H]Dihydropristinamycin I_A([^{3}H]H_2PI_A) and II_A([^{3}H]H_2PII_A) were synthesized as described previously[9].

Buffer A contained Tris-HCl 10 mM (pH 7.6), $Mg(OAc)_2$ 8 mM, NH_4Cl 100 mM. Buffer B contained Tris-HCl 10 mM (pH 7.6), $Mg(OAc)_2$ 10 mM, NH_4Cl 100 mM. Buffer C contained Tris-HCl 10 mM

List of abbreviations: PI_A; pristinamycin I_A, PII_A; pristinamycin II_A, [^{3}H]H_2PI_A; tritiated dihydropristinamycin I_A, [^{3}H]H_2PII_A; tritiated dihydropristinamycin II_A, MIC; minimal inhibitory concentration.

Table 1. Minimal inhibitory concentrations of pristinamycins determined by clonical agar dilution technique.

Test organism	MIC (μg/ml)		
	PI_A	PII_A	PI_A+PII_A
Staphylococcus aureus 209P	8	1	0.2
S. aureus "SCH"	>60	2	0.5

(pH 7.6), $Mg(OAc)_2$ 10 mM, KCl 150 mM. Buffer D contained Tris-HCl 300 mM (pH 7.4), $Mg(OAc)_2$ 10 mM, KCl 100 mM.

Bacterial Strain

S. aureus "SCH" a constitutive macrolide-lincosamide-streptogramine (MLS) resistant strain was a clinical isolate supplied by D. BOUANCHAUD (Rhône-Poulenc Santé, Paris, France). It expresses MLS resistance constitutively to a level greater than 100 μg/ml of erythromycin, spiramycin and lincomycin, and to a level of 60 μg/ml of pristinamycin I_A.

S. aureus strain 209P was from our laboratory collection. The minimal inhibitory concentrations of pristinamycins, determined by the agar dilution technique are presented in Table 1.

Preparation of Crude (S-30) Extract of *S. aureus*

S. aureus cells were grown in Tryptic Soy Broth (Difco) at 37°C. In middle logarithmic phase, they were slowly cooled, harvested by centrifugation and stored as a frozen paste at −20°C.

The cell paste was suspended in a cooled buffer A (1 mg/ml), mixed with five times its weight of glass beads (110 μm diameter) and disrupted with a cell homogenizer MSK (Braun) cooled by solid CO_2 (shaking frequency=4,000 rpm, homogenization time=10×40 seconds).

Five μg of deoxyribonuclease I (grade I) (Boehringer) was added per ml of suspension. After vigorous shaking for 30 minutes at 4°C, the glass beads were removed by centrifugation for 10 minutes at 5,000×g. The cellular debris was removed by centrifugation for 20 minutes at 10,000×g and for 30 minutes at 30,000×g. The supernatants (S-30) were immediately used for the preparation of ribosomes or stored frozen at −70°C.

Preparation of Ribosomes

Tight and vacant ribosomes as specified by NOLL *et al.*[15] were prepared in buffer A by zonal centrifugation, from S-30 extracts of *S. aureus* 209P as described previously[16]. The ribosome preparations were concentrated in buffer B using polyethyleneglycol[17] and stored at −20°C.

Salt-washed 70S ribosomes were prepared from *S. aureus* "SCH" by repeated suspension and centrifugation (2 hours, 100,000×g) in buffer B containing 1 M NH_4Cl as described previously[18].

Pristinamycin II_A Binding to Ribosomes

Titration of bound PII_A by the Norit A technique was used[8]. To buffer D, variable amounts of $[^3H]H_2PII_A$ were added and the reaction started by addition of ribosomes (8.6×10^{-7} M) (total volume; 200 μl). After incubation at 37°C for 20 minutes, the reaction was cooled on ice and 30 μl of a 2.5% (w/v) suspension of Norit A was added per 200 μl. The samples were homogenized, kept at 4°C for 10 minutes and centrifuged at 4,000×g for 15 minutes at 4°C. 100 μl samples of supernatant were counted in 5 ml scintillation fluid (Beckman HP).

Pristinamycin I_A Binding to Ribosomes

Methods based on centrifugation through sucrose gradients[19] were used. A reaction mixture containing $[^3H]H_2PI_A$ (1.2 Ci/mmol) 1 μM and 1~2 A_{260} nm units ribosomes or S-30 extract in 200 μl of buffer A were incubated for 30 minutes at 37°C, and then chilled on ice. 150 μl of reaction mixture were layered on 11 ml linear 5~30% sucrose gradients made in buffer A. 50 μl of buffer A were then carefully added. Centrifugation was in an SW 41 rotor in the Beckman ultracentrifuge for 4 hours at 4°C and at 40,000 rpm. Gradients were fractionated from the top and monitored at 254 nm in an Isco fractionator. Fractions (250 μl) were collected and the radioactivity was determined in a

scintillation spectrometer.

Determination of the Binding Parameters by Fluorescence Polarization (Anisotropy)

Fluorimetric measurements were performed at 37°C in a quartz cell. Increasing amounts of stock solutions of PI_A were added to a given concentration (between 0.01 and 3 μM) of 70S ribosomes (0.2 μM) in 1 ml buffer C in the optical cell. Immediately before titration, the ribosomes were incubated at 37°C for 30 minutes. Fluorescence anisotropy was measured with a Model 400 polarization fluorimeter (SLM Instruments, Urbana Ill) (for details see ref 20~22). Polarized emission spectra were taken with polarized excitation at 320 nm. Emitted light was measured through Schott KV 450 filters. At each point background correction was applied by parallel anisotropy measurement with omission of PI_A. This instrument allowed the direct measurement of the polarized intensity ratio $I_{//}/I_{\perp}$ and averaged from 10 to 100 values. These ratios, obtained with a vertically polarized excitation $a=(I_{//}/I_{\perp})$ and with a horizontally polarized excitation $b=(I_{//}/I_{\perp})$, allow us to calculate fluorescence anisotropy r given by:

$$r=(b/a-1)/(b/a+2) \quad (1)$$

Fluorescence anisotropy was used since, as opposed to fluorescence polarization, it is additive for mixed systems[23] and thus can be directly applied to binding systems[24]. Since the binding of an antibiotic was not found to entail a change of its total fluorescence intensity, it was possible to use the following intensity-independent formula for calculating the fraction of the total ligand bound[25] at any point on the titration curve corresponding to an anisotropy r

$$S=(r-r_{min})/(r_{max}-r_{min}) \quad (2)$$

where r_{min} and r_{max} are the fluorescence anisotropy when the ligand is completely free and bound, respectively.

The binding data are plotted according to SCATCHARD[26] with the equation adapted for the direct use of the variables $S/(1-S)C_T$ and SL_T/C_T.

$$S/(1-S)C_T=(1/K_D)(n-SL_T/C_T) \quad (3)$$

where C_T is the total ribosome concentration, L_T the total antibiotic concentration, n the number of sites and K_D the dissociation constant.

Fig. 1. Saturation curve of 70S ribosome from *S. aureus* 209P with increasing concentration of $[^3H]H_2PII_A$ and corresponding SCATCHARD plots.

The data were obtained by the Norit A technique in the conditions described in Materials and Methods.

RL represents bound pristinamycin and $\bar{\nu}$ the ratio RL/R_t with R_t concentration of ribosomes.

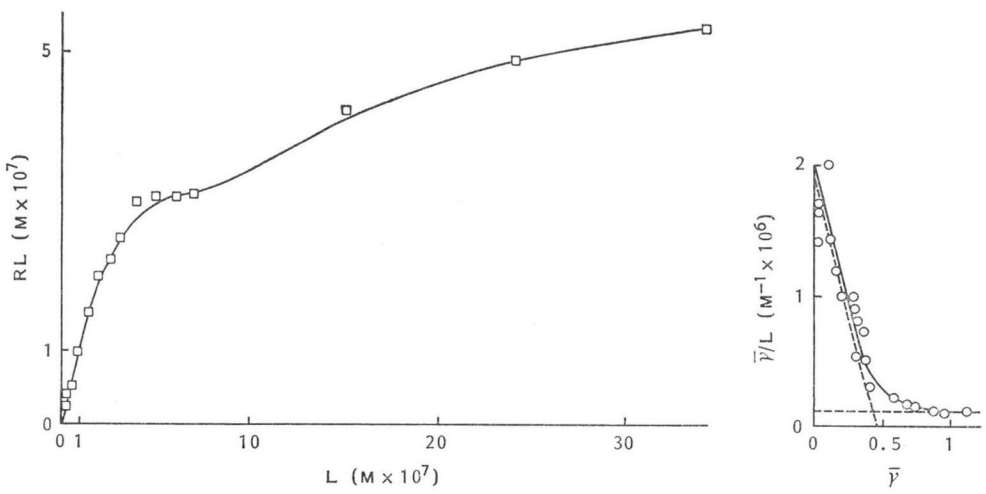
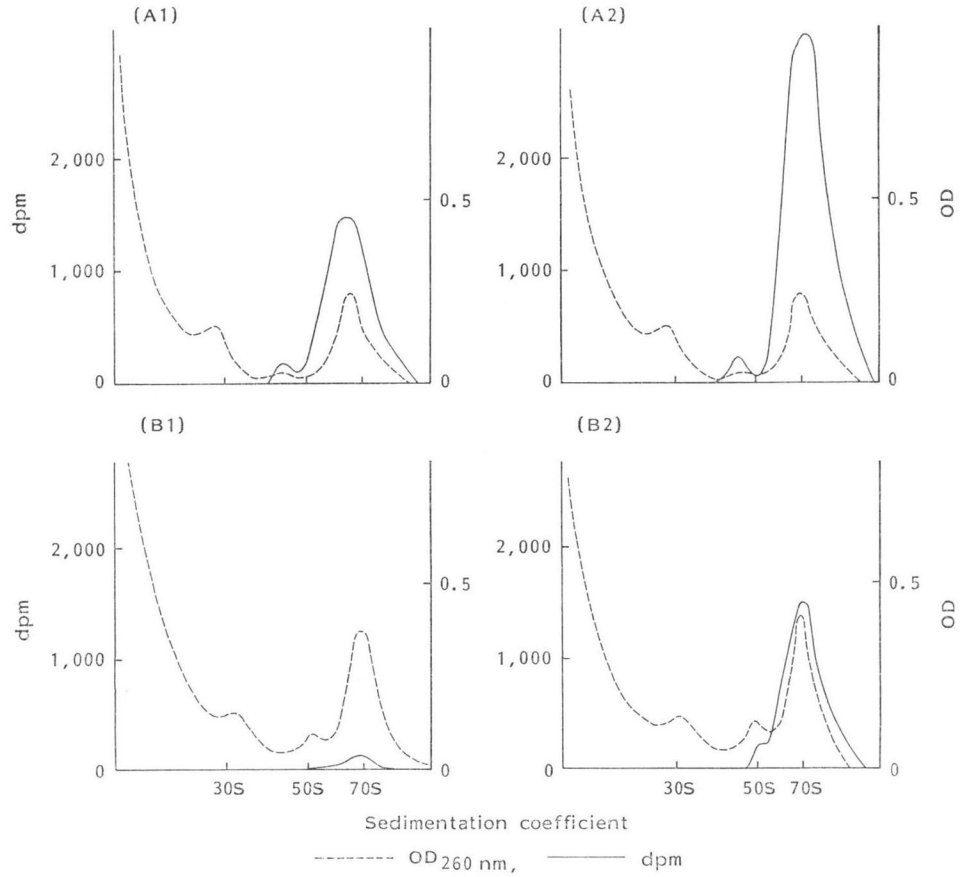


Fig. 2. Analytical gradient of S-30 incubated with radioactive PI_A in the absence (A1, B1) or presence (A2, B2) of pristinamycin II_A.
(A): Sensitive *S. aureus* strain, (B): PI_A resistant strain "SCH".
The experimental conditions are in Materials and Methods.



Accumulation Experiments

The accumulation of $[^3H]H_2PI_A$ by *S. aureus* "SCH" was assessed by partitioning in polymer aqueous phase system as described in previous papers[13,14].

Results

Binding of $[^3H]H_2PII_A$

We have shown previously that PII_A binds to the 70S ribosomes of *S. aureus* and *E. coli*[9] since association constants were only determined with *E. coli* ribosomes, we have measured binding parameters of PII_A with *S. aureus* 209P ribosomes. The technique used is based on the adsorption of unbound drug by Norit A[8]. Fig. 1 shows the retention of radioactive dihydropristinamycin II_A by *S. aureus* 209P 70S ribosomes. The SCATCHARD plot of the binding data suggests the presence of one high-affinity binding site with a K_D value of 5.6×10^{-7} M and multiple low-affinity binding sites.

Binding of $[^3H]H_2PI_A$

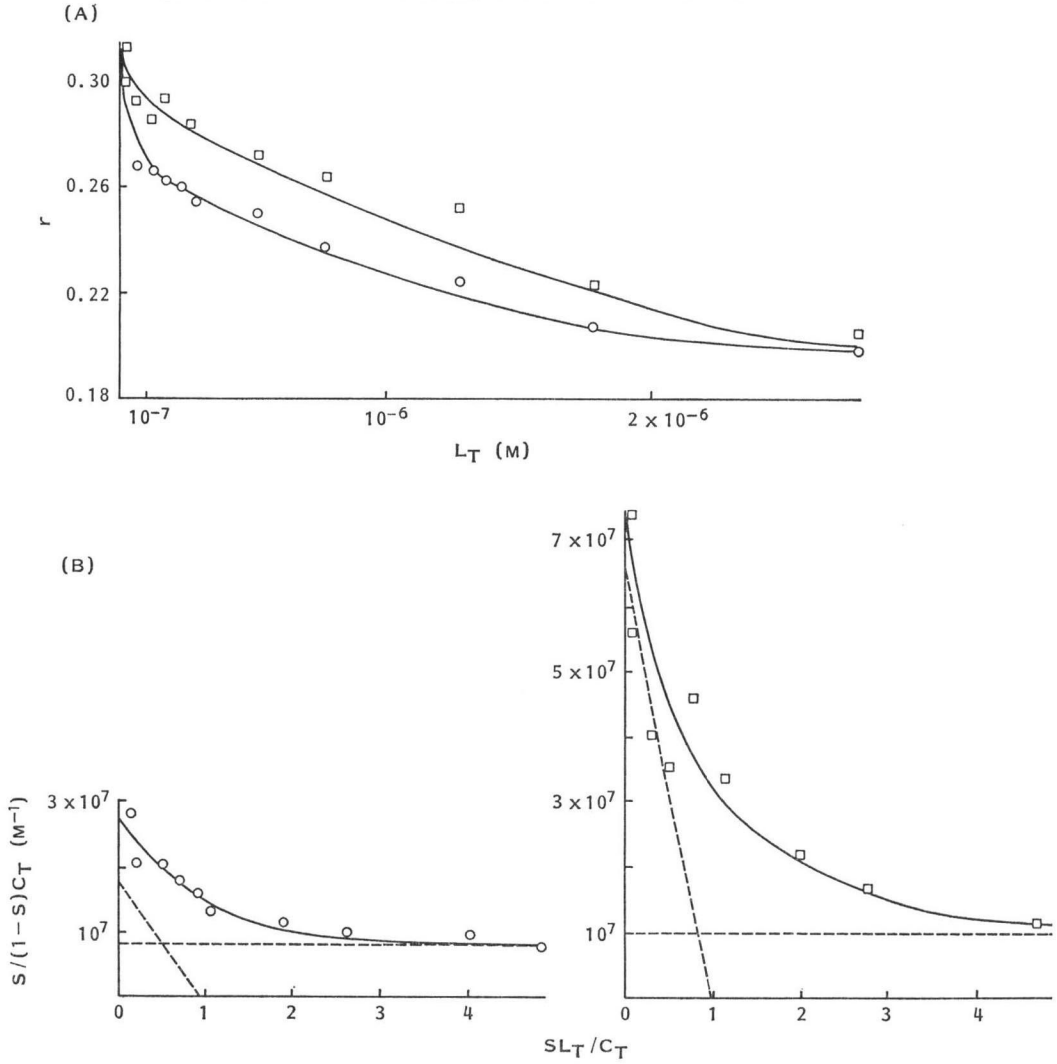
Formation of $[^3H]H_2PI_A$-ribosome complexes was studied by sucrose gradient centrifugation in the

Fig. 3.

(A): Evolution of the fluorescence anisotropy of PI_A with *S. aureus* 209P ribosome in the absence (○) and the presence of PII_A (□).

(B): SCATCHARD plot corresponding to the data of Fig. 3A.

The experimental conditions are indicated in Materials and Methods.



absence or the presence of PII_A, with PI_A sensitive and resistant *S. aureus* strains. As shown in Fig. 2A the amount of [^{3}H]H_2PI_A associated to ribosomes from the sensitive strain increases two-fold when PII_A is present in the medium whereas binding to ribosomes from the resistant "SCH" strain is only possible when PII_A is present in the medium (Fig. 2B).

Binding of the drug was also studied on 30S and 50S ribosomal particles. No binding of PI_A to 30S ribosomes was observed even in the presence of PII_A. The 50S particle from both strains behaved like the whole ribosome (results not shown).

PI_A-Ribosome Interaction Determined by Fluorescence Polarization (Anisotropy)

The fluorescence properties of PI_A have already been used for the determination of its binding parameters with *E. coli* ribosomes. An increase of its fluorescence intensity was observed when bound to

Fig. 4. Evolution of the fluorescence anisotropy of PI_A with PI_A resistant strain "SCH" ribosome in the absence (○) and the presence of PII_A (□) and related SCATCHARD plot.
The experimental conditions are in Materials and Methods.

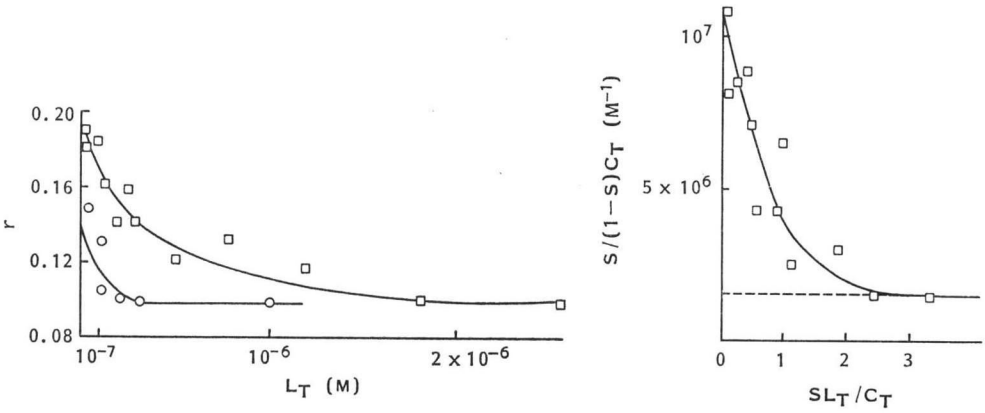
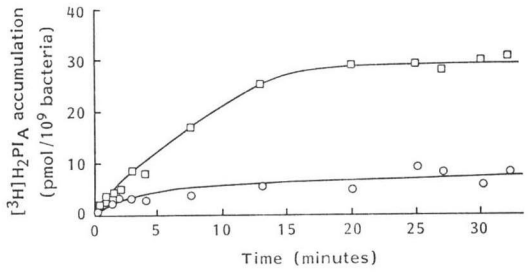


Table 2. Binding parameters of pristinamycins determinated with the SCATCHARD plots according to ROSENTHAL.

Antibiotic	H_2PII_A (α)	PI_A	
		Alone	$+PII_A$
Staphylococcus aureus 209P	$K_D=5.6\times10^{-7}$ M n=0.5	$K_D=6.3\times10^{-8}$ M n=1	$K_D=1.4\times10^{-8}$ M n=1
S. aureus "SCH"			$K_D=1.1\times10^{-7}$ M n=0.9

Fig. 5. Accumulation curve of PI_A in PI_A resistant strain "SCH" in the absence (○) and the presence of PII_A (□).



E. coli ribosomes[4,9]. However, when PI_A is added to *S. aureus* ribosomes, such a phenomenon is not observed. Therefore to determine the interaction parameters of PI_A *S. aureus* ribosome complexes, fluorescence anisotropy was measured.

Anisotropy was measured for increasing concentrations of PI_A added to a constant ribosome concentration in the absence or the presence of PII_A (2 μM). Fig. 3A shows the decrease of fluorescence anisotropy of PI_A alone with *S. aureus* 209P ribosomes or in the presence of PII_A.

For each experimental point, the fraction of bound PI_A is calculated using equation (2). The binding data are then plotted according to SCATCHARD with equation (3). Our results are shown in Fig. 3B. They show a non linear-plot in both cases suggesting the presence of one high-affinity binding site and multiple low-affinity binding sites. The binding parameters thus determined according to ROSENTHAL[27] are shown in Table 2.

Fig. 4 shows the results obtained with *S. aureus* "SCH" (pristinamycin I_A resistant) ribosomes. PI_A alone is unable to bind these ribosomes whereas in associaiton with PII_A, its binding site is formed leading to a SCATCHARD representation which also reveals a high-affinity binding site with parameters in-

dicated in Table 2.

Accumulation of [^{3}H]H_2PI_A by *S. aureus* "SCH"

To extend our studies, we also examined the accumulation of PI_A by *S. aureus* "SCH", a PI_A resistant strain which is synergistically sensitive to the mixture of PI_A and PII_A (Fig. 5). With PI_A (1 μM) alone in the medium, the *S. aureus* "SCH" concentrated the antibiotic 4-fold which is correlated to the lack of affinity of PI_A for "SCH" ribosomes. Conversely, in presence of PII_A, the presence of a high-affinity site for PI_A on *S. aureus* "SCH" induced an increase of the intracellular concentration of the antibiotic by some 27-fold.

Discussion

We present binding studies of pristinamycins to ribosomes and ribosomal subunits from *S. aureus* 209P a strain sensitive to both PI_A, PII_A components and also highly sensitive to their mixture and to *S. aureus* "SCH", a clinical isolate resistant to PI_A, sensitive to PII_A and highly sensitive to their mixture.

Based on the use of fluorescence anisotropy and Norit methods we have determined PI_A and PII_A binding parameters on these two types of ribosomes. The values we have obtained are close to those previously found by COCITO[7] and ourself[9] with *E. coli* ribosomes. The same remark can also be made concerning the increase of the affinity of PI_A for the ribosomes when PII_A is present in the medium; with *E. coli* ribosomes this affinity is increased by a factor of three whereas in *S. aureus* 209P ribosomes this increase is 5-fold. An important experimental finding is that resistant *S. aureus* "SCH" ribosomes appear to have no binding site for PI_A but acquire a high binding specificity for this drug in the presence of PII_A. The increase of affinity between the ribosomes and PI_A has been studied more accurately by using [^{3}H]H_2PI_A distribution on a sucrose gradient after incubation with a S-30 preparation obtained from *S. aureus* 209P and *S. aureus* "SCH" in the absence or the presence of PII_A. This type of experiment shows the relationship between the sensitivity of a strain for PI_A and the binding of this drug on the ribosome.

The uptake experiments performed with strain "SCH" also show the prime importance of the ribosome for PI_A accumulation within the cell (pumping-in effect) and confirm the hypothesis formulated previously[13]. It is likely that the phenomenon can be generalized to other macrolide antibiotic such as erythromycin for example[28].

These studies illuminate relationship between the synergistic effect (bactericidical) *in vivo* and the reinforced affinity of PI_A on the ribosome in the presence of PII_A.

Acknowledgments

The authors wish to thank Dr. D. BOUANCHAUD for supplying the resistant strain and Drs. J. GALLAY and M. VINCENT for their help and discussions with fluorescent experiments.

This research was supported by the CNRS and SPECIA laboratories.

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