# Mechanism of Action of Sparsomycin in Protein Synthesis†

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ABSTRACT: In a cell-free system derived from *Escherichia coli*, peptide bonds are formed between the donor Ac-Phe-tRNA (D) and the acceptor puromycin (S). D reacts in the form of a ternary complex (DRM) comprising also the 70S ribosome (R) and the message (M) which is poly(U). Complex DRM, called complex C, reacts with excess S according to the pseudo-first-order reaction given in reaction 1, and sparsomycin (I) inhibits reaction 1 by combining with C in the two-step equilibrium given in reaction 2:

$$C + S \overset{K_4}{\rightleftharpoons} CS \xrightarrow{k_3} P + C' \quad (1)$$

$$C + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} CI \underset{k_7}{\overset{k_6}{\rightleftharpoons}} C^*I \quad (2)$$

Before CI isomerizes to C*I, we detect a competitive phase of inhibition ($K_i = k_5/k_4 = 0.05$ μM) which eventually, by increasing the concentration of I, becomes linear mixed noncompetitive and involves C*I in place of CI. The equilibration of C and I according to reaction 2 is much slower than the equilibration between C and S in reaction 1 (time-dependent inhibition). The inactivation plots obey reaction 2 and allow us to estimate $k_6$ as equal to 2.2 $min^{-1}$. The isomerized C*I, free of excess I, can be studied as a mixture with complex C. From the kinetics of the regeneration of C from C*I, in the presence of puromycin, we can estimate $k_7$ to be between 0.22 $min^{-1}$ and 0.06 $min^{-1}$. Although the isomerized C*I survives after adsorption on cellulose nitrate filter disks, it does not survive after gel chromatography on a Sepharose CL-4B column but is converted quantitatively to complex C containing D of unchanged reactivity. This result does not support the proposed [Flynn, G. A., & Ash, R. J., (1990) *Biochem. Biophys. Res. Commun. 166*, 673–680] chemical reaction between D and I toward new products. The isomerized C*I can be obtained not only from the already-made complex C but also de novo from D, R, and M. In the latter case, the reactions which lead to C are represented by the following hypothetical scheme: D + R + M ⇌ DRM or C (*binding reaction*). When C*I is formed de novo, this reaction is coupled to reaction 2 and the ultimate product is a mixture of C and C*I. The amount of D bound, when the binding reaction is carried out coupled to reaction 2, is greater (up to 60% more) than when the binding reaction is carried out alone in the absence of I. This increase in bound donor has been called by others "stabilization of Ac-Phe-tRNA binding to ribosomes by sparsomycin" [Herner, A. E., Goldberg, I. H., & Cohen, L. B. (1969) *Biochemistry 8*, 1335–1344] and is currently considered to be one of the main characteristics of sparsomycin in its action as inhibitor of protein synthesis. We suggest that the "stabilization" is indirect. Driven by the coupling of the binding reaction to reaction 2, sparsomycin stabilizes complex C*I because $k_7$ is much smaller than $k_6$. We also suggest that sparsomycin binds initially to the A-site of the ribosome competing with puromycin or, by inference, with aminoacyl-tRNA. However, after the isomerization of the encounter complex CI to C*I, the overall kinetics of inhibition become complex and implicate C*I in further interactions with S as well as with I.

Sparsomycin is a sulfoxide-containing antibiotic that inhibits protein synthesis in sensitive prokaryotic and eukaryotic systems (Ottenheijm et al., 1986; Cundliffe, 1981). It has also been investigated as a potential antitumor agent and is of current interest in several laboratories (van den Broek et al., 1987, 1989a,b; Flynn & Ash 1990; Lazaro et al., 1991). There is general agreement that sparsomycin acts on the large ribosomal subunit and inhibits peptide bond formation. The mechanism by which this inhibition is brought about is still unclear. It has been known that the whole ribosome, or the large ribosomal subunit, forms a "sparsomycin-induced complex" with N-blocked aminoacyl oligonucleotides or N-blocked aminoacyl-tRNA, i.e., the so-called donor substrates (Monro, 1969; Jimenez et al., 1970; Vogel et al., 1971). Other investigators expressed the formation of this complex as "stabilization of the binding of the donor substrate" (Herner et al., 1969) or as the "sparsomycin reaction" which was found to be inhibited by other antibiotics (Yukioka, 1975). This "stabilization" is currently considered to be one of the main characteristics of sparsomycin in its action as an inhibitor of protein synthesis. In a previous report (Coutsogeorgopoulos et al., 1975), we found that the degree of inhibition by sparsomycin was increased if the inhibitor was preincubated with the puromycin-reactive ribosomal complex before the substrate was added (*preincubation effect*). It appears in retrospect that, of the antibiotics that inhibit peptide bond formation, sparsomycin was the first example of a new class of inhibitors (Williams & Morrison 1979), the so-called slow-binding inhibitors. The observed preincubation effect could easily be explained by the slow equilibration between the reactive ribosomal complex and the inhibitor. However, other investigators (Flynn & Ash 1983, 1990; Ash et al., 1984) explained

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this preincubation effect by invoking a chemical reaction (Pummerer rearrangement) between sparsomycin and the P-site bound donor (peptidyl-tRNA). In the past, a confusion seems to have often existed between the irreversible inactivation of enzymes and slow-onset inhibition or inhibition by slow-binding inhibitors (Schloss, 1988). In the present study, in order to characterize more precisely the nature of the "stabilization of the donor substrate" by sparsomycin and to answer the question of whether a covalent intermediate involving the donor exists or not, we have investigated in detail the "sparsomycin-induced complex" when Ac-Phe-tRNA is the donor substrate. Moreover, sparsomycin assumes increased importance because of the current interest in the slow-binding inhibitors as drugs (Morrison & Walsh, 1988; Schloss, 1988, 1989).

## Materials and Methods

*Materials.* Sepharose CL-4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). [$^3$H]Phenylalanine, poly(U), GTP (disodium salt), ATP (disodium salt), puromycin dihydrochloride, and transfer ribonucleic acid from *Escherichia coli* strain W were purchased from Sigma Chemical Co. [2,3-$^3$H]-L-Phenylalanine was from Amersham (U.K.). Zwittergent 3-12 (ZW) detergent (*N*-dodecyl-*N*,*N*-dimethyl-3-ammonium-1-propanesulfonate) was obtained from Calbiochem AG. Cellulose nitrate filters (type HA, 24-mm diameter, 0.45-$\mu$m pore size) were purchased from Millipore Corp. Sparsomycin was a gift from the Upjohn Co. Ac-[$^3$H]Phe-tRNA charged with 15.3 pmol of [$^3$H]Phe (44 400 cpm total)/$A_{260}$ unit was prepared as described previously (Kalpaxis et al., 1986).

(*1*) *De Novo Formation of Complex C and of the Sparsomycin Complex.* The *binding mixture* (1.0 mL) was prepared at 0 °C (ice) by the addition of, in the following order, 100 $\mu$mol of Tris-HCl, pH 7.2, 100 $\mu$mol of $NH_4Cl$ from a solution adjusted with $NH_4OH$ to pH 7.2, 10 $\mu$mol of total $Mg^{2+}$ (acetate), 320 $\mu$g of poly(U), and 0.4 $\mu$mol of GTP. At this point, sparsomycin at a final concentration of 0.1 or 1.0 or 10.0 $\mu$M was added where indicated. Subsequently, the following were added: 32 $A_{260}$ units of washed ribosomes; 400 $\mu$g (protein) of FWR, and 25.6 $A_{260}$ units of Ac-[$^3$H]Phe-tRNA. After incubation at 25 °C for 8 min, the mixture (binding mixture) was kept in ice. Among other components, the binding mixture contains complex C and the excess Ac-[$^3$H]Phe-tRNA which did not bind to complex C. At this point, the incubation mixture could be directly gel-chromatographed (see below) before the next step.

(*2*) *Isolation of Complex C or of the Sparsomycin Complex.* The mixture in which complex C or the sparsomycin complex was formed de novo, i.e., after the incubation step (25 °C, 8 min), was cooled in ice, diluted with ice-cold *binding buffer* (100 mM Tris-HCl, pH 7.2, 50 mM KCl, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol) and filtered through cellulose nitrate filter disks which were washed with binding buffer. The adsorbed complexes can be desorbed with a buffered solution of Zwittergent as described elsewhere (Theocharis & Coutsogeorgopoulos, 1989; preparation of the ZW-extract). The complexes can be examined using the puromycin reaction. Complex C engaged 19% of the ribosomes added (32 $A_{260}$ units; 840 pmol) in the form of the disk-adsorbed complex C. Assuming a 1:1 combination, this complex contained also 39% of the input (25.6 $A_{260}$ units) Ac-[$^3$H]Phe-tRNA. Over 99% of the disk-bound Ac-[$^3$H]-Phe-tRNA was reactive toward puromycin.

(*3*) *Puromycin Reaction.* The puromycin reaction in the presence or absence of sparsomycin was carried out with reactive complex either adsorbed on a cellulose nitrate disk (Kalpaxis et al., 1986) or in solution (Theocharis & Coutsogeorgopoulos, 1989).

(*a*) *Without Preincubation with Sparsomycin.* Complex C (0.8 mL of ZW extract) was equilibrated at 25 °C for 5 min. A mixture (0.2 mL) containing puromycin and sparsomycin was then added, and the reaction was allowed to proceed at 25 °C for the time intervals indicated and was stopped by the addition of 1.0 mL of 1 M NaOH. The subsequent incubation at 25 °C for 30 min and the determination of the corrected percentage ($x/a = x'$) of the ribosome-bound Ac-[$^3$H]Phe-tRNA that reacted with puromycin were carried out as described in Theocharis and Coutsogeorgopoulos (1989) (condition B).

(*b*) *Formation of the $nK_i$-Sparsomycin-Modified Complex C.* Complex C was preincubated at 25 °C with sparsomycin at the indicated concentration expressed in $K_i$ units ($nK_i$) where $K_i$ equals 0.05 $\mu$M ($n$ varied from 0.2 to 200). The time of incubation depended on the information obtained from the "inactivation plot". Seven times the $t_{1/2}$ is a sufficient preincubation period before the puromycin reaction is carried out.

(*c*) *Puromycin Reaction with the $nK_i$-Sparsomycin-Modified Complex C.* Complex C (0.8 mL of ZW extract) was equilibrated at 25 °C for 5 min. Sparsomycin (0.1 mL) was added at the appropriate concentrations, and incubation at 25 °C continued until equilibration between complex C and sparsomycin was reached (see previous section). Puromycin (0.1 mL) was then added at the appropriate concentrations, and the reaction was allowed to proceed at 25 °C for the time interval indicated and then stopped by the addition of 1.0 mL of 1 M NaOH. The subsequent incubation at 25 °C for 30 min and the determination of the corrected percentage ($x/a = x'$) were carried out as described in the previous section (section a).

(*4*) *Determination of the Apparent $k^I_{obs}$ from the Inactivation Plot.* Complex C (0.8 mL ZW extract) was allowed to equilibrate at 25 °C for 5 min. Sparsomycin (0.1 mL) was added at the appropriate amounts, and at the indicated time intervals puromycin was added (0.1 mL, final concentration 2 mM, containing the appropriate amount of sparsomycin so that its final concentration remains constant). Puromycin was allowed to react (back-titration with puromycin) for 15 or 30 s, and then 1.0 mL of 1 M NaOH was added. Further incubation at 25 °C for 30 min (hydrolysis of unreacted complex C) and determination of the quantity $x/a$ which gives the percentage of unreacted C, were carried out as described in Theocharis and Coutsogeorgopoulos (1989) (puromycin reaction under condition B). A correction related to the inhibition exerted by the excess sparsomycin during the back-titration with 2 mM puromycin was carried out at this point. The initial slopes (15 and 30 s) were compared for 2 mM puromycin alone and for 2 mM puromycin plus sparsomycin (0.05 $\mu$M, 0.1 $\mu$M, 1.0 $\mu$M). The resulting correction factor is applied to the percentage $x/a = x'$ obtained for each time of exposure ($t$) and at each concentration of sparsomycin. The corrected $x'$ becomes the increased value $y$. The log of $y$ was finally plotted against the corresponding $t$, and the initial slope of this plot gave the apparent first-order rate constant ($k^I_{obs}$) for each concentration of sparsomycin.

(*5*) *First-Order Analysis of the Puromycin Reaction.* The reaction between complex C and excess puromycin at 25 °C displayed pseudo-first-order kinetics. At each concentration of puromycin [S] the first-order rate constant ($k_{obs}$) was determined by fitting the values of $x/a = x'$ into ln [100/(100

$-x')] = k_{obs}t$ for each time point $t$ and calculating the slope of this straight line. In the absence of the inhibitor, the entire course of the reaction satisfies this relationship at all concentrations of puromycin tested. The relationship between $k_{obs}$ and $S$ follows the equation $k_{obs} = k_3[S]/(K_S + [S])$, as explained elsewhere (Theocharis & Coutsogeorgopoulos, 1989). In the presence of the inhibitor, the time plots may be biphasic, depending on the concentrations of puromycin and the inhibitor. In such cases, the slope of the line going through the origin (initial slope of the time plot) is taken as the value of $k_{obs} = k$. The relationship between $k$ and [S] follows the equation $k = k_{max}[S]/(K_S' + [S])$, as explained previously (Kalpaxis et al., 1986; Theocharis et al., 1986; Drainas et al., 1987).

(*6*) *Gel Chromatography.* Aliquots of the binding mixture (0.2 mL), where complex C or the sparsomycin complex have been formed de novo (section 2), were applied to a column (55 × 0.8 cm) packed with Sepharose CL-4B and equilibrated with binding buffer at 4 °C. In other experiments, 0.8 mL of ZW extract (reaction mixture), containing the "complexes" which were previously isolated on a cellulose nitrate disk, was applied to this column. The ZW extract may have also reacted with puromycin. The column was eluted at 4 °C with binding buffer, and 0.9-mL fractions were collected. The fractions were monitored for radioactivity using a scintillation spectrometer. In order to isolate and examine ribosomal complexes containing Ac-[$^3$H]Phe-tRNA, the fractions of the peak appearing in the void volume were pooled and passed through a cellulose nitrate filter disk under vacuum (12–14 mmHg). The filter was washed quickly under vacuum with two 5.0-mL portions of ice-cold binding buffer without allowing air to pass through the filter.

## Results and Discussion

(*A*) *Kinetics in the Continuous Presence of Sparsomycin.* These experiments have been carried out with the $nK_i$-sparsomycin-modified complex C.

(*1*) *Time-Dependent Inhibition and the Preincubation Effect.* The time course of the puromycin reaction (reaction 1)

$$C + S \overset{K_s}{\rightleftharpoons} CS \xrightarrow{k_3} P + C' \qquad (1)$$

carried out in solution (ZW-extract) with complex C and puromycin (S) as a first-order reaction, is shown in Figure 1 (upper line). In the presence of sparsomycin there is inhibition (two middle lines for 0.1 μM and 1.0 μM sparsomycin) estimated on the basis of the *initial slopes* of these biphasic time plots. The degree of inhibition changes with time (time-dependent inhibition). If, at the same concentrations of the inhibitor, preincubation precedes the addition of puromycin, the inhibition is much greater as shown by the two lower lines of Figure 1. This is the so-called *preincubation effect.* This effect has also been observed when complex C reacts while adsorbed on cellulose nitrate [Figure 3a of Coutsogeorgopoulos et al. (1975)] as well as in other systems (Lee & Vince, 1978; Ash et al., 1984).

(*2*) *Initial Slope Analysis.* In Figure 1 we show that without preincubation of C and I, the progress curves (2 mM puromycin) for 0.1 μM and for 1.0 μM sparsomycin are biphasic whereas after preincubation the time plots become linear for up to 4 min. Of particular interest is the progress curve obtained after preincubation with just 0.01 μM (=0.2$K_i$ see below) sparsomycin (Figure 1; line that crosses the two middle lines). This progress curve is still biphasic within the time interval of 4 min; the late slope shows an impressive degree of inhibition (66%) that corresponds to a hypothetical competitive inhibition with $K_i$ = 0.83 nM, i.e., 60 times less than

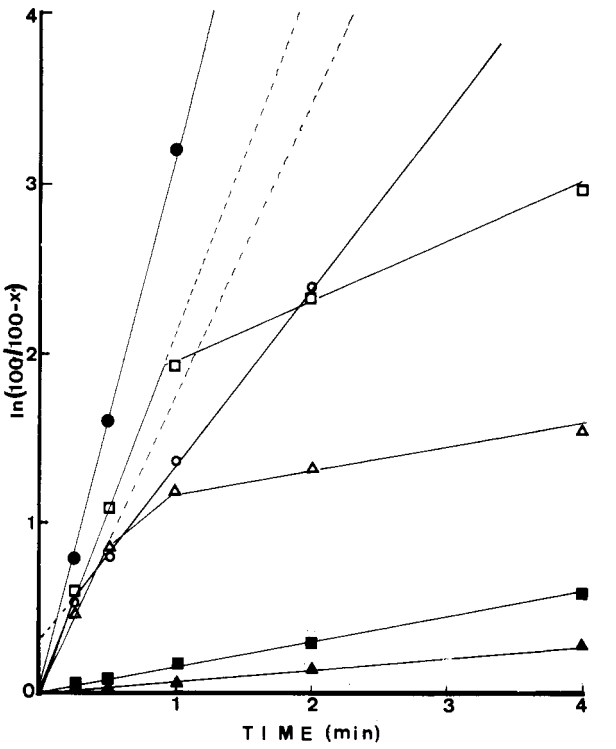


FIGURE 1: First-order time plots for Ac-Phe-puromycin formation in solution at 2 mM puromycin. Plots are shown for complex C under the following conditions: in the absence of sparsomycin (●; control); in the presence of sparsomycin at a final concentration of 0.1 μM (□) or 1.0 μM (△); preincubated for 8 min at 25 °C with sparomycin at 0.01 μM (○), 0.1 μM (■), or 1.0 μM (▲), and then reacted with puromycin.

the $K_i = k_5/k_4$ (see below). At several concentrations of puromycin and without preincubation with sparsomycin, the initial slopes ($k$) give linear double-reciprocal plots showing competitive kinetics from which a $K_i$ of 0.05 μM can be calculated (results not shown). If we preincubate C and I before we add S, the kinetics are not of the competitive type. The transition from competitive to another type of inhibition is also observed if, without preincubation, the concentration of I is increased; above a certain concentration ($10K_i$) we have linear intersecting mixed noncompetitive kinetics (Figure 2). The intercept replot ($1/k_{max}$ vs [I]) for the whole range of inhibitor concentrations, i.e., containing the competitive as well as the mixed noncompetitive phase, is not linear as shown in Figure 3. This replot becomes linear for values of [I] above $10K_i$ and the straight line, when extrapolated, cuts the vertical axis at 7.5. The reverse of this value (0.13 min$^{-1}$) corresponds to the hypothetical $k_{max}$ in the absence of I ($k_3$* of reaction 3, see below). The slope replot for the mixed noncompetitive phase (above $10K_i$) is also linear as shown in the inset of Figure 3. One model suggested by this kinetic analysis is the one given in a previous paper when the kinetics of inhibition by blasticidin S is analyzed (Kalpaxis et al., 1986). Of central importance is the formation of C*I according to the reaction

$$C + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} CI \underset{k_7}{\overset{k_6}{\rightleftharpoons}} C^*I \qquad (2)$$

The intercept with the vertical axis of the slope replot gives the ratio $K_S^*/k_3^*$ from which the value of 38 μM is obtained for $K_S^*$. The constants $K_S^*$ and $k_3^*$ belong to the scheme which describes the reaction of C*I with puromycin after a previous dissociation to free C* (reaction 3). The mixed

$$\left.\begin{aligned} C^*I &\rightleftharpoons C^* + I \\ C^* + S &\overset{K_S^*}{\rightleftharpoons} C^*S \xrightarrow{k_3^*} P + (C^*)' \end{aligned}\right\} \qquad (3)$$

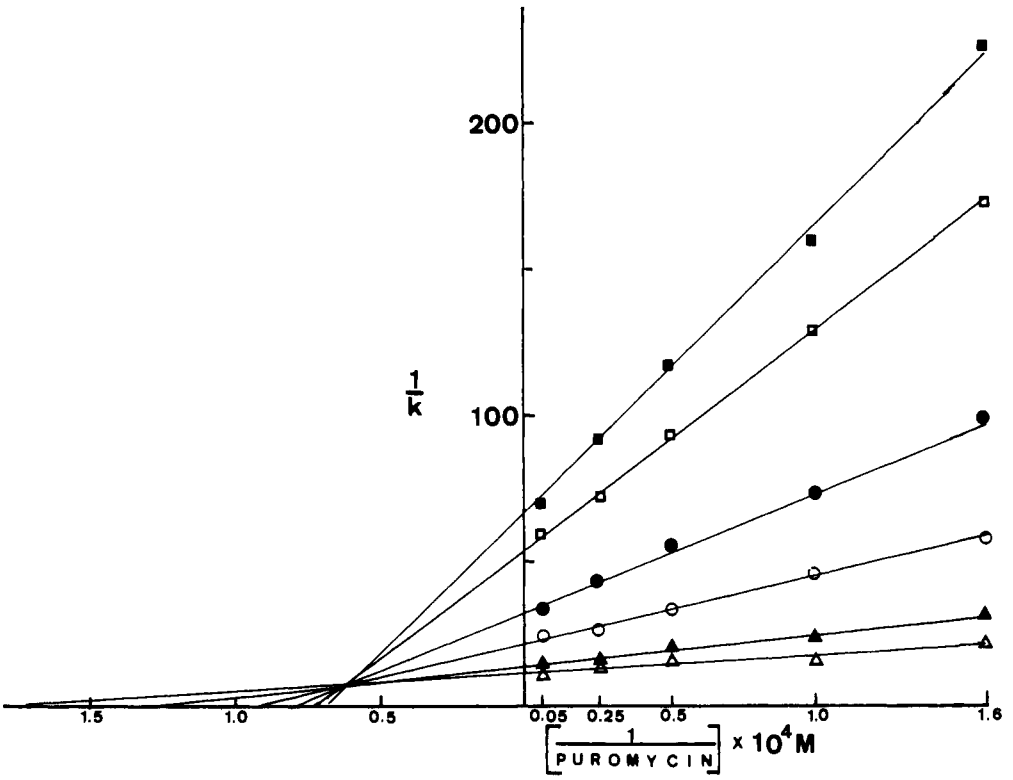


FIGURE 2: Double-reciprocal plot ($1/k$ versus 1/puromycin) for the puromycin reaction after preincubation of complex C for 8 min at 25 °C with sparsomycin at a final concentration of (△) 0.5 μM; (▲) 1.0 μM; (○) 2.5 μM; (●) 5.0 μM; (□) 10.0 μM; or (■) 50.0 μM.

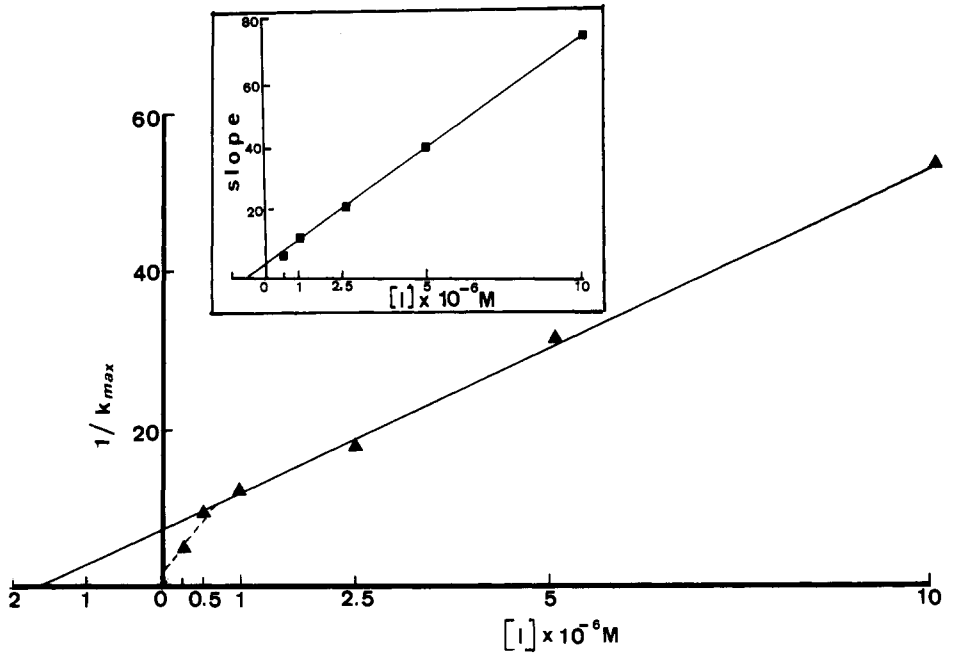


FIGURE 3: Intercept replot ($1/k_{max}$ versus sparsomycin). The data were taken from double-reciprocal plots of Figure 2 and from similar plots obtained after preincubation with sparsomycin at additional concentrations. The $1/k$ axis intercepts of these plots were replotted versus the concentrations of sparsomycin. The point plotted on the $1/k_{max}$ axis was taken from the intercept of the line in the absence of inhibitor (control). Inset: Slope replot. The data were taken from double-reciprocal plots obtained after preincubation with various concentrations of sparsomycin.

noncompetitive kinetics is explained in a previous paper (Kalpaxis et al., 1986). An analogous kinetic study was carried out with complex C adsorbed on cellulose nitrate filter disks. The kinetic and equilibrium constants obtained are presented in Table I.

(*3*) *The Inactivation Plot.* The nonlinear first-order time plots shown in Figure 1, the preincubation effect, and the development of time-dependent inhibition point out to a slowly equilibrating inhibitor. Some authors (Schönbrunn 1990) use also the term "inactivation" in order to describe slow equilibrations like ours. Schloss (1989) has also commented on the fact that the existence of very slow rates of inhibitor release (very low $k_7$ in reaction 2) tends to eliminate the classical distinction between reversible and irreversible inhibitors as giving noncovalent and covalent intermediates, respectively. To put it another way, the distinction between reversible and irreversible behavior is often only one of degree (Morrison & Walsh, 1988). In order to investigate the delayed equilibration,

Table I: Kinetic and Equilibrium Constants Derived from Primary and Secondary Kinetic Plots

| | reaction in solution | reaction on the filter disk |
|---|---|---|
| $K_i$ ($\mu$M) | 0.05 ± 0.013 | –[a] |
| $K_S$ ($\mu$M) | 400 ± 34 | 400 ± 34 |
| $k_3$ (min$^{-1}$) | 3.4 ± 0.32 | 2.0 ± 0.22 |
| $k_3$* (min$^{-1}$) | 0.13 zu 0.01 | 0.08 ± 0.009 |
| $K_S$* ($\mu$M) | 34.0 ± 4.60 | 28.0 ± 5.6 |

[a] At low concentrations of I, the double-reciprocal plots (without preincubation) are concave upward and $K_i$ cannot be calculated.

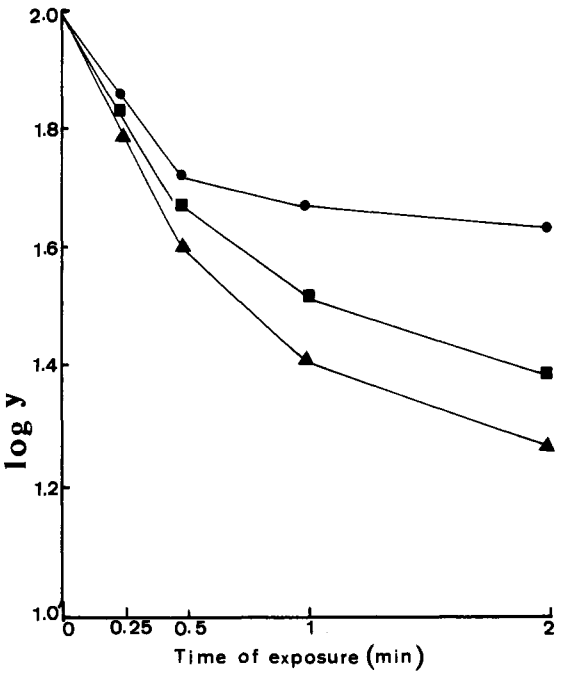


FIGURE 4: Inactivation plots. Sparsomycin reacted at a final concentration of (●) 0.05 $\mu$M, (■) 0.1 $\mu$M, or (▲) 1.0 $\mu$M, for the indicated times of exposure. Details for back-titrating the unreacted complex C and for the correction applied due to the presence of the inhibitor are given in the Materials and Methods Section.

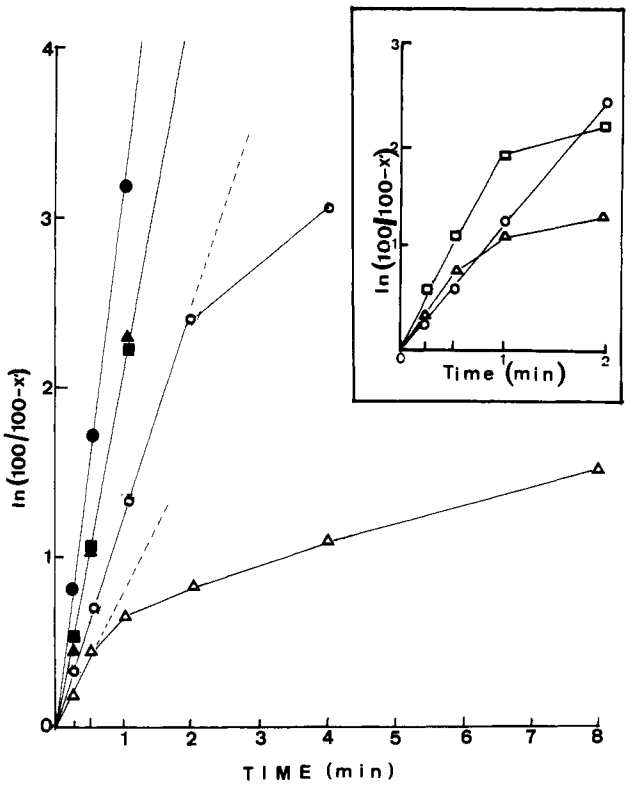


FIGURE 5: First-order time plots obtained with the control complex C or with the sparsomycin complex, and 2mM puromycin. Complex C (control) reacted with 2 mM puromycin (●) in solution and (■) in the adsorbed state. Sparsomycin complex, formed with 1.0 $\mu$M sparsomycin in the binding mixture and gel chromatographed, was isolated on cellulose nitrate filter disks and reacted in the adsorbed state (▲). The same sparsomycin complex, without gel chromatography, was isolated on cellulose nitrate and reacted with 2 mM puromycin (○) in solution after desorption and (△) in the adsorbed state. Inset: Comparison of progress curves with 2 mM puromycin in solution for the first 2 min: (△) complex C plus 1.0 $\mu$M sparsomycin; (□) complex C plus 0.1 $\mu$M sparsomycin; (○) sparsomycin complex prepared with 1.0 $\mu$M sparsomycin in the binding mixture, subsequently adsorbed on cellulose nitrate and finally desorbed and reacted with puromycin.

or inactivation, we carried out reaction 2 with several concentrations of I and treated the inactivation process as a pseudo-first-order reaction. Typical results are shown in Figure 4. The apparent $k^I_{obs}$ deduced from the initial slopes are 1.3 min$^{-1}$ (0.05 $\mu$M), 1.5 min$^{-1}$ (0.1 $\mu$M), and 2.2 min$^{-1}$ (1.0 $\mu$M). If we plot $1/k^I_{obs}$ versus $1/[I]$, we obtain a linear plot which is compatible with reaction 2 and corresponds to the equation $k^I_{obs} = k_7 + k_6[I]/(K_i + [I])$ (Halford et al., 1969; Morrison & Walsh, 1988), provided that the experimentally determined $k^I_{obs}$ is set equal to the difference ($k_{obs}{}^I - k_7$). In a subsequent section, we present evidence that $k_7$ is between 10 and 40 times smaller than $k_6$. Thus, the previous relationship becomes $k^I_{obs} = k_6[I]/(K_i + [I])$ and the plot of $1/k^I_{obs}$ versus $1/[I]$ gives $k_6 = 2.2$ min$^{-1}$ and $K_i = 0.04$ $\mu$M which is very close to the value obtained independently from the competitive kinetics in the presence of S.

*(B) Experiments with the Sparsomycin Complex.* The following experiments were carried out with the aim of studying complex C*I which was suggested by the initial slope analysis (reaction 2). The complex C*I can be studied in a mixture with C, either adsorbed on cellulose nitrate filter disks or desorbed therefrom and kept in the ZW extract. Typical progress curves are shown in Figure 5. The lowest curve is obtained with sparsomycin complex adsorbed on cellulose nitrate. The curve may be thought of as showing a mixture of two components: One fast-reacting and one slow-reacting (Kayastha & Gupta, 1987; Frost & Pearson, 1961). After some time, the curve becomes linear because the initially existing more reactive component (complex C) has disappeared. The linear part of the lowest line in Figure 5 obtained after 4 min (late slope) is retained at the same slope up to 32 min (results not shown beyond 8 min). The late slope ($k'$ = 0.11 min$^{-1}$) can be used in order to calculate $k_7$. We may assume that the late slope pertains to two pseudo-first-order parallel reactions which have a common starting material, i.e., C*I (Frost & Pearson, 1961). One of these reactions is reaction 3, and the other is the reversal of C*I toward free C (reaction 2) which, as soon as it is formed, reacts with S according to reaction 1 ($k_3 \gg k_7$, [S] = $5K_s$). Essentially, there is no free I to react with C. The sum of the two parallel reactions should give an overall $k'$ equal to the sum of the other two; i.e., $k' = k_3$* + $k_7$. Product formation according to reaction 3 proceeds at maximum capacity, i.e., with $k_3$* because [S] (2 mM) equals 70 times the $K_s$*. Since $k_3$* = 0.08 min$^{-1}$ (Table I), we can calculate that $k_7 = 0.03$ min$^{-1}$. In calculating $k_7$ under conditions in which the excess of I has been removed, the main reaction of concern could have been only reaction 2 which is supported (a) by the early competitive kinetics, (b) by the inactivation plots of Figure 4, and (c) by the results of the gel chromatography (see further). On the other hand, the kinetic analysis in the presence of excess I (above $10K_i$) and especially the nonlinearity of the intercept

replot (Figure 3) force us to take into account also reaction 3 in order to have a unified model which, however, implies the existence of free C*. Certainly, there may also be other interpretations for explaining the nonlinearity of the intercept replot, and until this matter is settled we consider the value assigned to $k_7$ as tentative; $k_7$ could have a value ranging from a maximum of 0.11 $min^{-1}$ (late slope of the lowest curve of Figure 5), obtained without taking into account reaction 3, to a minimum of 0.03 $min^{-1}$.

The second line from the bottom in Figure 5 shows the progress curve when the sparsomycin complex reacts in solution (ZW extract). The initial slope is higher than that of the lowest line. This is a consequence of the fact that the reaction in solution is faster (Table I) and that there is a higher percentage of C in the ZW extract than on the disk. The late slope is not clear enough to permit an estimate of $k_7$ because the reaction ends too soon.

In order to show that the sparsomycin complex contains a new species reactive toward puromycin and that it is not a mixture of C and some remnants of free sparsomycin that were stuck on the disk and then extracted in the ZW extract, we present the inset of Figure 5. In this inset, we reproduce the progress curve of the sparsomycin complex for the first 2 min (i.e., from the second line from the bottom; Figure 5) and the progress curves of the mixtures of complex C with 0.1 $\mu$M or 1.0 $\mu$M sparsomycin taken from the two middle lines of Figure 1. All the curves have been obtained with 2 mM puromycin. It may be seen that the progress curve of the sparsomycin complex does not match either of the other two curves. It is also worth noting that, although the sparsomycin complex has been prepared using 1.0 $\mu$M sparsomycin in the binding mixture, it nevertheless reacts slower (for the first half-minute) than the mixture of complex C and 1.0 $\mu$M sparsomycin.

*(C) Attempts To Calculate the $k_6/k_7$ Ratio.* Under the assumptions considered in a previous section, $k_6$ = 2.2 $min^{-1}$ for the reaction in solution. The value of $k_7$ for the reaction on the disk has also been estimated in a previous section to be between 0.11 $min^{-1}$ and 0.03 $min^{-1}$. It is our experience that the disk reactions and the corresponding reactions in solution may differ in rate by a factor of 2 at the most. This trend may also be seen by comparing the kinetic constants of Table I. Thus, we can assume that $k_7$ in solution is between 0.22 $min^{-1}$ and 0.06 $min^{-1}$. Taking the minimal value of 0.06 $min^{-1}$, it follows that the value for the ratio $k_6/k_7$ could be around 40. It can be calculated (Morrison & Walsh, 1988) that a $k_6/k_7$ ratio of 40 gives an overall $K_i'$ = [C][I]/([CI] + [C*I]) equal to 1.2 nM, indicating a great preference for the formation of C*I over C.

*(D) Gel Chromatography.* This method was applied with the following three objectives: (a) to examine whether C*I, that has been detected after filtration on cellulose nitrate disks, is a tight enough complex so as to survive gel chromatography; (b) to examine whether the donor D in C*I has suffered any changes, because the suggestion has been made (Flynn & Ash, 1983) that D reacts with I in a reaction in which the peptidyl portion (Ac-Phe in our case) of D is combined with I; and (c) to examine the mechanism by which sparsomycin "stabilizes and binding of Ac-Phe-tRNA to the ribosome" (Herner et al., 1969).

*(1) Gel Chromatography of the $20K_i$-Sparsomycin-Modified Complex C.* Comparative runs were carried out for the $20K_i$-sparsomycin-modified complex C, or plain complex C. Either of these complexes may have reacted with puromycin. The results are shown in Figure 6. We see (Figure 6A) that complex C, is eluted with the void volume. If reaction with

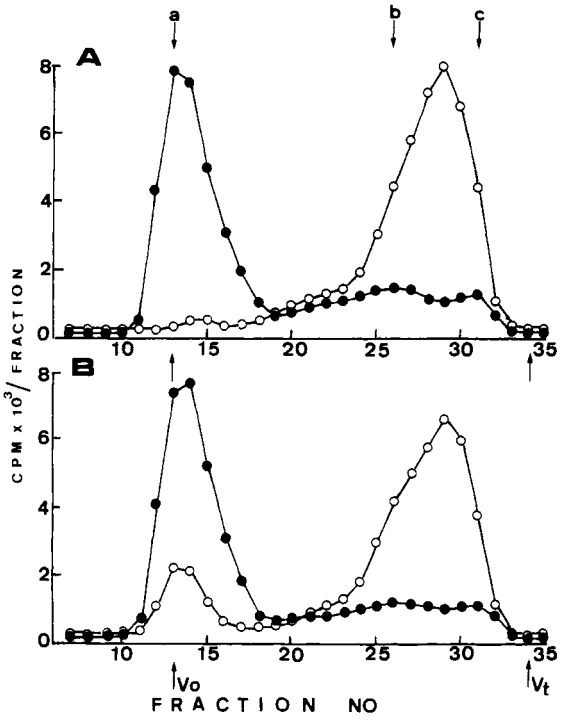


FIGURE 6: Gel chromatography of the reaction mixture (ZW extract) on a Sepharose CL-4B column. (A) Complex C in the reaction mixture (control) (●) before the addition of puromycin and (○) after the addition of puromycin at a final concentration of 2 mM, and reacting for 4 min at 25 °C. (B) Complex C in the reaction mixture made in the presence of sparsomycin (at 1.0 $\mu$M = $20K_i$, incubation for 8 min at 25 °C) (●) before the addition of puromycin and (○) after the addition of puromycin at a final concentration of 2 mM and reaction for 20 min at 25 °C. The letters a, b, and c indicate the positions of elution of complex C, Ac-Phe-tRNA, and Phe, respectively. $V_0$ and $V_t$ indicate void volume and total volume of the column, respectively.

puromycin precedes chromatography, then the first peak disappears and most of the radioactivity appears in the second peak where Ac-Phe-puromycin (P) is eluted. Without puromycin, the recovery of the radioactivity in the void volume was 80% of that applied to the column. The rest of the radioactivity appears as Ac-Phe-tRNA and Ac-Phe. The activity of complex C eluted in the void volume was the same as that of complex C which had not been chromatographed. The activity was measured by determining the $k_{obs}$ at 2 mM puromycin (reaction 1) after the column fractions were pooled, passed on to a cellulose nitrate disk, and then reacted with puromycin. The results of similar experiments carried out with the $20K_i$-sparsomycin-modified complex C in place of complex C are shown in Figure 6B. In the absence of puromycin, we can reason that, according to reaction 2, the mixture of complex C treated with sparsomycin (1.0 $\mu$M = $20K_i$) and remaining in the continuous presence of the inhibitor must have consisted mostly of C*I and almost no free C. Yet, after chromatography, we obtain only complex C, fully active, i.e., having the same $k_{obs}$ (2 mM puromycin) as the untreated complex C (Figure 6A). This result shows that the equilibrium of reaction 2 is disrupted by the gel chromatography and that, contrary to the proposal of Flynn and Ash (1983), D has suffered no changes by sparsomycin because it is found quantitatively bound to C in a puromycin-reactive state, as if no treatment with sparsomycin had occurred. Reaction with puromycin in the presence of 1.0 $\mu$M sparsomycin (Figure 6B) gives the puromycin product (P; Ac-Phe-puromycin) as it does in the absence of sparsomycin (Figure 6A). At this high concentration of sparsomycin, the puromycin reaction did not reach

Table II: Effect of Sparsomycin on the Binding of Ac-Phe-tRNA to Ribosomes

| conditions | percentage of input Ac-[$^3$H]Phe-tRNA determined after adsorption | percentage of input Ac-[$^3$H]Phe-tRNA determined after gel chromatography |
|---|---|---|
| in the absence of sparsomycin (control) | 30 | 29 (100)[a] |
| in the presence of Sparsomycin | | |
| 0.1 $\mu$M | 40 | 39 (+34) |
| 1.0 $\mu$M | 45 | 43 (+48) |
| 10.0 $\mu$M | 48 | 46 (+59) |

[a] Numbers in parentheses refer to the percentage of increase if the control is 100%.

in 20 min an extent as high as it reached in the absence of the inhibitor in 4 min.

(*2*) *Gel Chromatography of Complex C Formed de Novo.* The whole binding mixture, where complex C has been formed in the presence or in the absence of sparsomycin, was chromatographed as detailed in the previous section. In the de novo formation of C, the donor (D) reacts with ribosomes and poly(U) in a complex reaction which occurs in the presence of ribosomal wash and GTP. The end product is the ternary complex DRM [called complex C for short, where R is the 70S ribosome and M is the message poly(U)].

$$D + R + M \rightleftharpoons DRM \text{ or } C \qquad (4)$$

The overall reaction (*binding reaction*) is complex, but for our purposes it can be depicted with one overall reversible reaction (reaction 4). We carried out comparative gel chromatography runs with binding mixtures incubated at 25 °C for 8 min containing either D, R, and M or D, R, M, and I. In the latter case, reaction 4 is coupled with reaction 2. The examination of the binding mixture by gel chromatography takes place without using the intermediate step of removing the excess unbound D by filtration through a cellulose nitrate disk. The results of the experiments, carried out either with 1.0 $\mu$M sparsomycin or without the inhibitor, are shown in Figure 7. Complex C is eluted in the void volume of the column and separates satisfactorily from free D (Ac-Phe-tRNA). As shown in Table II, the amount of D bound to ribosomes and expressed as a percentage of input D increases when [I] is increased from 0.1 $\mu$M to 10 $\mu$M. As the concentration of I gets higher, reaction 2 is shifted toward more C*I. This shifting pulls reaction 4 to the right. Because of the 100-fold increase in I, the amount of bound D is eventually increased by 59% of the control value. Herner et al. (1969) reported that the half-maximal effect for the increased binding of D is at 0.1 $\mu$M sparsomycin which is close to the $K_i$ reported by others (0.16 $\mu$M; Pestka, 1972) and by us in the present work (0.05 $\mu$M).

(*E*) *General Discussion and Conclusions.* (1) We have studied C*I in two forms which are associated with two different types of environment. (A) One form exists in the continuous presence of free I. It is called the $nK_i$-sparsomycin-modified complex C ($n$ = 0.2–200) and is obtained by preincubating C and I before the addition S. This form permits kinetic analysis in the presence of S, which supports the kinetic scheme of reactions 2 and 3 (Kalpaxis et al., 1986). Depending on the value of $n$, the mixture may contain a very high proportion of C*I. (B) the other form is the isomerized C*I, free of excess I, which we call sparsomycin complex and which we study as a mixture with complex C. When adsorbed on a cellulose nitrate filter disk, this mixture is stabilized in favor of C*I and through its reaction with puromycin it permits an analysis that lends further support to reaction 2 and gives a value for $k_7$.

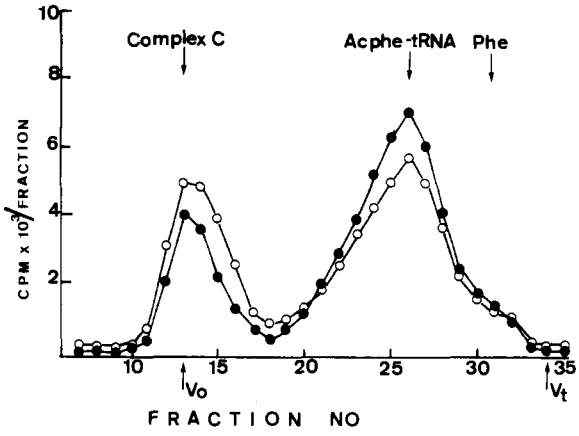


FIGURE 7: Gel chromatography of the binding mixture on a Sepharose CL-4B column. Binding mixture containing complex C was formed (○) in the presence of 1.0 $\mu$M sparsomycin and (●) in the absence of sparsomycin. $V_0$ and $V_t$ indicate void volume and total volume, respectively.

(2) The results obtained with both forms of C*I suggest that sparsomycin is a slowly-equilibrating ribosomal inhibitor, at least when the puromycin reaction is used as the reference reaction of peptide bond formation. During protein synthesis, peptide bonds are formed faster than in our system (Forchhammer & Lindahl, 1971; Gausing, 1972) and the characterization "slowly-equilibrating" is even more justified. The results are easily explained if we assume that reaction 2 involves a rapid equilibrium in which the encounter complex CI is formed and a further reversible but slower isomerization step, i.e., CI $\rightleftharpoons$ C*I.

(3) The isomerized complex C*I reverts to CI with the rate constant $k_7$ which is much smaller than $k_6$. Thus, the development of time-dependent inhibition and the "preincubation effect" are easily explained.

(4) The long-standing view of the dual effect of sparsomycin in inhibiting binding at the A-site but simultaneously stabilizing the binding at the P-site (Ottenheijm et al., 1986; Cundliffe, 1981; Pestka, 1969; Harris & Pestka, 1973) may be easily explained with our model. In the encounter complex (CI), the ribosome that has accepted sparsomycin readjusts its binding site and binds sparsomycin more efficiently. The result is C*I. The dual effect, according to our view, concerns the two forms CI and C*I. The first form (CI) is involved in competitive kinetics; i.e., the binding of the substrates at the A-site is excluded, whereas the second form (C*I) is involved in mixed noncompetitive kinetics. The stabilizing effect on the de novo binding of substrates at the P-site, reported in the literature (Herner et al., 1969; Monro et al., 1969; Jimenez et al., 1970) and considered to be part of the dual effect, appears to be an indirect effect of sparsomycin; it is due to the slow $k_7$ step when reaction 2 is coupled with reaction 4. It is interesting that sparsomycin can bind only to ribosomes that already bear donor substrate bound to the P-site (Cundliffe 1981, and references cited therein).

(5) We find no support for the proposal (Flynn & Ash, 1983) that sparsomycin enters into a chemical reaction with the P-site-bound donor catalyzed by the putative ribosomal peptidyltransferase. In our hands, the sparsomycin-treated ribosomal complex ($nK_i$-sparsomycin-modified complex C) retains all the bound Ac-Phe-tRNA intact, and after the removal of sparsomycin by gel chromatography, it is fully reactive toward puromycin. Following a completely different approach, Lazaro et al. (1991) concluded that there is no covalent association between sparsomycin and the ribosome. The preincubation effect was explained by Flynn and Ash

(1983) by invoking the above-mentioned chemical reaction. Much more recently, the same authors (Flynn & Ash, 1990) reiterated this principle of relating the preincubation effect to a chemical reaction. According to our view, the chemical step is unnecessary if we accept that $k_7$ is much lower than $k_6$ in reaction 2.

(6) Most probably, in the conversion CI $\rightleftharpoons$ C*I, we are dealing with conformational changes that involve macromolecules such as the ribosomal RNA(s), t-RNA, ribosomal proteins, etc. To our knowledge, values for apparent rate constants ($k_6$ and $k_7$) concerning such conformational changes in the presence of an antibiotic are given for the first time.

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