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Inhibitors of protein synthesis V.* Irreversible interaction of antibiotics with an initiation complex

C. Coutsogeorgopoulos, J. T. Miller and D. M. Hann

Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute, New York State Department of Health, 666 Elm Street, Buffalo, New York 14263, USA

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ABSTRACT

The initiation complex (t-complex) formed in a cell-free system (E. coli) from Ac-Phe-tRNA, poly(U) and washed ribosomes in the presence of initiation factors (ribosomal wash) and GTP, contains the Ac-Phe-tRNA bound quantitatively in a puromycin-reactive state. The t-complex is irreversibly inactivated by spiramycin with respect to its reactivity towards puromycin. The inactivated t-complex retains all of the Ac-Phe-tRNA bound, but it does not react with puromycin (2×10^{-3}M) within 32 min at 25°. In the case of another inhibitor of protein synthesis, sparsomycin, the permanently "modified" t-complex not only retains all the bound Ac-Phe-tRNA but it can still react with puromycin. In the continuous presence of sparsomycin (1×10^{-7}M) the bound Ac-Phe-tRNA reacts quantitatively at a rate which is one-tenth the rate at which the t-complex reacts with puromycin, at low (6.25×10^{-5}M) or high (2×10^{-3}M) concentrations. These results are not in agreement with current views according to which sparsomycin binds to the ribosome reversibly at a single site with a K_I in the range of 10^{-6} - 10^{-7} M and according to which this site is at the A'-site (puromycin site) of peptidyl transferase.

INTRODUCTION

Factors which are operative in controlling the rate of protein synthesis at the level of messenger RNA translation may be conveniently studied by analyzing the changes which occur in the size and composition of polyribosomes or in the chain length of the peptide products. Changes in these parameters can be also brought about by inhibitors of protein synthesis and the technique of modifying the

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Abbreviations used: poly(U), polyuridylic acid; Ac-Phe-tRNA, N-Acetyl-phenylalanyl-tRNA; Ac-Phe-pu, N-Acetyl-phenylalanyl puromycin; Ac-Phe, N-Acetyl-phenylalanine; t-complex, the Ac-Phe-tRNA-poly(U)-ribosome complex; "sparsomycin-modified t-complex", the hypothetical inactivated t-complex that exists in the continuous presence of sparsomycin; "sparsomycin complex", the disc-adsorbed t-complex which has been exposed to 1×10^{-7} or 1×10^{-6}M sparsomycin for at least 8 min at 25° and then washed.

rate of protein synthesis by means of antibiotics has been used recently by several investigators (1, 2, 3) in studies on transcription and translation. An inhibitor used in such studies is the antibiotic sparsomycin which interferes with protein synthesis in prokaryotic as well as in eukaryotic cells (4). The assumption has been made that sparsomycin inhibits equally the formation of all peptide bonds in a growing polypeptide chain regardless of their position in the chain. However, we have observed (5) in a cell-free system, that sparsomycin inhibits preferentially the formation of the longer peptide chains and leads to the accumulation of short peptides, a fact that would seem to contradict the notion of inhibiting equally the formation of all peptide bonds. We have also observed (ref. 6, p. 484) that the elongation of peptide chains initiated with N-protected aminoacyl-tRNA is inhibited by sparsomycin to a greater extent than the elongation of those initiated with an aminoacyl-tRNA bearing a free α-amino group which is analogous to the initiator in eukaryotes. In order to gain further information, we have first examined in detail the interaction of sparsomycin with an initiation complex containing a N-protected initiator, namely Ac-Phe-tRNA. The results reported here demonstrate that sparsomycin, as well as another antibiotic, spiramycin, interfere irreversibly with the formation of the peptide bond between Ac-Phe-tRNA and puromycin. These antibiotics may act in protein synthesis as irreversible or pseudo-irreversible inhibitors and the ultimate degree of inhibition may not develop instantly after the association of the drugs with the ribosome. These are the first examples of an irreversible interaction between antibiotic inhibitors of protein synthesis and a ribosomal complex. A preliminary report on these results has appeared (7).

MATERIALS AND METHODS

"Washed ribosomes" and ribosomal wash (fraction FWR) were prepared from frozen *E. coli* B cells, harvested before the middle of the logarithmic phase of their growth curve, as described previously (8). Ac-^{3}H-Phe-tRNA was also prepared as described previously (8). Cellulose nitrate filters (type HA 24 mm

diameter, 0.45 micron pore size) were purchased from Millipore Corp. Spiramycin was a mixture of spiramycins I, II and III and was a gift from Rhodia Inc., New York, N.Y. Sparsomycin (Lot No. 6891-NJM-8) was a gift from the Upjohn Co.

The formation of Ac-^{3}H-Phe-pu was followed under four different conditions:

Condition A. An incubation mixture (0.25 ml) was prepared at 0° (ice) by adding in the following sequence: 25 μmoles of Tris-HCl (pH 7.2); 25 μmoles NH_4Cl from a solution adjusted with NH_4OH to pH 7.2; 2.5 μmoles total Mg^{++} (acetate); 80 μg poly(U); 0.1 μmole GTP; 8.0 A_{260} units of washed ribosomes; 100 μg (protein) ribosomal wash; puromycin alone or together with sparsomycin or spiramycin at the indicated concentrations; 3.2 A_{260} units Ac-^{3}H-Phe-tRNA charged with 49 pmoles of H^{3}-Phe (142,000 cpm total). After incubation at 25° for the specified time intervals, the mixture was cooled in ice and the reaction stopped by adding 25 μl of 4N KOH. A 200 μl aliquot was transferred to 1.0 ml of 0.1M sodium acetate pH 5.5 buffer, and Ac-^{3}H-Phe-pu was extracted into 2.0 ml of ethyl acetate essentially as described by Leder and Bursztyn (9). An aliquot (1.5 ml) of the ethyl acetate phase was mixed with 15 ml of Bray's (10) phosphor and the radioactivity was determined with a Packard liquid scintillation spectrometer equipped with automatic external standardization which was used in correcting for quenching. Controls without puromycin were included in each experiment and the values subtracted. Such results are given on the ordinate of Figures 1 and 2. In order to estimate the total amount of Ac-^{3}-H-Phe-pu formed in the whole reaction mixture the values must be multiplied by 1.83.

Condition B. The incubation mixture was the same as that in Condition A except that twice the amount of Ac-^{3}H-Phe-tRNA was used and puromycin was omitted. Where indicated, sparsomycin or spiramycin were included. The incubation was carried out in 0.20 ml with the Mg^{++} reduced to 2.0 μmoles total. After incubation at 25° for 8 min, in order to preform the t-complex, the mixture was cooled

in ice. Fifty μl of an ice-cold solution containing 0.5 μmole of magnesium acetate and puromycin were then added. After reincubation at 25° for the specified time intervals the reaction was stopped by re-cooling in ice and adding 25 μl of 4 N KOH. The subsequent steps were identical to those described under Condition A.

Condition C. (Disc reaction) This assay (11) measures the reaction between the isolated t-complex and puromycin, thus separating this reaction from that in which the t-complex is formed.

I. Formation of the t-complex. The t-complex was first formed in a mixture identical to that described under Condition B by incubating at 25° for 8 or 16 min, thus allowing the binding of Ac-^{3}H-Phe-tRNA into the t-complex to become maximal. The 50 μl with 0.5 μmole magnesium acetate without puromycin were then added and the mixture was kept at 0°. The preparation of the t-complex can be scaled up according to the needs of the experiment. From a 250 μl incubation mixture a single disc containing the adsorbed t-complex was prepared as follows: To a 200 μl aliquot, four ml of ice-cold "binding buffer" (100 mM Tris-HCl, pH 7.2, 50 mM KCl, 10 mM $MgCl_2$) were added. After mixing, the solution was immediately passed through a cellulose nitrate filter disc (24 mm diameter, 0.45 micron pore size) and the filter was washed quickly with three 4 ml portions of the "binding buffer", without allowing any air to pass through the filter. These operations (filtering and washings) were carried out within 2 min from the time of dilution of the preincubated mixture. The disc was then immersed quickly into ice-cold "reaction buffer" (100 mM tris-HCl, pH 7.2, 100 mM NH_4Cl adjusted with NH_4OH to pH 7.2, 10 mM $MgCl_2$, 6.0 mM β-mercaptoethanol). The discs were cut in half, left in the cold buffer and used within two hours. The amount of the t-complex per half disc is in the order of 10^{-9} mmole assuming that Ac-^{3}H-Phe-tRNA is bound to the t-complex in a 1:1 ratio.

II. Puromycin reaction. The disc-adsorbed t-complex was incubated at 25° with puromycin in 1.0 ml "reaction buffer" in the presence or absence of

inhibitor (sparsomycin or spiramycin) and the percent (α) of the bound Ac-^{3}H-Phe-tRNA that reacted with puromycin was determined as described previously (ref. 8, Legend to Table I). Briefly, after the puromycin reaction proceeded for the desired time interval, an equal volume (1.0 ml) of 1 N NaOH was added to stop the reaction and the incubation continued at 25° for at least 30 min in order to hydrolyze the unreacted Ac-^{3}H-Phe-tRNA to Ac-^{3}H-Phe. One aliquot (0.80 ml) was extracted with ethyl acetate in order to determine the amount of Ac-^{3}H-Phe-pu (P) formed and an equal aliquot was counted to give the sum (N_o) of Ac-^{3}H-Phe-pu plus Ac-^{3}H-Phe, which represents the total amount of Ac-^{3}H-Phe-tRNA bound to the disc as part of the t-complex. By dividing P by N_o and multiplying by 100, the quantity α was obtained.

Condition D. Exposure of the disc-adsorbed t-complex to sparsomycin or spiramycin. This process was carried out in two ways: I. Without re-washing the exposed disc. The half-disc with the adsorbed t-complex was exposed to the inhibitor in 0.8 ml "reaction buffer" at 25° for the specified time interval in the absence of puromycin. Puromycin was then added in a volume of 0.2 ml, the reaction allowed to proceed for the desired time interval and then stopped by adding 1.0 ml of 1 N NaOH. The percent (α) of the bound N-Ac-^{3}H-Phe-tRNA that reacted with puromycin was determined as described under Condition C.

II. After re-washing the exposed disc. In an alternate procedure, after exposing the whole disc to the inhibitor in "reaction buffer" the reaction was stopped by adding a large excess (8 ml) of cold "binding buffer" and the disc was washed two times with 4 ml portions of cold "binding buffer" as described under Condition C (part I). The disc was then left to soak for less than 2 min in cold "binding buffer" in order to remove traces of mechanically-held inhibitor and washed once more with the same buffer. The disc was finally cut in two halves, left in cold "reaction buffer" and brought into reaction at 25° with puromycin exactly as described under Condition C (part II).

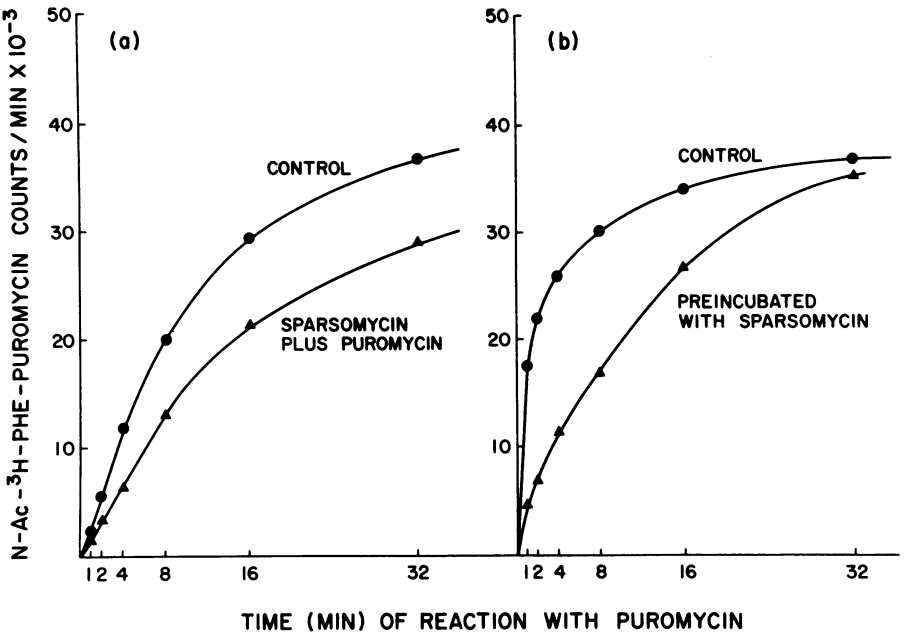

Fig. 1 Ac-Phe-pu formation in solution in the presence or absence of sparsomycin. (a) The assay was carried out under Condition A; (-●-●-●-) in the absence of inhibitor with 6.25×10^{-5} M puromycin; (-▲-▲-▲-) in the presence of 1×10^{-7} M sparsomycin and 6.25×10^{-5} M puromycin. (b) The assay was carried out under Condition B; (-●-●-●-) in the absence of inhibitor with 6.25×10^{-5} M puromycin added after the t-complex was preformed by pre-incubating at 25° for 8 min; (-▲-▲-▲-) the t-complex was preformed by preincubating at 25° for 8 min in the presence of 1×10^{-7} M sparsomycin. Puromycin (6.25×10^{-5} M) was added subsequently and the mixture was reincubated at 25°.

RESULTS

As shown in Fig. 1, the degree of inhibition of N-Ac-Phe-pu formation by sparsomycin (1×10^{-7} M) varied depending on the conditions of the reaction. There was a lower degree of inhibition when puromycin and sparsomycin were added together (Condition A) than when sparsomycin was already present while the t-complex was formed before the addition of puromycin (Condition B). The reaction in solution does not lend itself to kinetic analysis because, as pointed out elsewhere (12, 13), initial rates cannot be easily followed. For example, it can be calculated (Fig. 1b, control) that 45% of the bound Ac-^{3}H-Phe-tRNA has reacted with puromycin within the first minute under Condition B (in this case, it has been

determined separately that ca 50% of the input Ac-^{3}H-Phe-tRNA had been prebound to the t-complex before puromycin was added). However, it can still be seen that for the first 8 min and at each time-point the differences in the amounts of Ac-Phe-pu formed in the presence and absence of the inhibitor were smaller under Condition A than under Condition B. Similar results were obtained with spiramycin (1×10^{-6}M) as shown in Fig. 2. A striking difference between the data in Fig. 1 and Fig. 2 is that in the case of sparsomycin the total amount of N-Ac-Phe-pu formed in 32 min did not differ much whether sparsomycin was present or not. In contrast, in the case of spiramycin the total amount of Ac-Phe-pu formed was about 50% of that formed in the control, suggesting irreversible inhibition.

The characteristics of the inhibition exerted by sparsomycin and spiramycin were examined in more detail with the aid of the disc reaction (Condition C) which is amenable to kinetic analysis. Under Condition C, Ac-Phe-pu formation is kinetically of the (pseudo-) first order, with the t-complex decreasing exponentially and the concentration of puromycin remaining essentially constant, because of its presence in large excess (12, 13). Thus, there is no need to follow initial rates because the apparent rate constant can be determined as explained in the legend to Fig. 3. Only the reaction of the bound Ac-Phe-tRNA with puromycin is measured and there is no free Ac-Phe-tRNA in the medium to rebind. There is less than 10% 'un-binding' of Ac-^{3}H-Phe-tRNA from the disc within 32 min at 25°. Assuming a 1:1:1 ratio of ribosomes: Ac-^{3}H-Phe-tRNA: poly(U) in the t-complex, each ribosome which is part of the t-complex reacts with puromycin only once. The top line in Fig. 3a shows the full course of the reaction between the t-complex and puromycin (6.25×10^{-5} M) in the absence of sparsomycin. This is clearly a first order reaction with an apparent rate constant, $k_{obs} = 0.23$ min^{-1}. When the disc with the adsorbed t-complex was added to a mixture of puromycin (6.25×10^{-5}M) and sparsomycin (1×10^{-7}M), the course of the reaction is no longer that of a homogeneous first order reaction (Fig. 3a, middle line). An inspection at

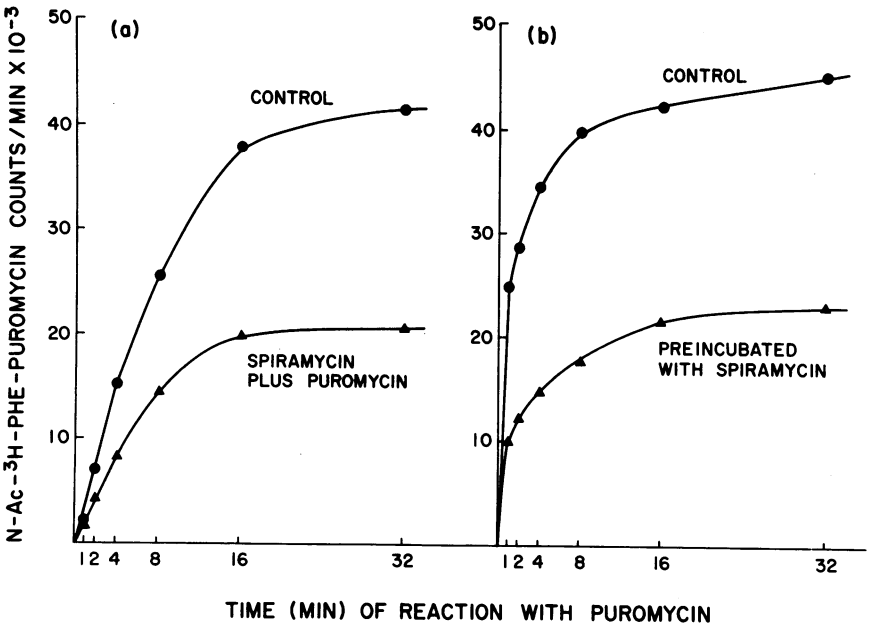

Fig. 2. Ac-Phe-pu formation in solution in the presence or absence of spiramycin. Conditions were exactly like in the legend of Fig. 1 except that sparsomycin was replaced by 1 x 10^{-6} M spiramycin.

each time-point reveals that the degree of inhibition varies with time and this is in accord with the pattern observed with the reaction in solution (Fig. 1a). In contrast, if the disc-adsorbed t-complex was allowed to react first with sparsomycin (25° for 8 min or longer up to 32 min) and then puromycin was added, a homogeneous first order reaction (k_{obs} = 0.02 min^{-1}) occurred (Fig. 3a lower line). The reaction proceeded linearly to completion and in 32 min, 70% of the bound t-complex had reacted. The degree of inhibition was over 90% (the k_{obs} dropped from 0.23 to 0.02 min^{-1}) and this result is unexpected in view of the current thinking (14, 15, 16) that sparsomycin acts as a reversible inhibitor with a K_I in the range of 10^{-6} - 10^{-7}M. If sparsomycin at 1 x 10^{-7} M were acting as a reversible inhibitor with a K_I equal to 1 x 10^{-7}M, the expected degree of inhibition would have been (17) 50%, for non-competitive, or 46% for competitive inhibition taking into account that in the latter case puromycin is present at 6.25 x 10^{-5}M

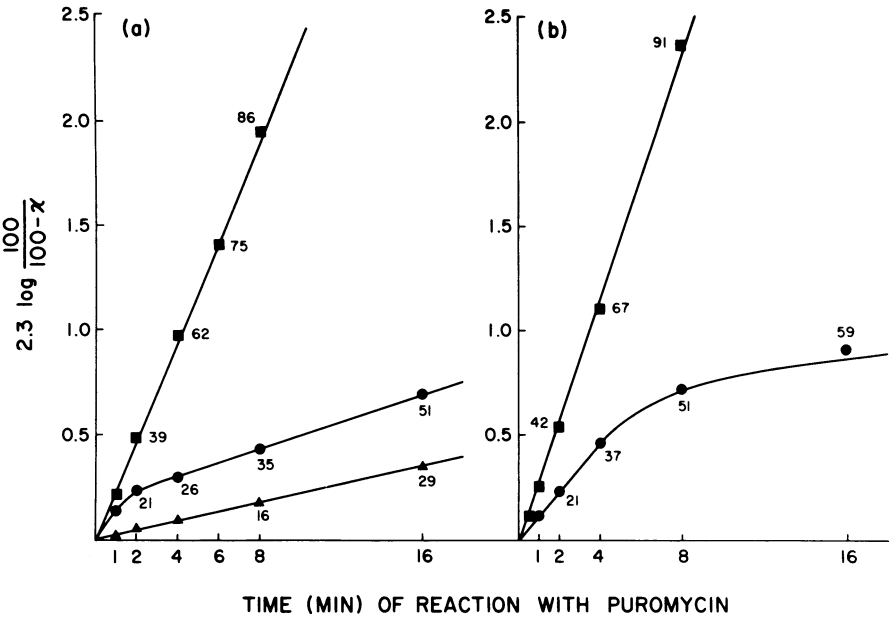

Fig. 3. Reaction between the disc-adsorbed t-complex and puromycin (6.25×10^{-5}M) in the absence or in the presence of sparsomycin or spiramycin. The percent of the bound Ac-^{3}H-Phe-tRNA that reacted with puromycin (α) was calculated as mentioned in Methods (Condition C). The values of α, which are given next to some of the points, at the various time intervals (t) were fitted into the integrated form of a first order reaction:

$$k_{obs} \cdot t = 2.3 \log \frac{100}{100 - \alpha}$$

k_{obs} is the apparent rate constant whose value depends on the concentration of puromycin. The slope of a straight line passing through the origin gives the value of k_{obs}.

(a) (-■-■-■-) control with 6.25×10^{-5}M puromycin in the absence of inhibitor (Condition C). (-●-●-●-) reaction with puromycin (6.25×10^{-5}M) and sparsomycin (1×10^{-7}M) being present simultaneously; (-▲-▲-▲-) the half discs with the adsorbed t-complex were first preincubated with sparsomycin (1×10^{-7}M) at 25° for 8 min and then puromycin (6.25×10^{-5}M) was added and the incubation was continued (Condition D, I).

(b) (-■-■-■-) control exactly like in (a); (-●-●-●-) reaction with puromycin (6.25×10^{-5}M) and spiramycin (1×10^{-6}M) being present simultaneously.

and the K_m is 3.13×10^{-4}M (11). Thus, since the inhibitor caused 90% inhibition at 1×10^{-7}M, sparsomycin is either a reversible inhibitor with a K_I much lower than 10^{-7}M, or it is not acting as a reversible inhibitor. As a matter of fact,

experiments carried out under Condition D strongly suggest the second possibility (see further). Patterns analogous to those shown in Fig. 1 and Fig. 3a for sparsomycin, were also obtained with amicetin (4×10^{-5}M) and with blasticidin S (5×10^{-7}M) and will be the subject of a separate publication.

In agreement with the results shown in Fig. 2, spiramycin at 1×10^{-6}M

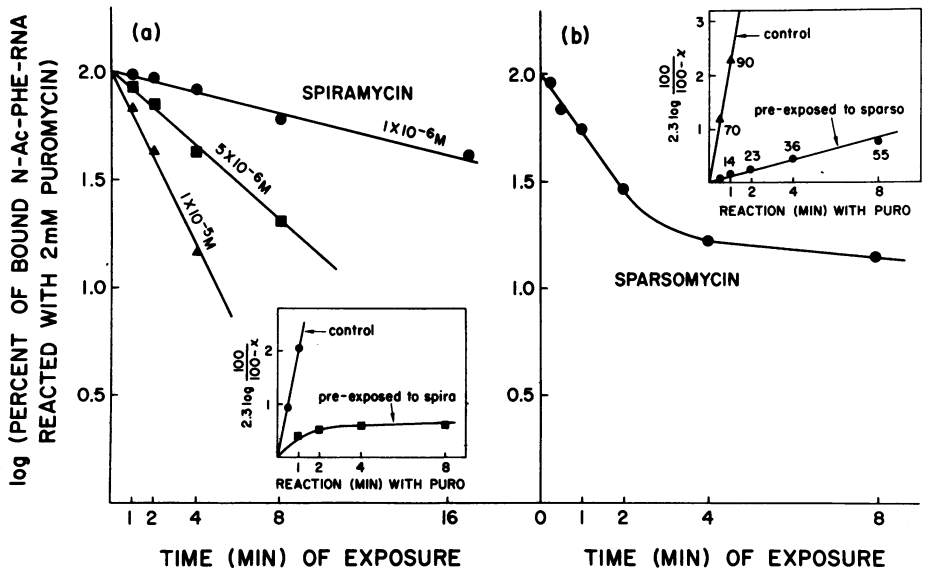

Fig. 4. Course of reaction between the disc-adsorbed t-complex and spiramycin or sparsomycin (a): whole discs were exposed at 25° to spiramycin, at the indicated time intervals and concentrations, in reaction buffer. The discs were then washed (Condition D, II) and added to "reaction buffer" (25°) containing puromycin at 2×10^{-3}M. Ac-^{3}H-Phe-pu formation was determined at 4 min. The quantity α, i.e. the percent of the bound Ac-^{3}H-Phe-tRNA that reacted with puromycin, was determined (see legend to Fig. 3) and its log was plotted on the ordinate. Inset (a): Reaction at 2×10^{-3}M puromycin of (-●-●-●-) control t-complex not exposed to spiramycin, and of (-■—■—■—) the t-complex after exposing to 5×10^{-6}M spiramycin for 4 min and washing the discs; the data were fitted into the equation of a first order reaction (see legend to Fig. 3).

(b): Half discs were exposed at 25° to 1×10^{-7}M sparsomycin in "reaction buffer" for the indicated time intervals. Puromycin was then added at 2×10^{-3}M and the incubation continued for 1 min (Condition D, I). The quantity α was calculated and plotted as explained in (a). Inset (b) Reaction at 2×10^{-3}M puromycin of (-▲-▲-▲-) the control and of (-●-●-●-) the t-complex exposed to 1×10^{-7}M sparsomycin for 8 min at 25° and reacted with 2×10^{-3}M puromycin without washing the disc. The numbers next to some of the points are the values of the quantity α which were fitted into the equation of a first order reaction (see legend to Fig. 3).

clearly showed the characteristics of an irreversible inhibitor (Fig. 3b). When the disc with the adsorbed t-complex was added to a mixture of puromycin (6.25×10^{-5}M) and spiramycin (1×10^{-6}M) (Fig. 3b, lower line) the reaction was first order only during the first 4 min and proceeded at about half the rate of the control (Fig. 3b, upper line). However, after 4 min the reaction proceeded very slowly. In fact, if the t-complex was first exposed to spiramycin (1×10^{-6}M), and puromycin (6.25×10^{-5}M) was then added, the activity of the t-complex was lost to a great extent depending on the duration of the exposure. A more detailed study of the inactivation of the t-complex by spiramycin is shown in Fig. 4a. After the disc-adsorbed t-complex was exposed to spiramycin for various times the discs were washed in order to remove the excess of inhibitor. The disc (half) was then added to a reaction mixture containing puromycin at a high concentration (2×10^{-3}M) and Ac-Phe-pu formation was measured at 4 min (Condition D, II). Actually, titration for 2 or even 1 min would have been sufficient, because the t-complex exposed to buffer alone, in the absence of spiramycin, reacts with 2 mM puromycin quantitatively in less than 2 min (Fig. 4a, inset, upper line). It can be seen that after exposure to 1×10^{-6}M spiramycin for 16 min, approx. 44% of the t-complex remains active (Fig. 4a top line). Inactivation of the t-complex up to 95% has been observed with this procedure (e.g. exposure to 1×10^{-5}M spiramycin for 8 min). It should be noted that once the t-complex is inactivated it cannot be reactivated by incubating at 25° with 2 mM puromycin for as long as 32 min. In fact, the "recovery" of activity under these conditions was estimated to be less than 0.003 min^{-1} for a first order decomposition. In examining the nature of the t-complex after its inactivation with spiramycin, two facts should be borne in mind. First, after incubation in "reaction buffer" at 25° for 32 min there is no more than 10% of the bound radioactivity (^{3}H-Phe) released from the disc, bearing spiramycin-inactivated t-complex, to the medium. Second, non-inactivated t-complex under the same conditions shows loss of radioactivity to the same extent, i.e. less than 10% of the bound radioactivity in 32 min at 25°.

Thus, the fraction of the disc-bound t-complex that is not reacting with 2 mM puromycin at 25° for up to 32 min seems to remain bound to the disc in the form of an inactive complex still bearing the ^{3}H-Phe-moiety of Ac-H^{3}-Phe-tRNA. Fig. 4a (inset, lower line) shows the results of an experiment in which the disc was exposed to 5 x 10^{-6}M spiramycin for 4 min (Fig. 4a, middle line). After washing off the excess inhibitor, the disc was brought in "reaction buffer" containing 2 mM puromycin at 25°. It can be seen that approx. 44% of the bound Ac-Phe-tRNA could react in either 2, 4 or 8 min. The incubation with puromycin was prolonged up to 32 min (results not shown) upon which time the extent of Ac-Phe-pu formation was found to be approx. 51% of the bound Ac-Phe-tRNA.

Experiments analogous to those presented in Fig. 4a were carried out with sparsomycin. Two major differences were encountered. First, as already shown in Fig. 3a, after its reaction with sparsomycin, the "sparsomycin-modified t-complex" reacts with puromycin quantitatively although at a rate one-tenth that of control. Second, if after the end of its reaction with sparsomycin the disc-adsorbed t-complex is washed, it seems to be more active than it is in the continuous presence of the inhibitor. Thus, if the disc-adsorbed t-complex was exposed to 1 x 10^{-7} or 1 x 10^{-6} or 1 x 10^{-5}M sparsomycin for 8 min, and the discs were washed before reacting with 2 x 10^{-3}M puromycin (Condition D, II), the residual activity in 1 min was approx. 36-40% in all three cases. If, on the other hand, the t-complex remained in the continuous presence of sparsomycin (1 x 10^{-7}M) the residual activity was only 10% (Fig. 4b). The full time course of the reaction between the disc-adsorbed t-complex and 1 x 10^{-7}M sparsomycin, is shown in Fig. 4b. After exposure for the specified time and without washing off the excess of the inhibitor (Condition D, I), puromycin at a high concentration (2 x 10^{-3}M) was added and the residual activity was measured by determining the percent of the bound Ac-^{3}H-Phe-tRNA that reacted with puromycin at 1 min. The loss of activity proceeds according to a first order reaction with t 1/2 = 1.1 min corresponding to an apparent rate constant of 0.63 min^{-1}. The lowest residual activity

(ca 10%) was found after exposure for 8 min and did not change if the exposure was continued for 12, 16, 24 or 32 min (results not shown beyond 8 min). This demonstrates that, at 1 x 10^{-7}M, sparsomycin is not rendered inactive during this prolonged incubation.

If the titration of the residual activity is carried out for 2 or 4 min, the end point corresponds to ca 23% or 36% residual activity, respectively. The whole time course of the reaction of the "sparsomycin-modified t-complex" (preincubation with 1 x 10^{-7}M sparsomycin at 25° for 8 min) with 2 x 10^{-3}M puromycin in the continuous presence of sparsomycin is shown in the inset of Fig. 4b (lower line). There occurs a first order reaction (k_{obs} = 0.11 min^{-1}) in which the "modified" t-complex can react quantitatively. In 16 and 32 min the percent of the bound Ac-^{3}H-Phe-tRNA that reacted with puromycin was 78% and 90%, respectively, and the two points (not shown) fall on the same straight line. It has been ascertained that after exposure to sparsomycin all of the Ac-^{3}H-Phe-tRNA remains bound to the disc. The non-inactivated t-complex reacted with puromycin twenty times faster (k_{obs} = 2.3 min^{-1}) as shown in Fig. 4b inset (upper line). This large difference in the rate of the puromycin reaction with the "modified" as compared to the non-exposed t-complex and the fact that the rate constant of the inactivation is at least three times smaller (maximum 0.63 min^{-1}) than that of the reaction of the t-complex with 2 x 10^{-3}M puromycin (k_{obs} = 2.3 min^{-1}) justify an examination of the reaction with 1 x 10^{-7}M sparsomycin without washing off the excess inhibitor. However, there may be competition between puromycin and sparsomycin especially at the early stages of the reaction when a large proportion of non-reacted t-complex is present, In such a case, the true rate constant of the reaction in which the activity decreases will be smaller than 0.63 min^{-1} and the titration of the non-reacted t-complex will be affected because both sparsomycin and puromycin will compete for the t-complex although, most probably, not at the same site. Due to these uncertainties and since we could not conveniently remove the excess of inhibitor without bringing an apparent change to the "sparsomycin-modified t-

complex", we chose not to present inactivation rates at concentrations of sparsomycin higher than 1 x 10^{-7}M. After the reaction between the t-complex and sparsomycin has ended, there seems to be no competition between sparsomycin (1 x 10^{-7}M) and puromycin at a possible second site where reversible binding could be occurring. As already mentioned, there is 90% inhibition of the puromycin reaction at low (6.25 x 10^{-5}M) puromycin (Fig. 3a, upper vs lower line) and there is more than 90% inhibition when the concentration of puromycin is increased 32-fold (2 x 10^{-3}M, Fig. 4b, inset).

These results indicate that there is a time-dependent modification of the t-complex by sparsomycin. The reactivity of the t-complex towards puromycin has been modified permanently as evidenced by the following additional data: (a) If, after its exposure to 1 x 10^{-7}M sparsomycin (25°, 8 min), the disc-adsorbed t-complex is washed (Condition D, II) the "sparsomycin complex" does not recover its activity in terms of the rate of its reaction with puromycin. It reacts with 6.25 x 10^{-5}M puromycin quantitatively in a kinetically homogeneous first order reaction and at a rate (k_{obs} = 0.13 min^{-1}) which is 52% that of the t-complex which has been exposed (25°, 8 min) to "reaction buffer" alone (k_{obs} = 0.25 min^{-1}). (b) The "sparsomycin complex" can be further reacted with sparsomycin as evidenced by the fact that, if added to a mixture of puromycin (6.25 x 10^{-5}M) and sparsomycin (1 x 10^{-7}M), it reacts in kinetically homogenous first order reaction with k_{obs} = 0.04 min^{-1} which corresponds to a rate 30% that of its control (k_{obs} = 0.13 min^{-1}). This is in sharp contrast to the heterogeneous kinetics (Fig. 3a, middle line) obtained when the t-complex reacted with a mixture of puromycin (6.25 x 10^{-5}M) and sparsomycin (1 x 10^{-7}M). (c) The "sparsomycin complex" does not show substantial decomposition to active t-complex when heated at 25° for periods up to 32 min in "reaction buffer" and in the absence of puromycin. The increase in the activity of the "sparsomycin complex" due to this treatment corresponded to less than 0.01 min^{-1} for a first order decomposition. (d) The rate of inactivation of the "sparsomycin complex" by 5 x 10^{-6}M spiramycin was

found to be 1.6 times slower than that of the control t-complex which was not "pre-treated" with sparsomycin. (e) These characteristics of the "sparsomycin complex" are not altered if sparsomycin at a ten-fold higher concentration (1×10^{-6}M) is used in preparing this new complex. Taken together, these results suggest that the "sparsomycin-modified t-complex" which is formed by exposure to sparsomycin and which, in the continuous presence of sparsomycin, reacts with puromycin (Fig. 3a, lower line, Fig. 4b inset, lower line) is a new reactive species different from the unexposed t-complex. Furthermore, the complex that is obtained after washing off the excess of sparsomycin ("sparsomycin complex") is also a new reactive species which is different from the unexposed t-complex, but one which can be converted to the "sparsomycin-modified t-complex" by re-exposure to sparsomycin.

The chemical nature of the ethyl acetate-extractable puromycin product obtained from the "sparsomycin-modified t-complex" (Fig. 3a, lower line; Fig. 4b, inset, lower line) remains to be determined, although it is most probably Ac-^{3}H-Phe-pu. For the present investigation it is sufficient to have shown that this product: a) contains all of the radioactivity (^{3}H-Phe) present in the Ac-^{3}H-Phe-tRNA initially bound to the unexposed t-complex, and b) can be formed quantitatively from the bound Ac-^{3}H-Phe-tRNA, according to a first order reaction; the value of the apparent rate constant (k_{obs}) of this reaction depends on the concentration of puromycin ($1.56 \times 10^{-5} \rightarrow 2 \times 10^{-3}$M). The K_m of this reaction, in the continuous presence of sparsomycin (1×10^{-7}M), was found to be 2.7×10^{-4}M which is close to the K_m (3.13×10^{-4}M, ref. 11) of Ac-^{3}H-Phe-pu formation.

DISCUSSION

The initiation complex used in this study, namely the Ac-Phe-tRNA-poly(U)-ribosome complex (t-complex), is known to have properties very similar to those of the naturally occurring initiation complexes (18, 19, 20). The observations presented revealed an, as yet, unknown property of the t-complex, namely its irreversible interaction with sparsomycin and spiramycin. Whether this inter-

action entails covalent or "tight" binding remains to be determined.

Previous studies have demonstrated that sparsomycin: (a) stabilizes the t-complex (21), (b) is bound to the t-complex (22), and (c) can induce binding of Ac-Phe-tRNA to washed ribosomes even in the absence of poly (U) (23). Thus, it became evident that sparsomycin may bind to the t-complex and interfere with the reaction between the Ac-Phe-moiety of Ac-Phe-tRNA and puromycin. However, in all studies where the interaction between puromycin and sparsomycin was examined by following the kinetics of the inhibition of the puromycin reaction, it was assumed that sparsomycin acted as a reversible inhibitor. On this basis, the analysis showed competitive kinetics (14, 15, 24, 25, 26, 27) and the concept has developed that sparsomycin binds at the A' site of peptidyl transferase where puromycin supposedly binds before forming a peptide bond (for reviews see ref. 4, 28 and 29). In support of this concept it was shown (16, 30, 31) that sparsomycin inhibited the binding of the C-A-C-C-A (Phe) fragment which is derived from the 3'-end of Phe-tRNA. On the other hand, it was also shown (16) that sparsomycin can stimulate the binding of the C-A-C-C-A-(Phe-Ac) fragment which is derived from the 3'-end of Ac-Phe-tRNA. Since the 3'-end of Ac-Phe-tRNA, or the fragment therefrom, supposedly bind to the P' site of peptidyl-transferase, and if a single site of action is accepted, it becomes questionable whether the site at which sparsomycin acts is the A' site or the P' site. The conciliatory view has been that (see e.g., 4, 28, 29) sparsomycin binds at the A' site, inhibits the binding of the amino acyl end of aminoacyl-tRNA and simultaneously stimulates the formation of an inactive complex between the peptidyl end of peptidyl-tRNA and the ribosome. In this scheme, when sparsomycin inhibits the binding of the 3'-end of amino acyl-tRNA, it is assumed to interact with the ribosome reversibly (16).

This investigation provides new information which suggests that modifications are required in the current scheme relating to the mechanism of action of sparsomycin. This is more so, because the present experiments were carried out under mild conditions in the absence of methanol or ethanol which were used

at high concentrations (30-50%) in some of the previous studies. The results presented strongly suggest the existence of a ribosomal site at which sparsomycin interacts irreversibly, at least during the formation of Ac-Phe-pu from Ac-Phe-tRNA and puromycin. This may also be true when sparsomycin interacts with other initiation complexes as well as with polysomes. Further experiments are necessary in order to investigate these points including the nature of the interaction of sparsomycin with eukaryotic ribosomes. The permanent nature of the "modification" in the activity of the t-complex, brought about by sparsomycin, is also supported by the finding (32) that ribosomes isolated from the livers of sparsomycin-injected mice have lower activity as compared to ribosomes from control animals.

The t-complex which has been exposed to sparsomycin, and remains in its presence ("sparsomycin-modified t-complex") is not inert as was thought previously (for a review see ref. 4). It reacts with puromycin as a new species which is different from the unexposed t-complex. It has been reported (22), that sparsomycin is bound on the t-complex which is formed in its presence and, as our experiments suggest, the drug could probably also bind after the t-complex has been formed. Thus, it can be suggested that sparsomycin and puromycin bind at two different sites of the same ribosome which is part of the "sparsomycin-modified t-complex". It remains to be established whether they compete for the same site(s) before sparsomycin binds irreversibly or whether they compete reversibly at a second site after the first site is occupied. The second possibility does not seem likely because there is no decrease in the degree of inhibition when the concentration of puromycin is increased thirty-two fold (Fig. 3a and 4b, inset).

Within the time scale of the present experiments, the t-complex that has been inactivated by spiramycin does not react with puromycin. Because of this non-reactivity, it cannot be assessed whether or not spiramycin has occupied the puromycin site. Further experiments are needed to investigate this point and to

examine whether spiramycin is present on the inactivated t-complex. In fact, it has been reported (33) that radioactive spiramycin I was isolated in a complex with ribosomes. The view has been expressed (ref. 29, p. 337) that the ability of spiramycin to inhibit protein synthesis may depend critically upon the length of the nascent peptide on a given ribosome. Since in the present study we have used an initiation complex bearing a peptidyl-tRNA of minimal length (Ac-Phe-tRNA), it remains to be clarified what effect the chain length would have on the irreversible inactivation. In analogy to sparsomycin, we have observed (34) that in a cell-free system spiramycin led to the accumulation of short peptides, whereas it inhibited the formation of the longer ones.

The irreversible interaction between a ribosomal complex and antibiotics, reported here, is of considerable interest and raises some questions as to the validity of previous interpretations of studies using antibiotics as probes for ribosome topography. Based on such studies, models have been proposed which define specific areas on the "amino acid" site (A site) of the ribosome where antibiotics such as sparsomycin, spiramycin or chloramphenicol bind (see e.g. Fig. 3 of ref. 28 and Fig. 6.3 of ref. 29). These models have been constructed mainly on the basis of analyses (e.g. Scatchard, Lineweaver-Burk , Dixon plots, etc.) which are based on the assumption of reversible interactions. In turn, the proposed antibiotic binding sites have served as the primary basis for "mapping" ribosomal proteins. For example, ribosomal proteins that bind chloramphenicol are currently "mapped" at the A' site of peptidyl transferase (e.g. ref. 35, p. 30), because this antibiotic supposedly acts at this site. The A' site is also the site at which sparsomycin is believed to act, although at present the question has not been resolved whether, as could be expected in such a case, sparsomycin interferes with the binding of chloramphenicol (22, 36, 37, 38, 39). This investigation provides evidence which suggests that, because the "sparsomycin-modified t-complex" is capable of reacting with puromycin, it is unlikely that the sites at which sparsomycin and puromycin bind overlap. This assumes, of course, that as suggested (see e.g.

Table I of ref. 16) only one sparsomycin site exists on the E. coli ribosome and that the same is true for puromycin. If so, it may be necessary to reassign the location of the sparsomycin binding site away from the A' site (puromycin site).

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