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The Specificity of Lincomycin Binding to Ribosomes*

F. N. Chang and B. Weisblum

ABSTRACT: The binding of [^{14}C]lincomycin to ribosomes of *Bacillus stearothermophilus* was studied with respect to ribosomal subunit specificity and the stability of the lincomycin–ribosome complex. Ammonium or potassium, but not calcium or magnesium ions, are required for optimal binding. Lincomycin binds to the 50S subunit of *B. stearothermophilus*, but not to the 30S subunit, nor to the 70S ribosome of *Escherichia coli*. Between 0 and 37° both the rate and extent of lincomycin binding by ribosomes increase, whereas only the rate (but not the final extent) of [^{14}C]phenylalanyl soluble ribonucleic acid binding increases. Thus at 25° lincomycin is a more effective inhibitor of phenylalanyl soluble ribonucleic acid binding than at 15°, although the capacity of the ribosomes to bind phenylalanyl soluble ribonucleic acid is the same at these two temperatures. The [^{14}C]lincomycin–ribosome complex dissociates partially upon dilution indicating that, at least, one phase of the binding reaction is reversible. [^{14}C]-Lincomycin can be displaced from the ribosomes by [^{12}C]lincomycin or erythromycin, but not by chlortetracycline. These results suggest that the antagonism between erythromycin and lincomycin observed in intact cells may be owing to a breakdown of the lincomycin–ribosome complex in the presence of erythromycin.

Lincomycin, at low concentrations, inhibits protein synthesis in Gram-positive, but not Gram-negative, organisms (Josten and Allen, 1964). It has been shown to act on the 50S subunit of the ribosomes of a Gram-positive organism but not on the corresponding subunit of a Gram-negative organism (Chang *et al.*, 1966). In order to characterize further the model of action of this antibiotic, kinetics, ionic requirements, and subunit specificity of the binding of [^{14}C]lincomycin to ribosomes were studied.

Lincomycin-sensitive, erythromycin-resistant strains of *Staphylococcus aureus* are (phenotypically) lincomycin resistant when grown in the presence of erythromycin (Barber and Waterworth, 1964; Griffith *et al.*, 1965). In the present studies, the interaction of lincomycin and erythromycin with *Bacillus stearothermophilus* ribosomes was examined in cell-free preparations. The reversibility of the [^{14}C]lincomycin–ribosome complex in the presence of erythromycin suggests a mechanism for the antagonism between lincomycin and erythromycin which has been reported for intact cells *in vitro*.

Materials and Methods

Bacterial cells, ribosomes, ribosomal subunits, and aminoacyl-sRNA were prepared as described previously (Chang *et al.*, 1966). For the strain of *B. stearothermophilus* used, the minimum inhibitory concentrations (by tube dilution assay) were 10^{-8} to 10^{-7} M for erythromycin and 10^{-7} to 10^{-6} M for lincomycin. The nitrocellulose membrane assay (Nirenberg and Leder, 1964) was used as described below. Under these conditions, at least four A_{260} units of ribosomes of *B. stearothermophilus* or *Escherichia coli* could be adsorbed from a solution of 3 ml with an efficiency greater than 85%. This was verified using [^{14}C]uracil-labeled *E. coli* ribosomes as well as by determining the A_{260} recoverable from the nitrocellulose membrane filter by extraction with 5% TCA[1] for 30 min at 90°. Incubation of *E. coli* or *B. stearothermophilus* ribosomes with lincomycin had no effect on the amount of A_{260} subsequently recovered from the membrane filter by hot TCA extraction. In calculating the stoichiometry of binding, one A_{260} unit of 70S ribosomes corresponds to 24 $\mu\mu$moles and 100 cpm of lincomycin corresponds to 22 $\mu\mu$moles.

[^{14}C]Lincomycin (specific activity, 5 μc/μmole), prepared by reductive alkylation of *N*-demethyllincomycin treated with [^{14}C]formaldehyde, was generously provided by Drs. B. Margerlein and D. J. Mason of the Upjohn Co. *N*-Demethyllincomycin, a presumed precursor of lincomycin which lacks the methyl substituent on the ring nitrogen, is obtained as a by-

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[1] Abbreviations: PEP, phosphoenolpyruvate; TCA, trichloroacetic acid; ATP and GTP, adenosine and guanosine triphosphates.

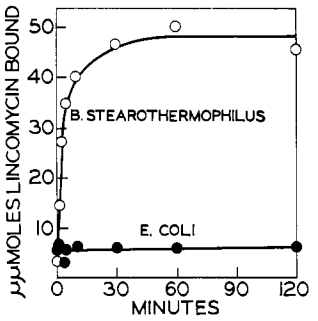

FIGURE 1: Binding of [^{14}C]lincomycin to ribosomes from *B. stearothermophilus* and *E. coli*. The incubations were performed in a total volume of 0.02 ml containing: Tris-HCl, pH 7.4, 0.05 M; $MgCl_2$, 0.02 M; KCl, 0.05 M; β-mercaptoethanol, 0.007 M (assay buffer); *E. coli* or *B. stearothermophilus* ribosomes, 4.0×10^{-6} M (3.3 A_{260} units/0.02 ml of reaction mixture); and [^{14}C]lincomycin, 3.3×10^{-4} M (30,000 cpm/0.02 ml of reaction mixture). The reaction mixtures were incubated at 25° for various lengths of time as indicated above, diluted with 3 ml of cold assay buffer, filtered, and washed with three 5-ml portions of cold assay buffer. A zero-time blank was determined by adding the same amount of [^{14}C]lincomycin to ribosomes diluted in 3 ml of cold standard buffer and filtering as above. For *B. stearothermophilus* and *E. coli*, this blank was 3 μμmoles (15 cpm).

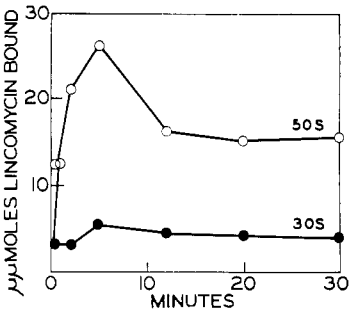

FIGURE 2: Comparison of binding of [^{14}C]lincomycin to 30S and 50S *B. stearothermophilus* ribosomal subunits. The incubations were performed in a total volume of 0.05 ml containing: Tris-HCl, pH 7.8, 0.01 M; $MgCl_2$, 10^{-4} M; KCl, 0.05 M; β-mercaptoethanol, 0.007 M; 30S subunits, 3.7×10^{-7} M (0.25 A_{260} unit/0.05 ml) or 50S subunits, 3.7×10^{-7} M (0.5 A_{260} unit/0.05 ml of reaction mixture); and [^{14}C]lincomycin, 1.2×10^{-4} M (27,000 cpm/0.05 ml of reaction mixture). The reaction mixtures were incubated at 25° and at times indicated chilled by addition of 3 ml of cold assay buffer, filtered, washed, and counted as in Figure 1. The zero-time blank for 30S subunits was 3 μμmoles (15 cpm) and for 50S subunits, 5.5 μμmoles (23 cpm).

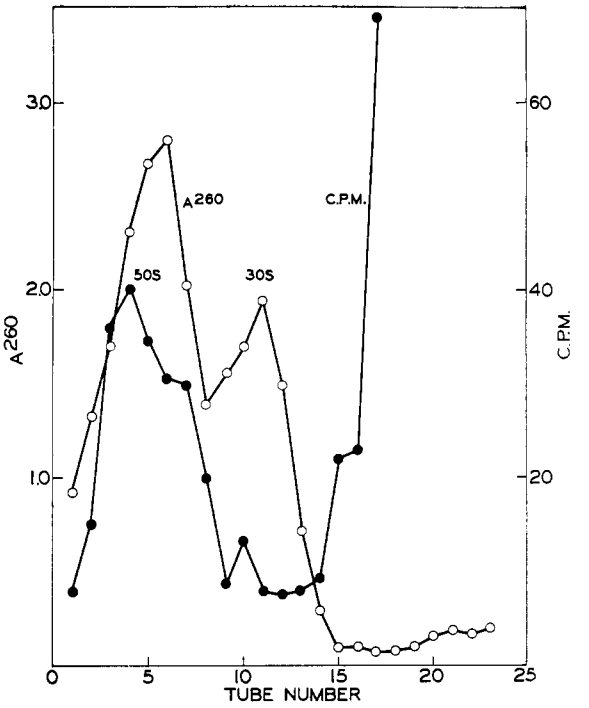

FIGURE 3: Determination of the ribosomal subunit specificity of [^{14}C]lincomycin binding by sucrose gradient centrifugation. Ribosomes used below were first dialyzed against Tris-HCl, pH 7.8, 0.01 M; $MgCl_2$, 10^{-4} M; NH_4Cl, 0.1 M; and β-mercaptoethanol, 0.007 M at 4° for 3 hr. The incubation was performed in a total volume of 0.1 ml containing: Tris-HCl, pH 7.8, 0.01 M; $MgCl_2$, 10^{-4} M; NH_4Cl, 0.01 M; β-mercaptoethanol, 0.007 M; *B. stearothermophilus* ribosomes, 7.9×10^{-6} M (33 A_{260} units/0.1 ml of reaction mixture); and [^{14}C]lincomycin, 10^{-4} M (50,000 cpm/0.1 ml of reaction mixture). The mixture was incubated at 25° for 1 hr, layered on top of a 5–20% linear sucrose gradient, total volume 4.5 ml, and centrifuged at 37,000*g* for 2 hr in the Spinco SW39 rotor. Fractions containing 2 drops each were collected after piercing the bottom of the tube and assayed for A_{260} and radioactivity using a gas-flow counter with 20% counting efficiency.

product of the lincomycin fermentation. Dr. G. Savage of the Upjohn Co. also provided generous supplies of unlabeled lincomycin and erythromycin used in these studies, and chlortetracycline was donated by the Lederle Co.

Measurements of radioactivity were performed using a liquid scintillation counter (Packard) which counted ^{14}C with an efficiency of 60%. The medium used for counting contained 10 g of 2,5-diphenyloxazole, 0.5 g of 1,4-bis-2-(5-phenyloxazolyl)benzene, 500 ml of ethanol, and 1500 ml of toluene.

Results

Requirements for Formation of the Lincomycin-

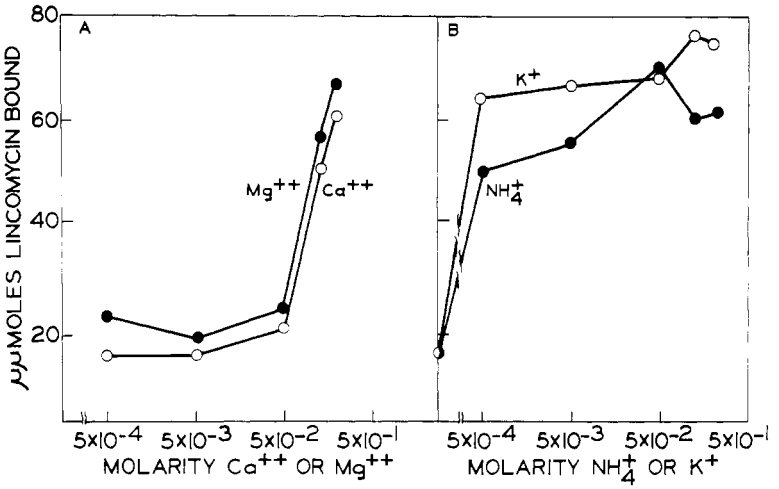

FIGURE 4: Ionic requirements for the binding of [^{14}C]lincomycin to the ribosomes. For determination of calcium or magnesium dependence, the incubation was performed in a total volume of 0.02 ml and contained: Tris-HCl, pH 7.4, 0.05 M; $CaCl_2$ or $MgCl_2$, molarity indicated on the abscissa; ribosomes (*B. stearothermophilus*), 4.0×10^{-6} M (3.3 A_{260} units/0.02 ml of reaction mixture); and [^{14}C]lincomycin, 5×10^{-4} M (45,000 cpm/0.02 ml of reaction mixture). For determination of ammonium or potassium dependence, the incubation was performed in a total volume of 0.02 ml and contained: Tris-HCl, pH 7.4, 0.05 M; $MgCl_2$, 0.0015 M; KCl or NH_4Cl, molarity as indicated on abscissa; ribosomes (*B. stearothermophilus*), 4.0×10^{-6} M (3.3 A_{260} units/0.02 ml of reaction mixture); and [^{14}C]lincomycin, 5×10^{-4} M (45,000 cpm/0.02 ml of reaction mixture). Samples were incubated at 25° for 30 min, then chilled by dilution into 3 ml of cold assay buffer, filtered, washed, and counted as in Figure 1.

Ribosome Complex. Studies on ribosomal binding of sRNA and mRNA have been facilitated by the nitrocellulose membrane filter method of Nirenberg and Leder (1964). An attempt was made to determine whether this method could also be applied to studies on the binding of [^{14}C]lincomycin to ribosomes and their subunits. [^{14}C]Lincomycin was incubated with ribosomes or subunits. At various times, the reaction mixtures were filtered through a nitrocellulose membrane and the amount of radioactivity bound to the membrane was determined. It was observed that lincomycin could be bound by ribosomes from *B. stearothermophilus*, but not by ribosomes from *E. coli* (Figure 1). An average binding of 0.7 mole of lincomycin/mole of ribosome at 25°, or 0.9 mole of lincomycin/mole of ribosome at 37°, was observed (Figure 5). In a similar analysis, using purified ribosomal subunits, binding was observed only to the 50S subunit with respect to both initial rate and final extent (Figure 2).

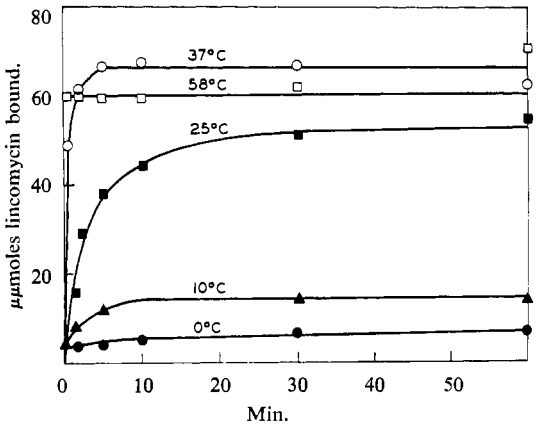

FIGURE 5: Temperature dependence of [^{14}C]lincomycin binding to *B. stearothermophilus* ribosomes. The incubation mixture was the same as that described in Figure 1, except that different incubation temperatures were used as indicated.

The subunit specificity of binding was also determined by a second independent method. *B. stearothermophilus* ribosomes were incubated with [^{14}C]lincomycin at 10^{-4} M $MgCl_2$ as described, and then fractionated by sucrose gradient centrifugation. In agreement with the subunit specificity determined by the membrane filtration method, binding of [^{14}C]lincomycin was observed only to the 50S subunit (Figure 3). Moreover, no trailing of radioactivity was detected indicating that the 50S lincomycin complex did not dissociate during centrifugation through sucrose.

The binding of sRNA to the ribosome requires magnesium (Nirenberg and Leder, 1964) as well as ammonium or potassium (Spyrides, 1964). As shown in Figure 4, magnesium has no effect on [^{14}C]lincomycin binding below 5×10^{-2} M, whereas potassium or ammonium has a profound effect at a level of 5×10^{-4} M. A potassium requirement has also been reported by Vazquez (1964) for [^{14}C]chloramphenicol binding to *E. coli* ribosomes. The binding of lincomycin was not markedly influenced by pH over a range of 5–9 obtained by using Tris-maleate and Tris-hydrochloride

buffers (data not shown).

The rate of binding was studied at five different temperatures (Figure 5). It was found that both the initial rate and final extent of binding are temperature dependent between 0 and 58°. This binding, however, differs from [^{14}C]phenylalanyl-sRNA binding to *E. coli* ribosomes which has been found to be temperature dependent only with respect to initial rate, but not final extent, between 20 and 45 °(C. G. Kurland, personal communication). The difference may be owing, in part, to temperature variation of the equilibrium constant for lincomycin binding. The extent of aminoacyl-sRNA binding to *B. stearothermophilus* ribosomes is the same at 15 and 25° (see Table I), but the extent of lincomycin binding varies over this range. One might, therefore, expect to observe a positive correlation between temperature and inhibition of aminoacyl-sRNA binding. Ribosomes were preincubated at 15 and 25°, respectively, for 30 min (sufficient to reach a plateau level of lincomycin binding characteristic of these two temperatures). [^{14}C]Phenylalanyl-sRNA was added to the incubation mixtures which were further incubated at 15 and 25°, respectively, until a plateau level of [^{14}C]phenylalanyl-sRNA binding was attained. The results of this experiment are tabulated in Table I.

TABLE I: The Effect of Temperature on the Inhibition by Lincomycin of [^{14}C]Phenylalanyl-sRNA Binding to Ribosomes.[a]

Time (min)	Cpm of [^{14}C]Phenylalanyl-sRNA Bound at 15° −Lincomycin	+Lincomycin	% Inhibn	Cpm of [^{14}C]Phenylalanyl-sRNA Bound at 25° −Lincomycin	+Lincomycin	% Inhibn
0	20	—	—	30	—	—
20	316	272	14	352	262	26
40	384	395	0	360	284	21
60	380	368	5	—	—	—

[a] The incubations were performed in a total volume of 0.07 ml containing: Tris-HCl, pH 7.4, 0.05 M; $MgCl_2$, 0.02 M; KCl, 0.05 M; β-mercaptoethanol, 0.006 M; *B. stearothermophilus* ribosomes, 1×10^{-6} M (3 A_{260} units/0.07 ml of reaction mixture); lincomycin, 3×10^{-4} M (where indicated); [^{14}C]phenylalanyl-sRNA (6000 cpm/A_{260} unit), 0.25 A_{260} unit; and poly U, 20 μg. The reaction mixtures were incubated at 15 and 25°, respectively, for the lengths of time indicated, chilled by dilution with 3 ml of cold standard buffer, filtered, and counted. Ribosomes tested in the presence of lincomycin were preincubated at a concentration of 1.0×10^{-6} M in assay buffer containing 3.0×10^{-4} M lincomycin for 30 min at 15 and 25°, respectively.

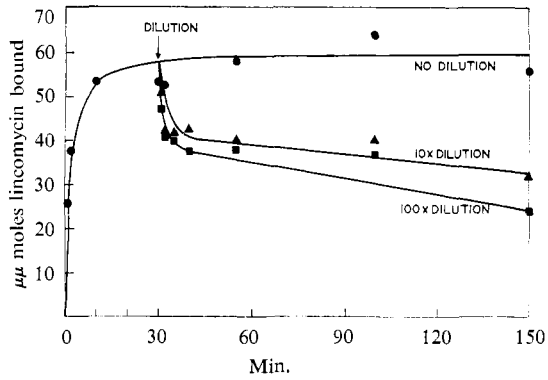

FIGURE 6: Effect of dilution on the [^{14}C]lincomycin–ribosomes complex. The incubation was performed in a total volume of 0.03 ml and contained: Tris-HCl, pH 7.4, 0.05 M; $MgCl_2$, 0.02 M; β-mercaptoethanol, 0.007 M; ribosomes (*B. stearothermophilus*), 2.4×10^{-6} M (3.0 A_{260} units/0.03 ml of reaction mixture); and [^{14}C]-lincomycin, 2.2×10^{-4} M (30,000 cpm/0.03 ml of reaction mixture). All samples were incubated at 25° for 30 min. The undiluted control samples were incubated for an additional 120 min. At various times, as indicated, the samples were chilled by dilution into 3 ml of cold assay buffer, filtered, washed, and counted as in Figure 1. The tenfold diluted samples were prepared by diluting 0.3 ml of original incubation mixture with 2.7 ml of standard buffer and incubated for an additional 120 min at 25°. Samples were taken at the times indicated by filtration and washed with cold standard buffer as above. The 100-fold diluted samples were diluted with 29.7 ml of assay buffer incubated for an additional 120 min at 25°, filtered, washed, and counted as above. The zero-time blank was 14 μμmoles (60 cpm).

Greater inhibition in the binding reaction performed at the higher temperature was observed (consistent with the higher level of lincomycin binding observed at 25°).

Stability of the Lincomycin–Ribosome Complex. In the previous section some requirements for formation of the lincomycin–ribosome complex were determined. The complex does not dissociate appreciably during centrifugation through sucrose or during washing on the membrane filter at 4°. Since the complex can be isolated efficiently by the membrane filtration technique, we examined some properties of the lincomycin–ribosome complex using this method. The rate of binding at 25° is sufficiently slow to permit measurement of initial rates (Figure 5). Therefore, these experiments were performed at this temperature.

The reversibility of the complex was examined. After 30 min at 25° a plateau level of binding was attained. Samples were diluted as indicated and further incubated at 25°, and the amount of complex was determined at various times. Over a period of 150 min the amount of complex in undiluted samples remained

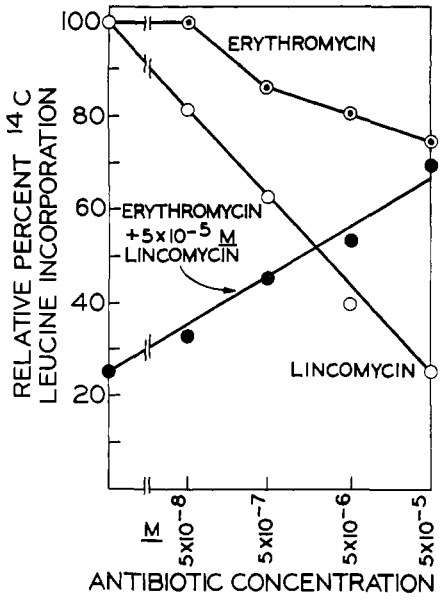

FIGURE 7: The inhibition of poly UC directed [^{14}C]-leucine incorporation in a cell-free extract of *B. stearothermophilus* by lincomycin and the antagonistic action of erythromycin. The incubation mixture was performed in a total volume of 0.25 ml and contained: Tris-HCl, pH 7.8, 0.1 M; $MgCl_2$, 0.013 M; KCl, 0.05 M; β-mercaptoethanol, 0.007 M; ATP, 0.001 M; GTP, 3×10^{-5} M; PEP, 5×10^{-3} M; PEP kinase, 20 μg; [^{14}C]leucine (sp act. 220 μc/μmole), 0.05 μc; 19 unlabeled amino acids (leucine omitted), 2×10^{-4} M each; poly UC (5:1), 10 μg; and iS-30, 0.75 mg of protein. The reaction mixtures were incubated at 37° for 30 min and precipitated with cold 5% TCA. The precipitates were extracted with 5% TCA at 90° for 20 min, filtered, washed, and counted. Antibiotic concentrations were as indicated. The antibiotics were added to the incubation mixture before poly UC. On the ordinate, 100% corresponds to 1971 cpm.

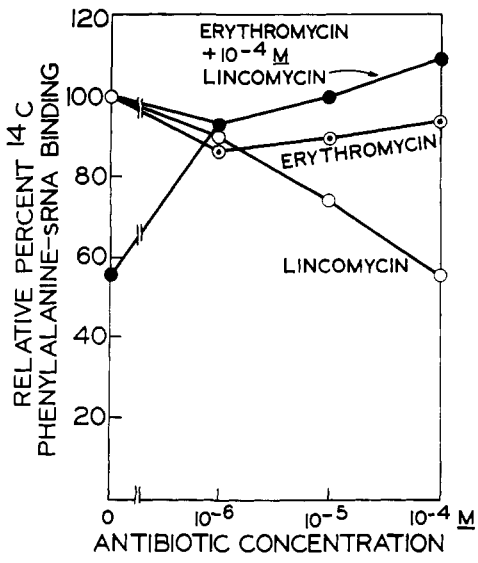

FIGURE 8: The antagonistic action of erythromycin in relation to inhibition by lincomycin of poly U directed [^{14}C]phenylalanyl-sRNA binding to *B stearothermophilus* ribosomes. The incubation was performed in a total volume of 0.1 ml and contained: Tris-HCl, pH 7.4, 0.05 M; $MgCl_2$, 0.02 M; KCl, 0.05 M; β-mercaptoethanol, 0.007 M; lincomycin and erythromycin, final molarity as indicated; poly U, 20 μg; ribosomes (*B. stearothermophilus*), 6.0 $\times$ 10^{-7} M (2.5 A_{260} units/0.1 ml of reaction mixture); and [^{14}C]phenylalanyl-sRNA, 2.0 A_{260} units containing 1800 cpm. The reaction mixtures were incubated at 25° for 15 min, chilled, diluted, filtered, washed, and counted as in Figure 1. On the ordinate, 100% corresponds to 337 cpm.

constant, whereas the amount of complex remaining in the diluted samples decreased, depending on the extent of dilution (Figure 6). Thus, the lincomycin–ribosome complex can dissociate at 25°.

It has been reported by Griffith *et al.* (1965) that erythromycin can act as an antagonist of lincomycin in intact erythromycin-resistant cells of *S. aureus* growing *in vitro*. Since a possible effect of erythromycin on the stability of the lincomycin–ribosome complex may be reflected in the antagonism observed between erythromycin and lincomycin, studies using a cell-free system *in vitro* were undertaken.

Reaction mixtures were prepared (poly UC directed leucine incorporation and poly U directed [^{14}C]-phenylalanyl-sRNA binding to ribosomes). The reactions were supplemented with lincomycin, erythromycin, or both, and the extent of incorporation or binding was determined as indicated. It was observed that the inhibition by lincomycin of poly UC directed leucine incorporation as well as of phenylalanyl-sRNA binding to ribosomes could be reversed by erythromycin (Figures 7 and 8).

The effects of erythromycin on the stability of the lincomycin–ribosome complex were examined next. [^{14}C]Lincomycin and ribosomes were incubated and, after a plateau was reached, [^{12}C]lincomycin or erythromycin was added. It was found in both cases that the amount of [^{14}C]lincomycin bound to the ribosomes decreased (Figure 9). The magnitude of the decrease after a plateau level was attained was found to be dependent on the concentration of added erythromycin or [^{12}C]lincomycin (Figure 10). The effect of chlortetracyline, an inhibitor of aminoacyl-sRNA binding (Hierowski, 1965; Suarez and Nathans, 1965), was also examined. Chlortetracycline, however, does not reverse [^{14}C]lincomycin binding to ribosomes (Figure 10). This is consistent with the localization of chlortetracycline action on the 30S ribosomal subunit (Connamacher and Mandel, 1965; Suzuka *et al.*, 1966).

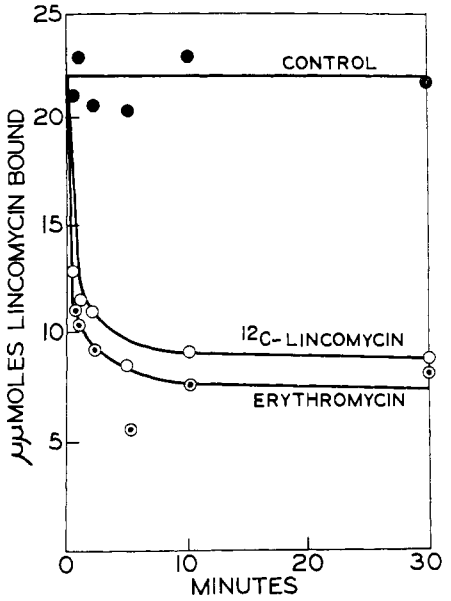

FIGURE 9: Breakdown of [^{14}C]lincomycin–ribosome complex upon addition of [^{12}C]lincomycin or erythromycin. An incubation was performed in a total volume of 0.40 ml containing: Tris-HCl, pH 7.4, 0.05 M; $MgCl_2$, 0.002 M; KCl, 0.05 M; β-mercaptoethanol, 0.007 M; ribosomes (*B. stearothermophilus*), 2.4 × 10^{-6} M (40 A_{260} units/0.4 ml of reaction mixture); and [^{14}C]lincomycin, 3.3 × 10^{-4} M (150,000 cpm/0.4 ml of reaction mixture). The mixture was incubated at 25° for 30 min at which time three 0.10-ml aliquots were added to 0.01 ml of water, 0.05 M lincomycin, or 0.05 M erythromycin, respectively, and incubated further at 25° (final antibiotic concentration, 5 × 10^{-3} M). At various times, as indicated, 0.015-ml aliquots were taken, chilled by dilution into 3 ml of cold assay buffer, filtered, washed, and counted as in Figure 1.

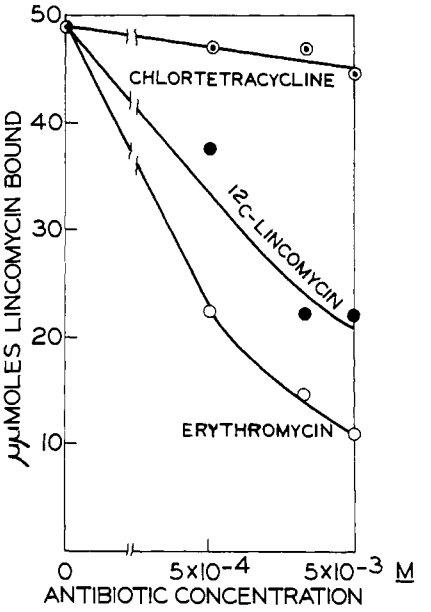

FIGURE 10: Antibiotic concentration dependence of [^{14}C]lincomycin–ribosome complex breakdown. The reaction mixtures were the same as Figure 1. After incubation at 25° for 30 min, chlortetracycline, unlabeled lincomycin, or erythromycin were added to a final concentration as indicated in the figure. The reaction mixtures were incubated for an additional 20 min at 25°, chilled by addition of 3 ml of cold assay buffer, filtered, washed, and counted as in Figure 1.

Discussion

The binding of [^{14}C]lincomycin to ribosomes and ribosomal subunits has been studied and some of the requirements for this reaction were determined. Binding to *B. stearothermophilus* but not to *E. coli* ribosomes was observed. In studies with purified subunits, [^{14}C]-lincomycin was bound by the 50S subunit, but not by the 30S subunit of *B. stearothermophilus*. These results are consistent with the spectrum of activity and subunit specificity of lincomycin observed in other studies (Chang *et al.*, 1966).

Vazquez (1966) has reported that lincomycin inhibited the binding of [^{14}C]chloramphenicol to ribosomes of *E. coli* (determined by the equilibrium dialysis method). Lincomycin at 10^{-4} M was also observed to inhibit poly A directed lysine incorporation and poly C directed proline incorporation in *E. coli*. The former result has been confirmed in this laboratory. It appears, therefore, that lincomycin can interact with *E. coli* ribosomes, but that the binding may be too weak to produce as stable a complex as it does with *B. stearothermophilus* ribosomes. Thus, the minimal inhibitory concentration of lincomycin for *B. stearothermophilus* might not suffice to inhibit protein synthesis in *E. coli*. Since inhibition of almost all protein synthesis would be possible if the incorporation of only one amino acid is inhibited, the observed inhibition of lysine and proline in cell-free extracts of *E. coli* cells seems to conflict with the observed resistance *in vitro* of intact *E. coli* cells. The observation that inhibition of protein synthesis in *E. coli* extracts is possible in the absence of demonstrable lincomycin binding raises the question of the relation, if any, between the observed lincomycin binding to ribosomes and the inhibition of aminoacyl-sRNA binding in *B. stearothermophilus*. Since the amount of lincomycin bound to the ribosome could be varied independently of the capacity of the ribosome to bind aminoacyl-sRNA, it would be a necessary condition that an inverse relation exist between lincomycin binding and aminoacyl-sRNA binding. By varying the temperature it was possible to demonstrate such a relationship. This provides further evidence that in *B. stearothermophilus* the mechanism of action of lincomycin is to inhibit aminoacyl-sRNA binding to ribosomes (Table I). The apparent stability of the lincomycin–ribosome complex at low temperatures contrasted with the apparent increased affinity between lincomycin

and the ribosome observed at higher temperature suggests that the interaction is complex and that various intermediates may occur during the binding reaction.

The inhibition by chloramphenicol of amino acid incorporation into protein in a cell-free system from *E. coli* has been found to be most marked in protein synthesis directed by synthetic messengers rich in A and C but low in U (Kucan and Lipmann, 1964; Speyer *et al.*, 1963). This type of amino acid specificity may be similar to that observed for the action of lincomycin in *E. coli*. We cannot yet interpret these effects unambiguously since we do not know of any step in protein synthesis, following activation, which is amino acid or sRNA specific, *per se*, with the exception of peptide chain initiation or termination. The observation by Vazquez (1966) that lincomycin inhibits [^{14}C]chloramphenicol binding to the 50S subunit of *Bacillus megaterium* further suggests that lincomycin may indeed be bound to *E. coli* ribosomes but that the difference in spectrum may be owing to the relatively lower affinity between lincomycin and its binding site on *E. coli* ribosome. It may be possible to test these hypotheses critically using a more potent derivative of lincomycin.

The ionic requirements for lincomycin binding to ribosomes differ from those for sRNA binding. For example, magnesium has no effect on lincomycin binding below 0.05 M, whereas near maximum effect of ammonium or potassium is observed at 5×10^{-4} M. On the other hand, optimal conditions for sRNA binding are 0.01–0.05 M magnesium (Nirenberg and Leder, 1964), 0.03–0.1 M ammonium (Spyrides, 1964). Thus, lincomycin binding is dependent on ammonium or potassium, but it has not been excluded that increased lincomycin binding at 0.05 M magnesium or calcium may reflect a requirement which *in vivo* is satisfied by a lower intracellular concentration of these ions. It has not yet been determined whether monovalent cations participate at the binding site or whether a generalized conformational change of ribosome is produced which makes it capable of binding lincomycin.

Dissociation of the lincomycin–ribosome complex can be demonstrated by means of the dilution experiment described in Figure 6. Decrease in the amount of complex was also observed on addition of erythromycin or [^{12}C]lincomycin to the reaction mixture after a plateau level of [^{14}C]lincomycin binding was obtained. Since the complex can dissociate, it can be inferred that the loss of complex which occurs upon addition of erythromycin or [^{12}C]lincomycin may be due to an equilibrium exchange reaction in which [^{14}C]lincomycin dissociates from the ribosome making the binding site available to a new molecule of antibiotic.

The inhibition of phenylalanyl-sRNA binding produced by 10^{-4} M lincomycin was completely reversed by 10^{-6} M erythromycin, whereas only partial reversal was observed in relation to leucine incorporation into protein. Based on the data presently available, we wish to propose that lincomycin has a lower affinity for the ribosome, but a higher efficiency in inhibiting cell-free protein synthesis, than does erythromycin. Although lincomycin can produce an 85% inhibition of poly UC directed leucine incorporation, further experiments (not shown) as well as extrapolation of the data present in Figure 7 to infinite erythromycin concentration by means of a reciprocal plot indicated that this inhibition cannot be reversed by erythromycin to a level greater than that produced by the corresponding inhibitory concentrations of erythromycin alone. These data suggest that the mechanism of erythromycin reversal depends on the replacement of lincomycin by erythromycin on the ribosome. Alternatively, a configurational alteration of the ribosome induced by erythromycin may preclude simultaneous binding of lincomycin at another site. Although Griffith *et al.* (1965) failed to detect any chemical modification of lincomycin or erythromycin, the possibility of other classes of direct interaction between these two antibiotics has not been excluded.

Recent experiments of Taubman *et al.* (1966) and Tanaka *et al.* (1966) using *Bacillus subtilis* have demonstrated a form of erythromycin resistance associated with decreased ability of the ribosome to bind labeled erythromycin. Assuming that the erythromycin-resistant mutant studied by Griffith *et al.* (1965) was of this type, the observed antagonism may be formulated in terms of a weak binding of erythromycin which nevertheless may suffice to prevent the binding of lincomycin to the ribosome. Competition experiments, preferably using both antibiotics simultaneously in a double-label experiment, would help to clarify further the nature of their interaction.

The results reported above reveal certain aspects of ribosome function. Considered together with data reported by Vazquez (1966) and by Griffith *et al.* (1965), mutual antagonism appears to be characteristic of a number of commonly used antibiotics which act on the 50S subunit. These investigations suggest how one might predict antagonistic antibiotic combinations on the basis of *in vitro* studies. Moreover, inhibition of [^{14}C]chloramphenicol or [^{14}C]lincomycin binding to ribosomes may be useful in providing preliminary evidence for localization of the site of action of newly isolated antibiotics.

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Optical Rotatory Dispersion of Nucleic Acid Derivatives. VIII. The Conformation of Pyrimidine Nucleosides in Solution*

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ABSTRACT: The optical rotatory dispersion data for an extended series of substituted uracil, thymine, and cytosine nucleosides are reported. The compounds studied include anomeric pairs, cyclouridines, azapyrimidine nucleosides, pseudouridine, and 2′,3′-unsaturated derivatives. Possible explanations of the sign and magnitude of the Cotton effects in relation to the conformation of these compounds are discussed, and it is concluded that normal pyrimidine β-nucleosides have the *anti* conformation in aqueous solution. A rule predicting the sign of the Cotton effect in pyrimidine furanose nucleosides is proposed. This rule is obeyed by all the compounds for which data are available.

The optical rotatory dispersion (ORD) of nucleic acids, both DNA and RNA, as well as of enzymatically synthesized polynucleotides, has been studied intensively for some time, although accurate results in the region below 300 mμ have only been obtained in the last 3 years, beginning with the work of Yang and Samejima on the four deoxyribonucleotides (Yang and Samejima, 1963) and on DNA and RNA (Samejima and Yang, 1964), and our own work on nucleosides (Ulbricht *et al.*, 1964) (for reviews see Ulbricht, 1964, 1965a). (Solutions of high absorbance can give rotatory artifacts simulating Cotton effects (Urnes and Doty, 1961), but necessity of using dilute solutions in the ultraviolet region still does not seem to be generally appreciated (Lamborg *et al.*, 1965).) These studies have been mainly concerned with the effects of temperature, solvent, and pH, factors known to affect the helical structure of polynucleotides. An interpretation of the ORD curves obtained was not possible until recently, for two reasons: first, because no data were available concerning the monomeric components (nucleosides and nucleotides) of these polymeric substances, and second, because the interaction between adjacent residues in a chain was not understood. Studies of oligo- and polynucleotides by various workers, including Tinoco, Yang, Fasman, Brahms, and their respective co-workers, and the interpretation of the ORD of diadenylic acid in terms of interaction between the two bases (Warshaw *et al.*, 1965) has provided a basis for the understanding of the ORD of these substances (for references, see Michelson *et al.*, 1966; Ulbricht, 1964, 1965a).

In previous papers[1] we reported that the ORD curves of pyrimidine α-nucleosides give negative

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[1] It should be noted that in earlier papers of this series a different numbering system was used for the pyrimidine ring. The atoms referred to in this paper as N-1, N-3, N-4, and C-6 were previously designated N-3, N-1, N-6, and C-4, respectively.