Studies of the Binding of Tetracycline to Ribosomes *in Vitro*

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SUMMARY

Ribosomes, isolated from *Bacillus megaterium* or from rat liver, were found capable of binding considerable quantities of tetracycline. The amount bound depended upon the concentrations of tetracycline and of Mg^{2+} and K^+ present. The highest level of binding observed was equivalent to about 300 molecules of tetracycline per ribosome.

Binding of the antibiotic was largely reversible, but, using tetracycline-^{3}H of high specific activity, it was possible to detect a low level of irreversible binding, amounting to less than one molecule per ribosome under the conditions used. This binding was observed to take place to both 30 S and 50 S ribosome subunits.

The possible relevance of the binding to inhibition of protein synthesis by tetracycline is discussed.

INTRODUCTION

The tetracyclines are a group of antibiotics which inhibit protein synthesis in intact bacteria (1–3) and in cell-free systems from bacterial (4–8) and mammalian cells (4, 8). They do not affect amino acid activation or attachment to transfer RNA (tRNA) but strongly inhibit the transfer of amino acids from amino acyl-tRNA to polypeptide on the ribosome (4, 5). Such inhibition might result from an inactivation of ribosomes, perhaps by binding of the antibiotic to essential sites on these particles. Preliminary experiments showed that tetracycline was capable of binding to ribosomes *in vitro*, and studies of this binding under various conditions are described in the present communication.

During the course of this work it has been reported by Connamacher and Mandel (9) and by Day (10, 11) that tetracycline binds irreversibly to ribosomes and ribosome subunits isolated from *Bacillus cereus* or *Escherichia coli*. The results presented below indicate that tetracycline binds to ribosomes reversibly in considerable quantity and irreversibly to a much lesser extent. The observations concerning the irreversible binding are similar to those of Day (10).

MATERIALS AND METHODS

Preparation of Ribosomes from Bacillus megaterium

1. The following method of preparation was employed for the ribosomes used in the experiments in which tetracycline was estimated spectrophotometrically.

Bacillus megaterium, strain KM, was grown in 5 liters of C medium (12) containing 1% w/v glucose and 1% w/v casamino acids (Difco Laboratories, vitamin-free) at 30° with vigorous aeration. The exponentially growing culture (0.5–

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[2] In Figs. 3, 4, and 5 and in Table 1 the concentrations of free and bound tetracycline are expressed in terms of micrograms of tetracycline hydrochloride.

0.7 mg dry weight of cells per milliliter) was chilled by addition of 500–1000 ml of frozen C medium and the cells were harvested at 0–4°. The pellets were rinsed with 10 mM Tris-HCl (pH 7.4), 10 mM magnesium acetate, 60 mM or 200 mM KCl, 10% w/v sucrose, and then the cells were resuspended in 40–45 ml of this medium. Egg-white lysozyme (Armour Pharmaceutical Company) was added (20 μg per milligram dry weight of cells) and the mixture was incubated at 37°. Conversion of the cells to protoplasts was complete within 20–30 min. Subsequent operations were carried out at 0–4°. The protoplasts were harvested by centrifugation and were then lysed by resuspension in 15 ml of Tris buffer of the same salt concentrations but lacking sucrose. DNase (Worthington Biochemical Corporation) was then added to the viscous mixture to give a concentration of 5–20 μg/ml. Sodium deoxycholate (final concentration 0.5% w/v) was added and then the mixture was centrifuged for 10 min at 23,000 rpm in a Spinco, model L ultracentrifuge. The resulting pellet was discarded and the supernatant was centrifuged for 60 min at 50,000 rpm to give a crude ribosome pellet. The latter was resuspended in 10 mM Tris HCl (pH 7.4) containing appropriate concentrations of magnesium acetate and KCl. Remaining debris was removed by centrifugation for 10 min at 23,000 rpm and then the ribosomes were again sedimented by centrifugation at 50,000 rpm for 60 minutes (10 mM Mg^{2+}) or 130 min (0.1 mM Mg^{2+}). The ribosome pellet was resuspended by hand, using a glass pestle, in 5–10 ml of the same buffer. After low-speed centrifugation to remove aggregated material, the suspension was dialyzed overnight against 50 volumes of buffer.

2. In later experiments, using radioactively labeled tetracycline, the ribosomes were prepared by a modification of the above procedure, described elsewhere (13). The modification allowed a much more rapid conversion of the cells to protoplasts and gave a final preparation containing a high proportion of polysomes. The method described above gave preparations containing mainly ribosome monomers and dimers.

Preparation of Ribosomes, Depleted of Messenger RNA

Exponentially growing cultures of *Bacillus megaterium* KM were incubated with actinomycin D (4 μg/ml) for 15 min before chilling and harvesting the cells. Ribosomes were then prepared from the cells by method 2, above. This procedure gave preparations containing ribosome monomers and dimers but very little polysome material.

Preparation of Ribosomes from Rat Liver

Rat liver ribosomes were prepared by the following method, based on that of Korner (14). Four rats were starved overnight and were then decapitated. The livers were rapidly removed into 50 ml of ice-cold 0.5 M sucrose, 3 mM $MgCl_2$, and were minced and then homogenized using a hand homogenizer. Subsequent operations were performed at 0–4°. The suspension was centrifuged for 6 minutes at 1500 rpm and then for 10 min at 11,000 rpm. The middle part of the supernatant was pipetted off and was centrifuged for 30 min at 50,000 rpm. The pellet of microsomes was resuspended in 10 mM Tris HCl (pH 7.4), 10 mM magnesium acetate, 200 mM KCl, and sodium deoxycholate was added to 0.5% w/v. The mixture was centrifuged for 60 min at 50,000 rpm, and the resulting pellet of ribosomes was resuspended in the same medium (lacking deoxycholate).

Measurement of Ribosome Concentrations

The absorbances of diluted suspensions of ribosomes were measured at wavelengths 260 and 280 mμ using a Unicam SP 500 spectrophotometer. The values obtained were related to RNA concentrations using the nomograph of Adams based on extinction coefficients for enolase and nucleic acid (15). This was a convenient means of determining ribosome concentration although the value obtained for RNA concentration could only be regarded as approximate.

Technique Used in Studying Binding of Unlabeled Tetracycline to Ribosomes

Tetracycline hydrochloride was obtained from Lederle Laboratories Division, American Cyanamid Company, New York.

Binding was studied in media containing 10 mM Tris HCl, pH 7.4 and various K^+ and Mg^{2+} concentrations. Measured quantities of ribosomes and of tetracycline, each in the appropriate medium, were mixed at 4° in cellulose nitrate centrifuge tubes. Control mixtures were prepared containing (a) tetracycline but no ribosomes, and (b) ribosomes but no tetracycline. The ribosomes were sedimented by centrifugation and tetracycline was estimated in the supernatants, diluted when necessary, by absorbance measurement as described below. Correction was applied for any absorbance of remaining ribosomal material, using control (b). This absorbance was very small, and it was necessary to apply this correction only when determining low concentrations of tetracycline, when measurements were made on the undiluted supernatants.

The tetracycline concentrations of controls (a) were determined after these also had been centrifuged. The uptake of the antibiotic by the ribosomes was calculated for each mixture from the difference between the tetracycline concentrations of the supernatant and of the corresponding control (a). The uptake could be calculated in this way with reasonable accuracy because a considerable proportion of the antibiotic was bound by the ribosomes. For each point shown in Figs. 3 and 4, the amount of tetracycline taken up was not less than 20% of that remaining in the supernatant. In Fig. 5 the least amount of tetracycline bound was approximately 7% of that remaining. Only one measurement of uptake was made for each tetracycline concentration, and so it was not possible to estimate a standard error. However, it is considered that the error in reading an absorbance of approximately 0.5 is not likely to be greater than ± 0.002. Thus the least accurate calculated uptake in the data presented in this paper is not considered likely to be subject to an error of more than $\pm 12\%$, approximately. On the same basis, where the uptake was 20% or more of the amount remaining in the supernatant, the error would not be more than $\pm 4\%$.

In most experiments the mixtures were prepared in a total volume of 3.5 ml in 4-ml tubes. These were centrifuged for 4 hr at 40,000 rpm in the 40 rotor of a Spinco Model L ultracentrifuge. In some experiments a total volume of 2.0 ml was used in 2-ml tubes. These were centrifuged for 130 min at 50,000 rpm in the 50 rotor. Centrifugation was started as soon as the mixtures had been prepared and was carried out at 4°. The upper part of each supernatant was pipetted off for tetracycline estimation, about 0.5 ml being left above the pellet. The remaining 0.5 ml was discarded and the tubes were inverted and the pellets were allowed to drain.

In order to study the reversibility of the binding, the pellets were resuspended in fresh buffer and the suspensions were made up to the same volumes as the original mixtures (usually 3.5 ml). The suspensions were allowed to stand at 4° for 30 min and then the ribosomes were again sedimented and tetracycline was estimated in the supernatants.

Spectrophotometric Determination of Tetracycline Concentration

At the time of commencement of this work isotopically labeled tetracycline was not available and so a spectrophotometric estimation for the antibiotic was developed. Tetracycline solutions in water show strong ultraviolet absorbance, with absorption maxima at wavelengths of approximately 275 and 360 mμ. The absorption at the latter wavelength offered the possibility of tetracycline estimation in the presence of small quantities of RNA which has negligible absorbance at this wavelength (16).

In the experiments described below, tetracycline was estimated in the presence of high and low Mg^{2+} concentrations and various K^+ concentrations. The absorption spectrum of the antibiotic did not change with the K^+ concentration, in the range

used, but was considerably affected by the Mg^{2+} concentration. Figure 1 shows that an increase in Mg^{2+} concentration from 0 to 10 mM results in a slight shift of the tetracycline absorption maximum to longer wavelengths and in a considerable increase in the maximum absorbance. The binding experiments were carried out (as described above) in media which contained either 0.1 mM or 10 mM Mg^{2+}. In supernatants

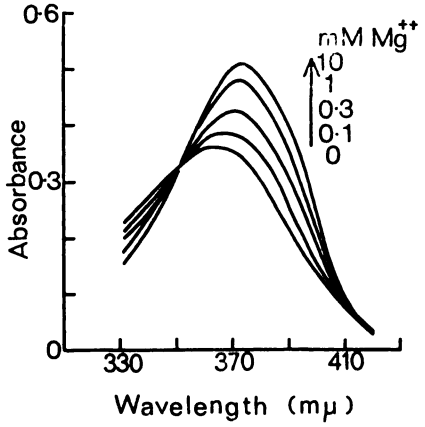

FIG. 1. *Effect of magnesium concentration on the absorption spectrum of tetracycline*

The absorption spectra of solutions of tetracycline HCl (10 μg/ml) in 10 mM Tris HCl (pH 7.4), 60 mM KCl, containing magnesium acetate (concentrations ranging from 0 to 10 mM) were measured using a Zeiss PMQ II spectrophotometer.

prepared at the lower Mg^{2+} concentration, a slight shift of the tetracycline spectrum was observed compared with the controls (a) which had not contained ribosomes. This was probably due to release of small quantities of Mg^{2+} by the ribosomes. No shift of spectrum was observed at the higher Mg^{2+} concentration.

The curves shown in Fig. 1 pass through an isobestic point at wavelength 350 mμ, at which the absorbance is independent of Mg^{2+} concentration. In order to eliminate the effect of slight release of Mg^{2+} by the ribosomes, tetracycline was estimated by absorbance measurement at this wavelength in supernatants obtained from mixtures containing 0.1 mM Mg^{2+}. Measurements were carried out at the absorption maximum, wavelength 374 mμ, with supernatants obtained from mixtures containing 10 mM Mg^{++}. Figure 2 shows calibration

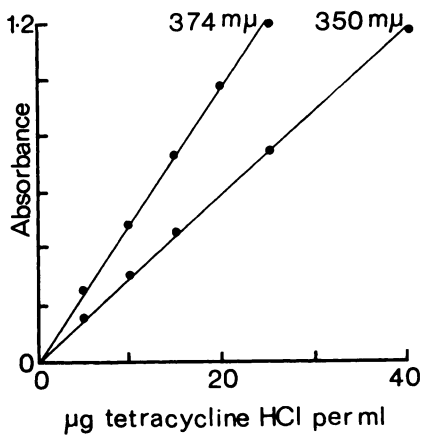

FIG. 2. *Calibration curves for spectrophotometric estimation of tetracycline*

The absorbances of standard solutions of tetracycline HCl in 10 mM Tris HCl (pH 7.4), 10 mM magnesium acetate, 60 mM KCl were measured at wavelengths 350 and 374 mμ using a Zeiss PMQ II spectrophotometer.

curves obtained by measuring the absorbance of standard solutions of tetracycline at these two wavelengths.

Technique Used in Studying Binding of Tetracycline-^3H to Ribosomes

Mixtures (0.20 or 0.25 ml) containing normal or messenger RNA-depleted ribosomes and tetracycline-7-^3H (240 mC/mmole, Radiochemical Centre, Amersham, England) were prepared in 10 mM Tris HCl (pH 7.4), 60 mM KCl, 1 mM or 10 mM magnesium acetate, and were allowed to stand for 15 min at 0°. Excess labeled tetracycline was removed from mixtures containing 1 mM Mg^{2+} by dialysis against 300 ml 10 mM Tris HCl (pH 7.4), 60 mM KCl, 1 mM magnesium acetate for 5 hours at 4°. Mixtures containing 10 mM Mg^{2+} were layered on top of 2 ml portions of 1 M sucrose, containing the same buffer and salt concentrations, in 4 ml centrifuge tubes. The tubes were carefully filled with the same medium (lacking sucrose) and were centrifuged for 90 minutes at 50,000 rpm in a Spinco, model L ultracentrifuge.

The supernatants were decanted and discarded. The small, colorless pellets were drained and were then resuspended in small volumes of 10 mM Tris-HCl (pH 7.4), 60 mM-KCl, 10 mM magnesium acetate.

Samples (0.05–0.20 ml) of the ribosome suspensions were layered on 4.6 ml linear 5–20% w/v sucrose gradients (17) containing appropriate buffer and salt concentrations. These were centrifuged at 37,000 rpm and 4° in the SW39 rotor of a Spinco Model L or L2 ultracentrifuge. In some experiments the gradient tubes were then punctured at the bottom with a needle and fractions (8 drops; approximately 0.14 ml) were collected. Alternate fractions were mixed with 2.5 ml Bray's solution (18) and were counted in a Nuclear Chicago, Model 725, liquid scintillation counter. Absorbances at 260 mμ of the remaining fractions after dilution with 2.5 ml water were measured with a Unicam SP 500 spectrophotometer. In other experiments the tubes were again punctured and then 50% w/v sucrose solution was pumped into the tubes through the needle, at a constant rate. The gradients were passed from the tops of the tubes through a flow cell attached to a Beckman continuous recording spectrophotometer, set to record absorbance at 260 mμ. Fractions (approximately 0.20 ml) were collected from the exit of the flow cell and radioactivity was determined in Bray's solution as above.

RESULTS

Experiments using Unlabeled Tetracycline

Binding of tetracycline to ribosomes in 10 mM Mg^{2+}. The medium used initially consisted of 10 mM Tris HCl (pH 7.4), 10 mM magnesium acetate, 60 mM KCl. Mixtures containing ribosomes (2 mg RNA per milliliter) from *Bacillus megaterium* and tetracycline HCl (concentrations ranging from 10 to 700 μg/ml) were centrifuged to sediment the ribosomes. The tetracycline remaining in the supernatants was estimated as described above.

Figure 3 shows the quantities of tetracycline bound by the ribosomes plotted against the concentrations of the antibiotic

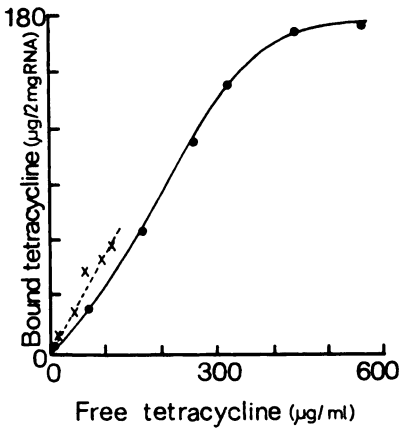

FIG. 3. *Binding of tetracycline by ribosomes from Bacillus megaterium in 10 mM Mg^{2+} and removal of the antibiotic on resuspension of the ribosomes in fresh medium*

Mixtures (3.5 ml) contained ribosomes (2 mg RNA per milliliter) and tetracycline at various concentrations, in 10 mM Tris HCl (pH 7.4), 60 mM KCl, 10 mM magnesium acetate. The ribosomes were sedimented by centrifugation at 40,000 rpm for 4 hours and the concentrations of tetracycline remaining in the supernatants were determined. The ribosome pellets were resuspended in fresh medium (3.5 ml) and were again sedimented by centrifugation. The quantities of tetracycline released into the supernatants were then estimated.[3] Binding ——●——; removal - - - × - - -.

remaining in the supernatant. A considerable proportion of the total tetracycline was bound, saturation apparently being reached at a free tetracycline concentration of about 500 μg/ml.

A rough calculation was made of the greatest number of tetracycline molecules bound per ribosome in this experiment. The greatest binding observed was equivalent to about 85 μg of tetracycline HCl per milligram of RNA of ribosomes. Assuming a molecular weight of 1.7×10^6 for the total RNA of a 70 S ribosome (19), this amount was equivalent to about 300 molecules of tetracycline bound per 70 S ribosome.

Chloramphenicol has been shown to bind optimally to ribosomes *in vitro* in 10 mM Mg^{2+} at a K^+ concentration of 200 mM (20). This K^+ concentration may be nearer to that pertaining in the cell than the con-

centration of 60 mM used in above experiment (21). It was therefore of interest to study the effect of K^+ concentration on the binding of tetracycline to ribosomes. Table 1 shows the amounts of the antibiotic which were bound to ribosomes in 10 mM Tris HCl (pH 7.4), 10 mM magnesium acetate in the presence of various K^+ concentrations. The binding of tetracycline to ribosomes, unlike that of chloramphenicol (20), was inhibited by the presence of KCl. It cannot be concluded definitely that this inhibition was due to K^+ rather than Cl^- ions, but it seems more likely that K^+ was the responsible factor since tetracycline itself is a cation.

TABLE 1

Effect of KCl concentration on the binding of tetracycline by ribosomes from Bacillus megaterium in 10 mM Mg^{2+}

Experimental conditions were as given for Fig. 3 except that the concentration of KCl was varied.[2]

KCl concentration (M)	Tetracycline bound (μg/2 mg RNA)	Tetracycline free (μg/ml)
0	145	232
0.0005	145	226
0.005	136	238
0.06	96	232
0.20	58	232

Binding of tetracycline to ribosomes in 0.1 mM Mg^{2+}. The experiment shown in Fig. 3 was repeated using medium containing 0.1 mM magnesium acetate (Fig. 4). The amount of the antibiotic bound was 0.6 to 0.7 times that bound at the higher magnesium concentration.

Reversibility of the binding of tetracycline to ribosomes. The ribosome pellets obtained in the experiments of Figs. 3 and 4 were used to study the reversibility of the binding, as described in Methods, by resuspending in fresh buffer and sedimenting again. The amounts of tetracycline remaining on the ribosomes were calculated as the difference between the amounts bound in the first experiments and the amounts released on resuspension. These calculated values were plotted against the

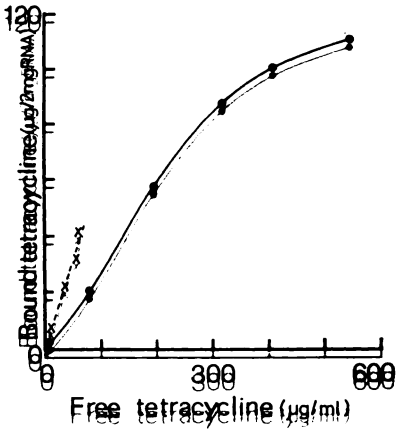

FIG. 4. *Binding of tetracycline by ribosomes from Bacillus megaterium in 0.1 mM Mg^{2+} and removal of the antibiotic on resuspension of the ribosomes in fresh medium*

Experimental details were as given for Fig. 3 except that the medium contained 0.1 mM magnesium acetate.[2] Binding ●—●; removal × - - - ×.

concentrations of tetracycline in the supernatants after the second sedimentation of the ribosomes in Figs. 3 and 4 (broken lines). If the binding were completely reversible, these points would be expected to lie on the same curves as the points from the binding experiments, provided none of the bound material was lost before resuspension of the pellets. The points did not lie on these curves but lay approximately on straight lines through the origin. The results can be explained if about 20% of the bound tetracycline was lost before the pellets were resuspended. This loss could have occurred in the last 0.5 ml quantities of the supernatants which were discarded after the binding experiments (see Methods). Inhomogeneity of refraction was observed when these last portions of supernatants were pipetted off, suggesting that some ribosomal material was being removed.

Binding of tetracycline to rat liver ribosomes. Higher concentrations of the tetracyclines are required to produce a given percentage inhibition of protein synthesis in cell-free systems from mammalian cells than in those from bacterial cells (4). It was therefore desirable to determine

whether different amounts of tetracycline were bound by the ribosomes from the two types of organism.

Mixtures were prepared in 10 mM Tris HCl (pH 7.4), 10 mM magnesium acetate, 200 mM KCl. They contained ribosomes isolated from *Bacillus megaterium* or from rat liver (1 mg RNA per milliliter) and tetracycline HCl (concentrations ranging from approximately 100 to 600 μg/ml). After sedimentation of the ribosomes, the concentrations of tetracycline remaining in the supernatants were determined.

The results are shown in Fig. 5. There

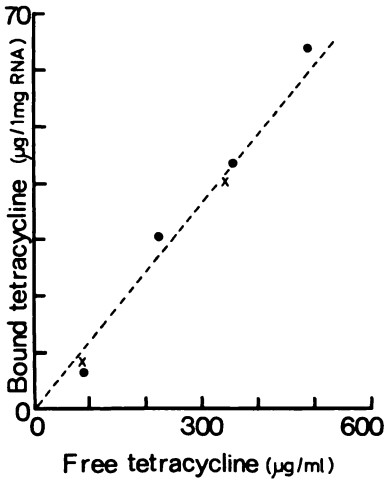

FIG. 5. *Binding of tetracycline by ribosomes isolated from Bacillus megaterium* (●) *and from rat liver* (×)

Experimental details are given in the text.[2]

was no significant difference between the amounts of tetracycline bound by the two types of ribosome.

Experiments Using Tetracycline-^3H

The experiments described above demonstrated that tetracycline was capable of binding reversibly to ribosomes in considerable quantities. However, the method would not have been sufficiently sensitive to detect an irreversible binding of small amounts of the antibiotic (of the order of one molecule per ribosome). Sucrose gradient centrifugation provides an efficient method of washing ribosomes, substances that bind only weakly being removed by this procedure. For example, chloramphenicol-^{14}C is not found on ribosomes centrifuged through sucrose gradients (22), while erythromycin-^3H remains bound (23). The following experiments were performed using this method to determine whether any irreversible binding of tetracycline-^3H to polysomes, ribosomes, or ribosome subunits could be detected.

a. *Binding of tetracycline-^3H to polysomes.* Figure 6 shows a sucrose gradient

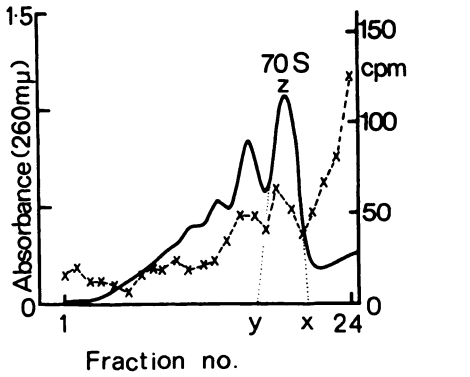

FIG. 6. *Sucrose gradient centrifugation analysis of polysomes from Bacillus megaterium after treatment with tetracycline-^3H*

The incubation mixture (0.25 ml) contained approximately 0.4 mg of RNA of polysomes and 1.5 μg (0.75 μC) tetracycline-^3H HCl in 10 mM Tris HCl (pH 7.4), 60 mM KCl, 10 mM magnesium acetate. After incubation at 0° for 15 min the polysomes were separated by centrifugation through 1 M sucrose and were suspended in 0.33 ml of 10 mM Tris HCl (pH 7.4), 60 mM KCl, 10 mM magnesium acetate. A sample (0.1 ml) was then subjected to sucrose gradient centrifugation as described in Methods. The gradient contained buffer and salts at the same concentrations as the incubation mixture and centrifugation was for 40 min. After centrifugation the gradient was passed through a flow cell attached to a continuous recording spectrophotometer and fractions were collected for determination of radioactivity. Absorbance ——; cpm × --- ×.

centrifugation analysis of polysomes of *Bacillus megaterium* after isolation from a mixture containing tetracycline-^3H. The top of the gradient is shown on the right. Peaks of radioactivity were observed associated with 70 S monomers and 100 S dimers. The counts present in the polysome

region were very low but were all significantly above background (58 cpm), probably representing binding of tetracycline-^{3}H to polysomes. It is not known why the counts increased in the lowest 5 fractions of the gradient, where the absorbance declined almost to zero. The 70 S ribosomes present probably resulted largely from mechanical breakdown of polysomes during resuspension. Much less 70 S material is observed when lysates of protoplasts of *Bacillus megaterium* are centrifuged directly into sucrose gradients (E. Cundliffe, personal communication). There is no reason to suppose that such mechanical breakdown would result in an increase in the ability of the ribosome particles to bind tetracycline.

b. *Binding of tetracycline-^{3}H to ribosomes, depleted of messenger RNA.* Actinomycin D prevents RNA synthesis in gram-positive bacteria while messenger RNA breakdown takes place, probably at the normal rate (24, 25). Incubation of cultures of *Bacillus megaterium* with actinomycin results in the breakdown of polysomes to ribosome monomers which are free of messenger RNA (26). With the concentrations of Mg^{2+} and K^+ used in the present experiments, the preparations of messenger RNA-depleted ribosomes contained monomers and dimers.

Figure 7 shows a sucrose gradient centrifugation analysis of messenger RNA-depleted ribosomes after isolation from a mixture containing tetracycline-^{3}H. Radioactivity was present as a distinct peak in the region of 70 S ribosome monomers and as a faster-sedimenting shoulder. The latter probably represented association of the antibiotic with ribosome dimers and with the small amount of polysomes remaining in the ribosome preparation.

c. *Binding of tetracycline-^{3}H to ribosome subunits in 0.1 mM Mg^{2+}.* The sucrose gradients used for the experiments of Figs. 6 and 7 contained 10 mM magnesium acetate. Further samples of the same preparations of ribosomes and polysomes, isolated from mixtures containing tetracycline-^{3}H were analyzed on sucrose gradients containing 0.1 mM magnesium acetate. This procedure resulted in dissociation of polysomes and 70 S ribosomes into ribosome subunits.

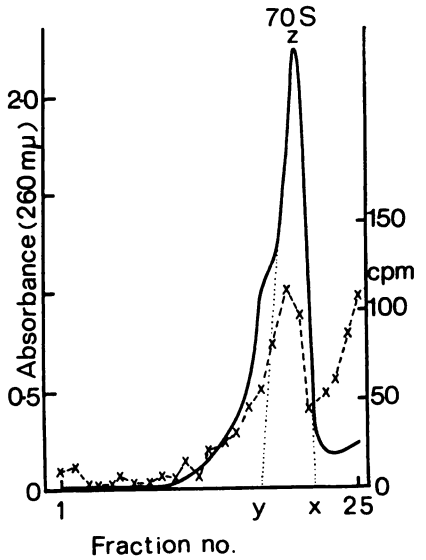

FIG. 7. *Sucrose gradient centrifugation analysis of messenger RNA-depleted ribosomes from Bacillus megaterium after treatment with tetracycline-^{3}H*

Experimental details were as given for Fig. 6 except that polysomes were replaced by ribosomes from actinomycin-treated cells. Absorbance ——; cpm × - - - ×.

The patterns obtained both from the polysomes and from the messenger RNA-depleted ribosomes (Fig. 8) showed a peak of radioactivity in the region of the smaller subunit. There was much less radioactivity in the region of the larger subunit. Similar results have been reported by Connamacher and Mandel (9).

There was some uncertainty as to the exact nature of the subunits observed in Fig. 8. Their sedimentation rates through the sucrose gradients, compared with 70 S ribosomes, were consistent with their being 50 S and 30 S particles. However, later analytical ultracentrifugation studies of ribosome preparations from *Bacillus megaterium* in 10 mM Tris HCl (pH 7.4), 60 mM KCl, 0.1 mM magnesium acetate showed the presence of particles sedimenting at about 40 S and 20 S. Ribosome suspensions in 10 mM Tris HCl (pH 7.4), 60 mM KCl, 1 mM magnesium acetate did,

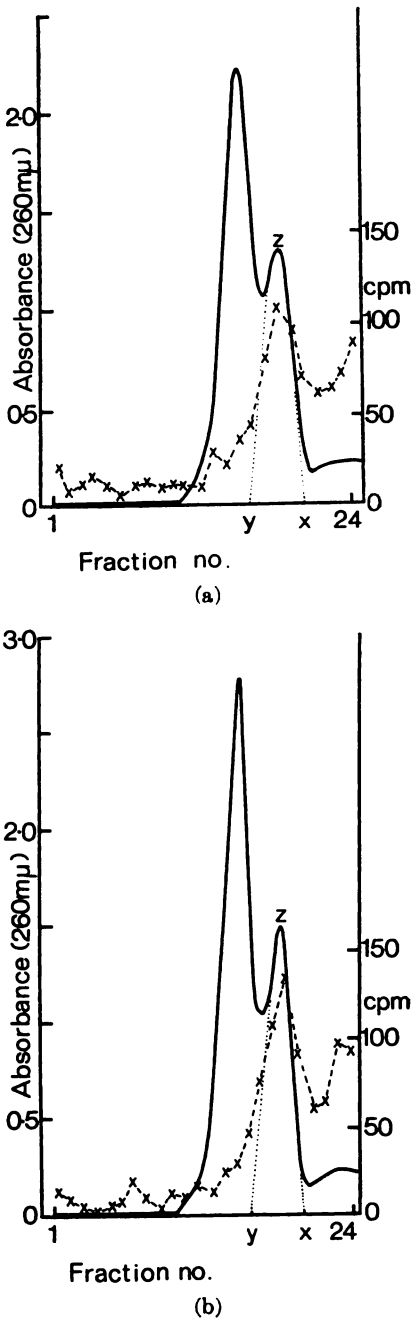

FIG. 8. *Dissociation of (a) polysomes and (b) messenger RNA-depleted ribosomes of Bacillus megaterium, after treatment with tetracycline-³H into subunits by centrifugation into sucrose gradients containing Mg^{2+} at low concentration*

Further samples (0.07 ml) of the suspensions of tetracycline-³H-treated polysomes or ribosomes, obtained as described under Fig. 6 were layered on 5–20% sucrose gradients containing 10 mM Tris-HCl (pH 7.4), 60 mM KCl, 0.1 mM magnesium acetate. Centrifugation was for 90 minutes. Absorbance ———; cpm × - - - ×.

however, contain particles sedimenting at about 50 S and 30 S (unpublished observations). [The 40 S and 20 S particles may perhaps have corresponded to the "cores" obtainable from *Escherichia coli* ribosomes (27).]

d. *Binding of tetracycline-³H to 50 S and 30 S ribosome subunits in 1 mM Mg^{2+}.* A sample (0.7 ml) of a suspension of messenger RNA-depleted ribosomes was dialyzed against 300 ml of 10 mM Tris HCl (pH 7.4), 60 mM KCl, 1 mM magnesium acetate at 4° for 16 hours. Analytical ultracentrifugation (Spinco, Model E ultracentrifuge) of the dialyzed suspension, using schlieren optics, showed two peaks only. The measured sedimentation coefficients (28) were 51 S and 33 S.

Figure 9 shows the sucrose gradient pattern of this preparation after treatment with tetracycline-³H. Excess labeled antibiotic had been removed by dialysis (see Methods) and the sucrose gradient contained 1 mM Mg^{2+}. The quantity of ribosomes loaded on the gradient was larger in this experiment than in the one shown in Fig. 8 and centrifugation was carried out for a longer time in order to obtain better resolution of the particles. Association of radioactivity with both subunits was observed, the ratio of counts per minute to absorbance being considerably greater for 30 S than for 50 S particles. Similar results have been reported by Day (10).

e. *Calculation of the number of tetracycline molecules bound per particle in the experiments described in (a) to (d).* The number of tetracycline molecules bound per particle in given regions of the gradients was calculated from the total counts per minute and the total absorbance in these regions.

The specific activity of the tetracycline-³H HCl was determined in terms of counts per minute per microgram by counting samples (10 μl) of a standard aqueous

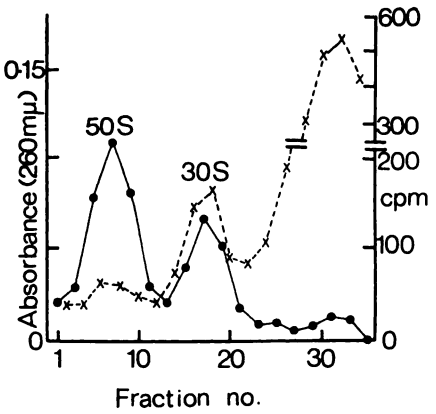

FIG. 9. *Binding of tetracycline-^{3}H by 30 S and 50 S ribosome subunits*

The incubation mixture (0.20 ml) contained approximately 0.32 mg RNA of 30 S and 50 S particles and 2.0 μg (1 μC) of tetracycline-^{3}H HCl in 10 mM Tris-HCl (pH 7.4), 60 mM KCl, 1 mM magnesium acetate. After removal of excess antibiotic by dialysis the mixture was layered on a 5–20% sucrose gradient containing buffer and salts at the same concentrations as in the incubation mixture. Centrifugation was carried out for 3 hours and then fractions were collected from the bottom of the gradient. Alternate fractions were used for measurement of absorbance and radioactivity. Absorbance ———; cpm ×---×.

solution in 2.5 ml quantities of Bray's solution containing a volume of 10 mM Tris HCl (pH 7.4), 60 mM KCl, 10 mM magnesium acetate, 5% w/v sucrose equal to that of one fraction from a gradient. Under these conditions, the counting efficiency was about 6%. The presence of higher sucrose concentrations, within the range used in the gradients, did not decrease the counting efficiency significantly. Using the molecular weight of tetracycline HCl (=481) it was calculated that 1000 cpm were equivalent to 34 μμmoles tetracycline.

The total absorbance was related to number of micromoles of ribosome particles using the assumptions that there are 1.7×10^6 daltons of RNA per 70 S ribosome, distributed between 50 S and 30 S subunits in the ratio 2:1 (19) and that a ribosome suspension containing 1 mg RNA per milliliter has an absorbance at 260 mμ of approximately 25 (K. McQuillen, personal communication). This calculation gave the result that 1 absorbance unit (260 mμ) was equivalent to 24 μμmoles of 70 S ribosomes.

For gradients passed through the Beckman continuous recording spectrophotometer, the total absorbances corresponding to given regions were obtained by measuring the areas of the triangles XYZ (Figs. 6–8). In Fig. 9, the 50 S and 30 S regions were taken as fractions 4 through 10 and 14 through 20, respectively.

The results of the calculations are shown in Table 2. Less than one molecule of tetracycline per particle was bound in each case.

TABLE 2

Calculated numbers of tetracycline molecules bound per particle in the experiments of Figs. 6–9

The method of calculation is described in the text.

Fig. No.	Type of particle	Molecules tetracycline bound per particle
6	70 S	0.62
7	70 S	0.54
8a	30 S (?)	0.34
8b	30 S (?)	0.32
9	30 S	0.48
9	50 S	0.26

DISCUSSION

In the first section, above, a reversible binding of considerable quantities of tetracycline to ribosomes (up to 300 molecules per 70 S particle) was demonstrated. The large number of molecules bound suggests that this binding was to a major constituent of the ribosome. Binding of tetracycline to proteins (29, 30), to DNA (30), to polyuridylic acid (9), to polyadenylic acid (11), and to soluble RNA (11) has been reported. It therefore seems likely that the antibiotic may bind both to the RNA and to the protein of the ribosome. If the binding occurred simply according to the law of mass action, to a fixed number of sites per ribosome, the curves plotted in Figs. 3 and 4 would have been rectangular hyperbolas. In fact these curves were

slightly sigmoid in shape, suggesting that the binding of a few molecules of tetracycline per ribosome facilitated further binding. The fact that the amount of tetracycline bound was less at the lower magnesium concentration (cf. Figs. 3 and 4) may mean that the antibiotic binds more strongly to 70 S ribosomes than to dissociated ribosomes. Alternatively, the difference may have been due to a direct effect of Mg^{2+} concentration on the binding reaction(s).

In the second section, binding of tetracycline-^{3}H to ribosome monomers, dimers, subunits and probably to polysomes was demonstrated. This binding was irreversible in that the bound antibiotic was not removed during centrifugation through sucrose gradients. It has been reported by Connamacher and Mandel (9) that tetracycline-^{3}H binds to 70 S ribosomes and to 30 S ribosome subunits of *Escherichia coli* or *Bacillus cereus*. These workers found that preincubation under conditions allowing protein synthesis lessened the ability of ribosomes of *E. coli* to bind tetracycline. They suggested that this effect was due to removal of messenger RNA from the ribosomes and that tetracycline was only capable of binding to ribosomes containing bound messenger RNA. The results presented in Figs. 6 and 7 indicate that tetracycline binds to ribosomes from normal cells and from actinomycin-treated cells of *Bacillus megaterium*. The calculated amounts of the antibiotic bound per 70 S ribosome (Table 2) in Figs. 6 and 7 are not regarded as significantly different since the counts per minute were low, particularly in Fig. 6. As noted earlier, the 70 S ribosomes in the polysome preparation (Fig. 6) probably resulted largely from mechanical breakdown of polysomes during resuspension and therefore probably contained bound messenger RNA. The 70 S particles (and the dimers) observed in Fig. 7 are believed to have been largely free of messenger RNA since they resulted from breakdown of polysomes *in vivo* when the cells were incubated with actinomycin. This breakdown is believed to depend on completion of polypeptide chains and release of ribosome monomers from messenger RNA. Schaechter *et al.* (26) showed that incubation of cultures of *Bacillus megaterium* KM with actinomycin D for 5 min after pulse-labeling with uridine-^{3}H resulted in breakdown of a large proportion of the polysomes to 70 S ribosomes which were almost free of ^{3}H label. In the present communication, the cells from which messenger RNA-depleted ribosomes were prepared had been incubated with actinomycin for 15 min. These results therefore indicate that the presence of messenger RNA is probably not required for the binding of tetracycline to ribosomes of *Bacillus megaterium*.

Association of the labeled antibiotic with subunits obtained by dissociation of polysomes and of messenger RNA-depleted ribosomes (Figs. 8 and 9) was observed. After dissociation of polysomes in 0.1 mM Mg^{2+} about one-third to one-half of the messenger RNA has been observed to sediment in sucrose gradients in the region of the smaller subunit, the remainder sedimenting at lower S values (unpublished observations). The same amounts of labeled antibiotic were bound by the smaller subunits in Figs. 8 and 9 (see Table 2).

Day (10) used sucrose gradient centrifugation to study the binding of tetracycline-^{3}H to ribosome subunits of *Escherichia coli*. He observed a binding of up to 1 molecule of the antibiotic per particle to both 30 S and 50 S subunits. The figures obtained above (Table 2) were considerably lower than this. However, the amount of antibiotic bound may depend on the concentrations of ribosome particles and labeled antibiotic in the incubation mixture. The effects of varying these concentrations have not been investigated. Also, it may be relevant that Day (10) incubated ribosome particles with tetracycline-^{3}H for 30 min at 37° while in the experiments reported above incubation was at 0°.

It seems very likely that tetracycline exerts its inhibitory effect on protein synthesis by binding to ribosomes. Suarez and Nathans (31) showed that the degree of

inhibition of polyphenylalanine synthesis in an *Escherichia coli* cell-free system by tetracycline at a fixed concentration could be lessened by increasing the concentration of ribosomes + polyuridylic acid. Also, Day showed (11) that preincubation of ribosomes with tetracycline impaired their subsequent ability to function in protein synthesis. In this experiment the ribosomes were sedimented twice through 10% sucrose after tetracycline treatment and before addition to the cell-free system. It was therefore presumably the irreversibly bound antibiotic which was responsible for the impairment.

Evidence is presented elsewhere (13) that tetracycline causes a partial removal of transfer RNA from polysomes engaged in protein synthesis *in vitro*. It has been suggested that the antibiotic may compete with amino acyl-transfer RNA for the recognition site of the ribosome-messenger RNA complex (13). This site apparently consists of the messenger RNA codon and part of the 30 S ribosome subunit (32). It may be that the observed binding of tetracycline to the 30 S subunit, (9, 10) and Fig. 9 above, represents association of the antibiotic with part of the recognition site, perhaps preventing access of amino acyl-transfer RNA. Specific, polynucleotide-directed binding of the latter to 30 S subunits in the absence of 50 S subunits is partially inhibited by tetracycline [(32), and D. Vazquez, personal communication]. Under the conditions used above (Fig. 9) and by other workers (9–11) the amount of tetracycline bound by the 50 S subunits was considerably less than by the 30 S. It may be that the binding to 50 S subunits is not directly concerned with inhibition of protein synthesis by the antibiotic.

Reversible binding of tetracycline took place to the same extent to ribosomes isolated from rat liver as to those isolated from *Bacillus megaterium* (Fig. 5). Protein synthesis in a rat liver cell-free system is less sensitive to the tetracyclines than that in bacterial systems (4). This again suggests that it is not the reversible binding of the antibiotic which is responsible for its action. It would be of considerable interest to study the irreversible binding of tetracycline-^{3}H to ribosomes from various sources.

The high level of reversible binding of tetracycline to ribosomes may not be entirely irrelevant to inhibition of protein synthesis by the antibiotic. The sigmoid shape of the binding curve (Fig. 3) suggests that binding of small amounts of tetracycline may cause some alteration in the conformation of the ribosome. This might constitute an additional effect in hindering the functioning of these particles in protein synthesis.

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