Antibiotics and Polyribosomes
Chlortetracycline and Polyribosomes of *Bacillus megaterium*

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SUMMARY

When added to suspensions of exponentially growing protoplasts, low concentrations of chlortetracycline (CTC) caused polyribosomes to break down to 70 S material. Increased concentrations of CTC gave less breakdown. With low drug concentrations the breakdown was complete within a few minutes and was followed by a process in which aggregation of ribosomes occurred. The aggregation process was sensitive to actinomycin D, and the product was degraded by RNase with the release of 70 S ribosomes.

INTRODUCTION

Many antibiotics (e.g., chloramphenicol, the tetracyclines, the macrolides) inhibit protein synthesis in sensitive organisms, and much work has been reported concerning the effects of these antibiotics in cell-free protein-synthesizing systems (1, 2). However, little information has been available concerning the interactions *in vivo* between such antibiotics and polyribosomes, whose role in protein synthesis is now well established, although it has been shown that certain antibiotics bind to ribosomes and polyribosomes *in vitro* (for references see 2).

Before this problem could be studied adequately using bacterial cells it was necessary to devise a method for quantitative release of undegraded polyribosomes. Protoplasts of *Bacillus megaterium* formed by the action of lysozyme are sensitive to gentle lysis by detergents, but this method is not applicable to antibiotic-treated cells since conversion to protoplasts is variable and incomplete.

In the present work this difficulty was overcome by converting cells to protoplasts, incubating the latter under conditions leading to exponential growth (3) and then treating them with antibiotic. Under these conditions the protoplasts were susceptible to lysis by traces of detergent, and it is believed that the released polyribosomes were less degraded than those obtained by more vigorous methods of cell breakage.

The effects of chlortetracycline (CTC) on the content and profile of polyribosomes of *Bacillus megaterium* have been studied under a variety of conditions. The effects of other antibiotics will be reported elsewhere.

MATERIALS AND METHODS

Cultivation. Lyophilized cultures of *Bacillus megaterium* KM were used to start new stocks at monthly intervals. The organism was maintained by subculturing daily in a liquid medium C (4) with 0.1% glucose (w/v) added. A basic liquid medium PRE was used. This is similar to medium PR of Yudkin (5) and contains per liter: KCl 4 g; $MgCl_2 \cdot 6\,H_2O$ 4 g; NH_4Cl 2 g; Na_2SO_4 150 mg; $Na_2HPO_4 \cdot 12\,H_2O$ 350 mg; glucose to 1% (w/v); Difco peptone to 0.1% (w/v); sucrose to 10% (w/v).

For steady-state labeling of cells with ^{32}P, the phosphate concentration was reduced 10-fold (to 10^{-4} M), and ^{32}P orthophosphate (4 μC/ml) was added. Cultures

were grown for 3 generations by shaking in a water bath at 37°. Under these conditions the optical density at 600 mμ (OD_{600}) doubled in 25–30 min (protoplasts in this medium showed a similar doubling time).

Preparation of protoplasts. ^{32}P-Labeled cells were harvested in the exponential phase of growth at densities of about 200 μg bacterial dry weight per milliliter. Suspensions (10 mg/ml in medium PRE) were rapidly and quantitatively converted to protoplasts at 37° by adding lysozyme (200 μg/ml) as described elsewhere (3). The protoplast suspension was then diluted to 200 μg/ml, dry weight, and incubated until the OD_{600} was increasing exponentially. (Protoplast dry weights are referred to the whole cells from which they were derived.) The protoplast density was then about 300 μg/ml, dry weight, and such a suspension of exponentially growing protoplasts was used in each of the experiments reported here.

Lysis of protoplasts. Samples of suspensions were pipetted into ice-cold glass vials containing deoxyribonuclease (DNase) and Triton X-100 (a neutral detergent) to final concentrations of 10 μg/ml and 0.1% (v/v), respectively. All subsequent treatments of lysates were carried out at temperatures not exceeding 4°. Lysates were centrifuged on sucrose density gradients as soon as possible after preparation but there was little or no alteration of the polyribosome profiles of control lysates after standing in ice for 30 min. Sometimes, as described in the text, pancreatic ribonuclease (RNase; 5 μg/ml) was added to protoplast lysates which were then stored in ice for at least 5 min prior to gradient analysis.

Sucrose density gradient analysis. Linear sucrose density gradients having a total volume of 4.6 ml were prepared at 3° and were allowed to equilibrate at that temperature for 5–18 hr before use. Each gradient ranged from 15 to 40% (w/v) sucrose in a buffer containing Tris 0.01 M; magnesium acetate 10^{-2} M; potassium chloride 10^{-1} M; HCl pH 7.5; 0.2 ml of protoplast lysate was layered on each gradient.

Centrifugation was carried out at 3° for 40 min using the Spinco SW 39 rotor at 38,000 rpm. The centrifuge was evacuated for 5 min prior to each run. After spinning, the bottom of each tube was pierced with a needle, and successive fractions of 8 drops (0.13 ml total) were collected into cold 5% (w/v) trichloroacetic acid. After all the liquid had been dripped out of a given gradient tube, 1 ml of 5% trichloroacetic acid was introduced into the bottom of the tube, which was then scraped with a glass rod. The trichloroacetic acid-precipitated ^{32}P-radioactivity recovered in this way was termed the "pelleted fraction." However this fraction undoubtedly contained a small amount of radioactivity which had not been pelleted, since small volumes of liquid remained in the tubes after dripping.

The trichloroacetic acid precipitates were collected on Oxoid membrane filters, rinsed with 1% acetic acid (v/v), dried at 80° for 30 min, and counted in a liquid-scintillation counter (Nuclear Chicago Corporation). A toluene-based scintillation fluid containing 4 g of 2,5-bis-(5′-*t*-butylbenzoxazolyl-2′)-thiophene (BBOT) per liter was used. Routinely 38 ± 1 fractions were collected. The distribution of ^{32}P from steady-state labeled systems was found to be an accurate indicator of the polyribosome profile and was more sensitive than the alternative of estimating absorbance at 260 mμ.

The sedimentation coefficients of materials running in the gradients were not directly measured. The term 70 S has been used to describe the material sedimenting in the same region as the major peak in RNase-treated ribosome preparations.

Inhibition of protein synthesis by CTC. Protoplasts (not labeled with ^{32}P) were prepared as usual and incubated until they were in the exponential phase of growth. The uptake of leucine-^{14}C into hot trichloroacetic acid-insoluble material was followed before and after the addition of CTC at various concentrations. L-Leucine-1-^{14}C (8.5 mC/mmole) was used at a final activity of 0.2 μC/ml. Then 0.5-ml samples were taken into 1.0 ml of 8% trichloroacetic acid (w/v) and held at 90° for 20 min prior to filtration, etc., as above.

MATERIALS

Deoxyribonuclease ("electrophoretically purified") was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey.

Actinomycin D (Dactinomycin) was a kind gift from Merck Sharp and Dohme Inc., Rahway, New Jersey.

CTC (Aureomycin) was obtained from Lederle Laboratories Division, American Cyanamid Company, New York, New York.

Egg white lysozyme was obtained from Armour Pharmaceutical Co., Ltd., Eastbourne, England.

^{32}P-orthophosphate and leucine-^{14}C were obtained from the Radiochemical Centre, Amersham, England.

BBOT was obtained from C.I.B.A. Ltd.

Analar grade reagents were used at all times, and standard solutions were made up in glass-distilled water.

RESULTS

A typical polyribosome profile from a lysate of exponentially growing protoplasts is shown in Fig. 1 together with the effect of treating such a lysate with RNase (5 μg/ml as described in the Materials and Methods section). It can be seen that the RNase removed all the polyribosomes, giving a massive 70 S peak. Assuming that this peak contained 100% of the ribosomes present, it was calculated from the relative numbers of ^{32}P counts recorded in the 70 S peaks, that 80–85% of the total ribosomes were present as polyribosomes in protoplasts in exponential growth.

Breakdown of Polyribosomes in the Presence of CTC

Figure 2 shows the effects of incubating exponentially growing protoplasts with CTC (5 μg/ml and 50 μg/ml) for 5 min prior to lysis and gradient analysis. In each instance breakdown of polyribosomes occurred together with accumulation of 70 S material. The amount of breakdown observed was less after treatment with the higher concentration of CTC than with the lower concentration. After incubation of

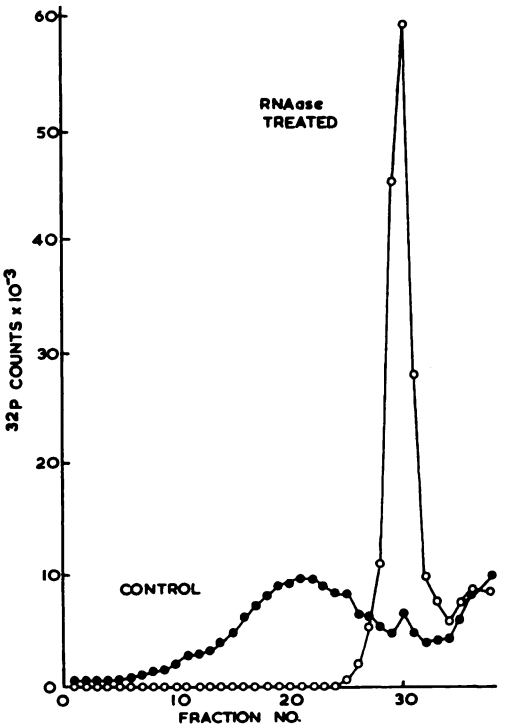

FIG. 1. *Polyribosome profiles from exponentially growing protoplasts: the effect of RNase*

^{32}P-labeled protoplasts were lysed during exponential growth. (●) A portion of the lysate was analyzed on a sucrose density gradient. (○) Another portion of the same lysate was treated with RNase (5 μg/ml) for 5 min at 0° prior to gradient analysis.

protoplasts with CTC (5μg/ml) for 5 min, about 55% of the ribosomes were present as 70 S material (calculated as above).

Figure 3 shows that when incubation with CTC (5 μg/ml) was prolonged beyond 5 min, the amount of 70 S material observed in gradients decreased. Protoplasts were subsequently incubated for up to 50 min with various concentrations of CTC (ranging from 1 to 150 μg/ml) (Fig. 4). Concentrations of CTC of 1–5 μg/ml all gave a maximum accumulation of 70 S material after 5–6 min, after this time the 70 S peak in the gradients began to diminish. The maximum amount of 70 S material accumulating represented about 60% of the total ribosomes present: the subsequent disappearance of 70 S material was, if any-

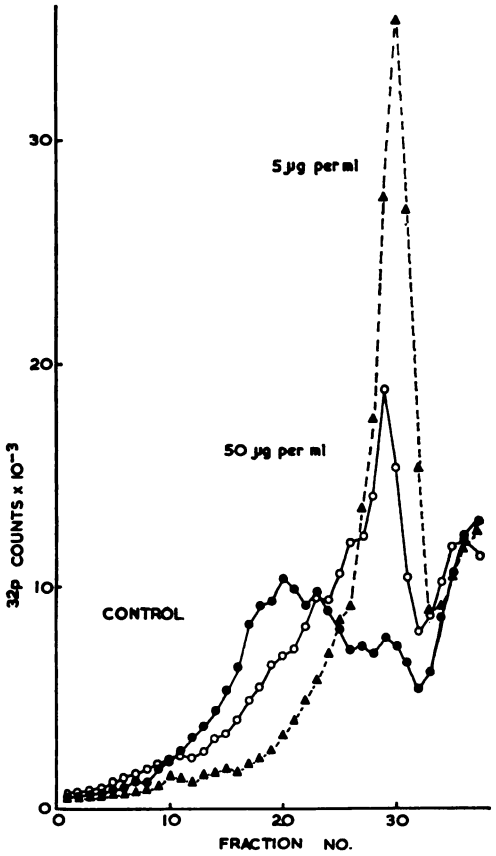

FIG. 2. *Polyribosome profiles after incubation of protoplasts for 5 min with different concentrations of CTC*

Lysates of ^{32}P-labeled protoplasts were analyzed on sucrose density gradients: (●) protoplasts lysed during the exponential phase of growth. (▲) protoplasts incubated with CTC (5 μg/ml) for 5 min prior to lysis. (○) protoplasts incubated with CTC (50 μg/ml) for 5 min prior to lysis.

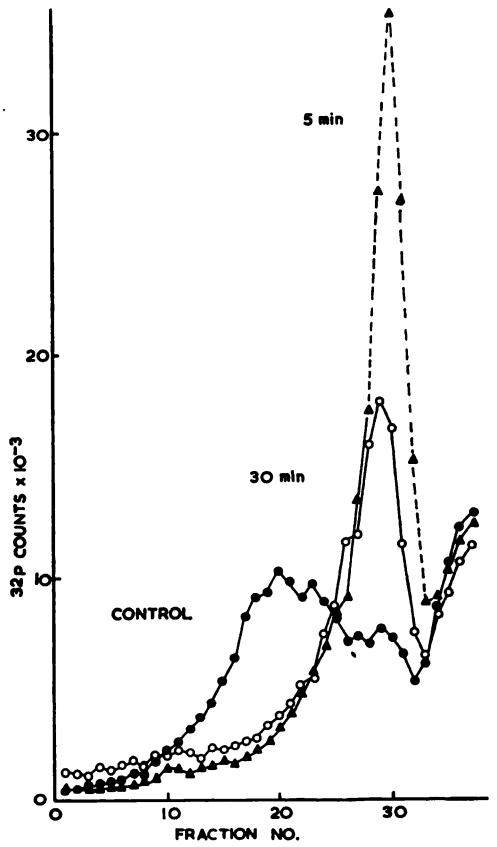

FIG. 3. *Polyribosome profiles after incubation of protoplasts with CTC (5 μg/ml) for different times*

Lysates of ^{32}P-labeled protoplasts were analyzed on sucrose density gradients: (●) protoplasts lysed during exponential growth. (▲) protoplasts incubated with CTC (5 μg/ml) for 5 min prior to lysis. (○) protoplasts incubated with CTC (5 μg/ml) for 30 min prior to lysis.

thing, favored by increased concentrations of CTC (see later). Increased concentrations of CTC gave less breakdown of polyribosomes, and at 150 μg/ml no breakdown was detected at all.

Breakdown of polyribosomes in the presence of CTC seemed only to occur in intact protoplasts. When samples of a lysate of exponentially growing protoplasts were centrifuged on two separate sucrose gradients, one of which contained CTC (5 μg/ml), the resultant polyribosome profiles were indistinguishable (Fig. 5). It does not appear, therefore, that the drug induces breakdown of polyribosomes *in vitro*.

Formation of Ribosome-Aggregates in the Presence of CTC

Protoplasts in exponential growth were incubated with CTC (5 μg/ml) for 5 min to give maximal CTC-induced breakdown of polyribosomes. The incubation of one half of the suspension was continued in the presence of CTC at 5 μg/ml for a further 25 min. The other half was incubated with

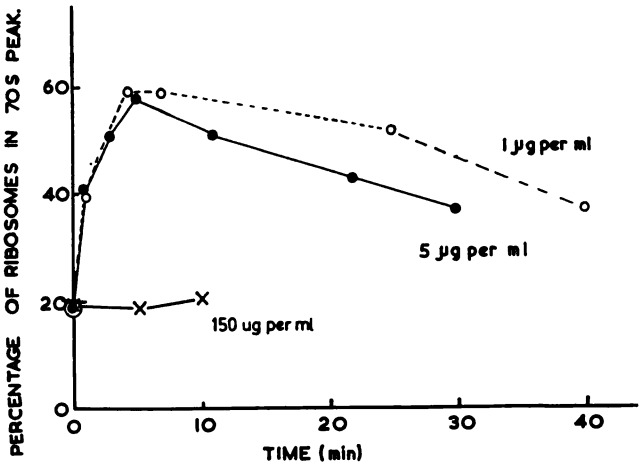

FIG. 4. *Variation in the percentage of the total ribosomes present as 70 S material during incubation of protoplasts with different concentrations of CTC*

^{32}P-labeled protoplasts were incubated with different concentrations of CTC, and samples were taken for lysis and sucrose gradient analysis at various times during the incubations: (○) protoplasts incubated with CTC (1 µg/ml); (●) protoplasts incubated with CTC (5 µg/ml); (×) protoplasts incubated with CTC (150 µg/ml).

The amounts of 70 S material were estimated as the ^{32}P-counts in gradient fractions 27–32 and are expressed as percentages of the corresponding values obtained from RNase-treated lysates (see Figs. 1–3 and Table 1).

CTC at 50 µg/ml for a further 25 min. Samples for gradient analysis were taken at various times during the incubations. Figure 6 shows that with CTC at 50 µg/ml a greater decrease in the amount of 70 S material occurred than at 5 µg/ml. Although not included in Fig. 6 the polyribosome profile of the protoplasts before CTC treatment was similar to that shown in Fig. 1. This applies also to those other figures presented here which do not show "control" protoplast lysates. From Fig. 6 it seemed that the process under study was favored by increased concentrations of CTC (see also Fig. 4), and subsequent experiments revealed that this process involved the aggregation of ribosomes rather than their degradation.

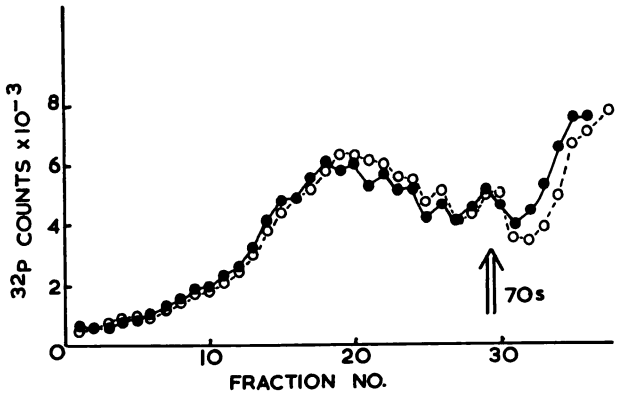

FIG. 5. *Analysis of a protoplast lysate on a sucrose gradient containing CTC (5 µg/ml)*

^{32}P-Labeled protoplasts were lysed during exponential growth. Portions of the lysate were analyzed on different sucrose gradients in the presence and absence of CTC: (●) control (no CTC present); (○) gradient containing CTC (5 µg/ml).

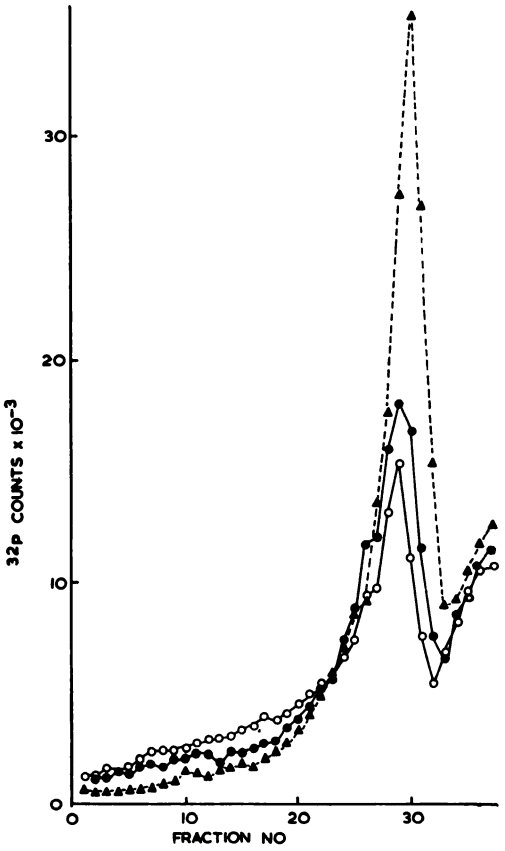

FIG. 6. *The effect of CTC concentration on the "aggregation of ribosomes"*

Lysates of ^{32}P-labeled protoplasts were analyzed on sucrose density gradients after various treatments with CTC: (▲) protoplasts incubated with CTC (5 μg/ml) for 5 min prior to lysis; (●) protoplasts incubated with CTC (5 μg/ml) for 30 min prior to lysis; (○) protoplasts incubated with CTC (5 μg/ml) for 5 min, then with CTC (50 μg/ml) for a further 25 min prior to lysis.

CTC Does Not Cause Aggregation of Ribosomes in Vitro at 0°

Exponentially growing protoplasts were incubated with CTC (5 μg/ml) for 5 min, and the suspension was divided into two fractions. One was incubated for a further 25 min in the presence of CTC at 50 μg/ml; the other was lysed, and portions of the lysate were allowed to stand in an ice bath in the presence and absence of CTC (50 μg/ml) for 60 min. As shown in Fig. 7a,b the addition of CTC at 50 μg/ml induced the usual formation of aggregates *in vivo* whereas there was no such effect when the same concentration of drug was added to chilled lysates.

Ribosome-Aggregates Are Degraded to 70 S Material by RNase

Exponentially growing protoplasts were incubated with CTC (5 μg/ml) for 5 min, then the drug concentration was increased to 50 μg/ml for a further 45 min. Samples were taken at various times during the incubations. Each sample was immediately lysed and the lysate was treated with RNase prior to gradient analysis. Figure 8 shows that each of these three preparations contained virtually the same amount of 70 S material. This indicates that the aggregates contained 70 S ribosomes held together by RNA.

The Formation of Ribosome-Aggregates Is Prevented by Actinomycin D

Exponentially growing protoplasts were incubated with CTC (5 μg/ml) for 5 min, and portions of the suspension were transferred to flasks containing CTC at 5 μg/ml in the presence and absence of actinomycin D at 10 μg/ml (final concentrations). The incubations were continued for a further 25 min. Samples for lysis were taken at intervals throughout the incubations. The lysates were analyzed on sucrose gradients. Figure 9 shows that actinomycin D completely prevented the disappearance of 70 S material. Synthesis of new RNA, therefore, seems to be a necessary prerequisite for the aggregation process. Exactly parallel results were obtained in experiments with other concentrations of CTC.

The amounts of radioactivity recovered from the bottom of the gradient tubes after the fractions had been collected (termed the "pelleted fraction"—see Materials and Methods section) are not shown on Figs. 1–9. However, a balance sheet (Table 1) is presented which accounts for the distribution of radioactivity between the different fractions and the "pellet" in gradients representing each of the effects demonstrated above. Some of the radioactivity recovered in the pellet is due to liquid remaining in the gradient tube after sam-

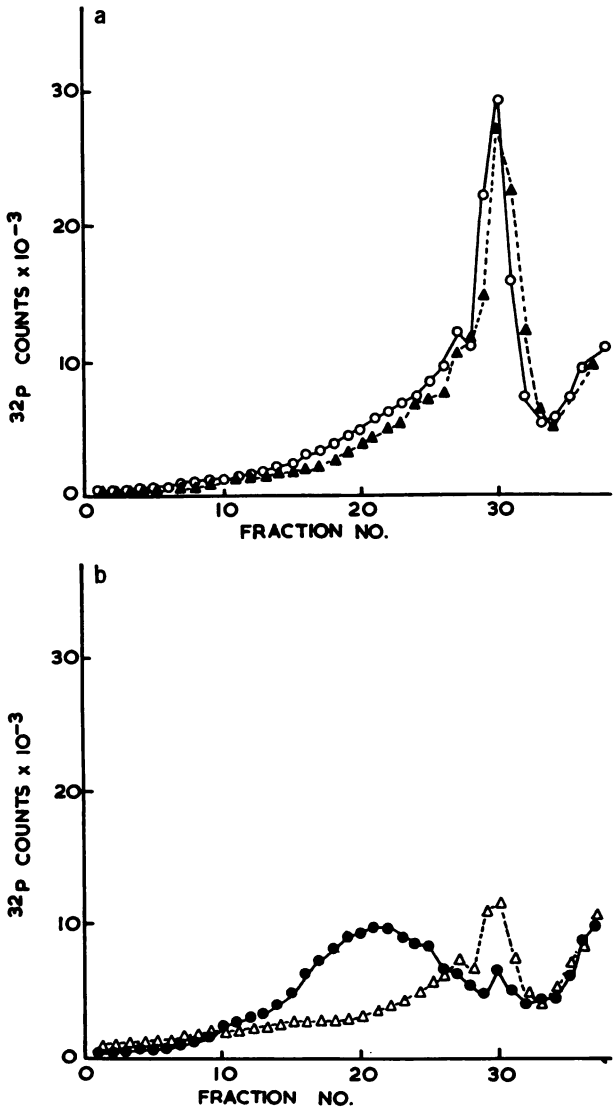

FIG. 7. *The absence of ribosome-aggregation in the presence of CTC in vitro*

Lysates of ^{32}P-labeled protoplasts were analyzed on sucrose density gradients. (a) (○) protoplasts incubated with CTC (5 μg/ml) for 5 min prior to lysis. (▲) protoplasts incubated with CTC (5 μg/ml) for 5 min then lysed and stood in ice in the presence of CTC, (50 μg/ml) for 60 min prior to gradient analysis. (b) (●) protoplasts lysed during exponential growth. (△) protoplasts incubated with CTC (5 μg/ml) for 5 min, then with CTC (50 μg/ml) for a further 35 min prior to lysis. i.e., the lysate analyzed contained "ribosome-aggregates."

pling; the amount of radioactivity recovered in this fraction after RNase-treatment possibly indicates the extent of this contamination. From the table it can be seen that recovery of radioactivity from the gradients was virtually quantitative, and that the same amount of 70 S material, was present after RNase treatment irrespective of the manner in which the protoplasts had been pretreated. Further, it is evident that the radioactivity not accounted for in gradients in Figs. 3, 6, and 7b had been pelleted rather than degraded to soluble material. It is therefore clear

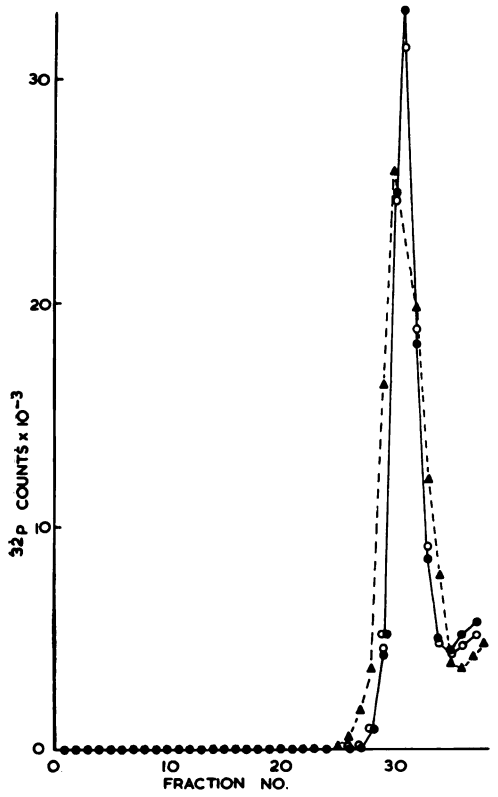

FIG. 8. *RNase treatment of protoplast lysates prepared before and after aggregation of ribosomes in the presence of CTC*

Lysates of ^{32}P-labeled protoplasts were treated with RNase (5 μg/ml) at 0° for 5 min prior to gradient analysis. (○) Protoplasts lysed during exponential growth. (▲) Protoplasts incubated with CTC (5 μg/ml) for 5 min prior to lysis. (●) Protoplasts incubated with CTC at 5 μg/ml for 5 min then at 50 μg/ml for a further 45 min prior to lysis.

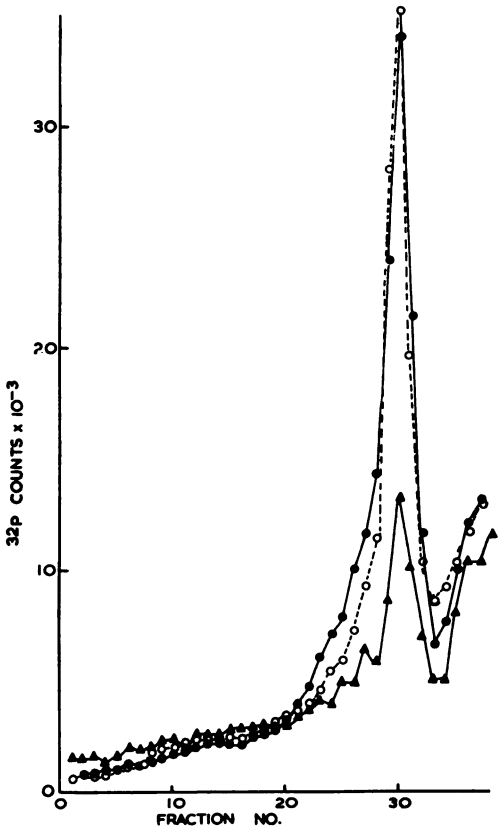

FIG. 9. *Effect of actinomycin D on aggregation of ribosomes in the presence of CTC*

^{32}P-labeled protoplasts were incubated with CTC (5 μg/ml) for 5 min and then treated in various ways: (●) protoplasts lysed immediately; (▲) protoplasts incubated for a further 45 min in the presence of CTC (50 μg/ml) prior to lysis. (○) protoplasts incubated for a further 45 min in the presence of CTC (50 μg/ml) and actinomycin D (10 μg/ml) prior to lysis. The lysates were analyzed on sucrose density gradients.

that the disappearance of 70 S material subsequent to CTC-induced breakdown of polyribosomes is due to aggregation of ribosomes.

Inhibition of Protein Synthesis by CTC

Exponentially growing protoplasts were incubated with leucine-^{14}C (0.2 μC/ml) for 2 min prior to the addition of CTC at various concentrations. The uptake of ^{14}C radioactivity into hot-trichloroacetic acid-insoluble material was followed before and after the addition of CTC, and in controls. Figure 10 shows that at 50 μg/ml CTC promptly and completely inhibited protein synthesis, whereas at 5 μg/ml protein synthesis was progressively slowed over a period of about 5 min before being completely inhibited.

DISCUSSION

Low concentrations of the tetracyclines specifically inhibit protein synthesis in bacteria (6) and in cell-free systems from bacterial and mammalian sources (7–11). These drugs have been variously reported

TABLE 1
BALANCE SHEET TO SHOW THE DISTRIBUTION OF ^{32}P-RADIOACTIVITY IN VARIOUS SUCROSE DENSITY GRADIENTS

Treatment	Total counts put on gradients in 0.2 ml of lysate	Total counts recovered (%)	Counts recovered in 70 S peak (%)	Counts recovered in pellet (%)
Control polyribosomes	143,070 100%	99	15	6
As above, RNase-treated	139,260 100%	97	70	2
Maximally degraded polyribosomes after CTC 5 μg/ml, 5 min	134,044 100%	101	35	7
Ribosome-aggregates after CTC 5 μg/ml, 30 min	141,220 100%	102	22	35
As above, RNase-treated	134,414 100%	98	67	3
After CTC 50 μg/ml, 50 min	146,213 100%	100	29	14
As above RNase-treated	139,150 100%	101	70	3

to bind to 70 S ribosomes, 50 S and 30 S ribosomal subunits, nucleic acids, and protein (12–16). The tetracyclines do not appear to prevent the binding of messenger RNA (mRNA) to ribosomes in sensitive bacteria (15, 17), but probably inhibit protein synthesis by preventing the binding of amino acyl-transfer RNA (aminoacyl-tRNA) to the ribosome-mRNA complex (15, 17–19).

Polyribosomes are thought to be formed by successive ribosomes being attached to the 5′ end of mRNA and progressing along it as protein is synthesized. Although there are various hypotheses about mechanisms, most involve some kind of relative movement between the ribosomes and mRNA (for discussion see 20).

The following hypothesis is tentatively put forward to account for the breakdown of polyribosomes in the presence of CTC. It is assumed that CTC interferes with the binding of incoming aminoacyl-tRNA to ribosomes present in polyribosomes. This would prevent peptide bond formation, and hence the subsequent GTP reaction and translocation of the ribosomes along the mRNA would not occur (see 21). Those ribosomes to the 3′ side of affected ribosomes would be free to continue along the mRNA to complete their nascent polypeptide chains and to be released. Those on the 5′ side would be impeded and would possibly remain *in situ*. Increasing the CTC concentration would increase the number of drug interactions per polyribosome thus allowing the release of fewer 70 S ribosomes. Since the CTC-ribosome interactions would be random, concentrations of CTC sufficient to cause, on average, one drug interaction per polyribosome would allow release of about half the ribosomes from polyribosomes. These, together with the 20% already free, would cause about 60% of the total ribosomes to be present as 70 S material. Such an effect was demonstrated with CTC at concentrations of 1–5 μg/ml (Fig. 4). This value of 60% must represent the maximum accumulation, since concentrations of drug giving, on average, less than one interaction per polyribosome would allow release of fewer 70 S ribosomes. This idea is supported by the fact that, although concentrations of CTC ranging from 1 to 150 μg/ml have been used for periods of time of up to 50 min, in no case has the amount of 70 S material accumulated been as great as that after treatment for 5 min with CTC at 1–5 μg/ml. The amino acid incorporation data also

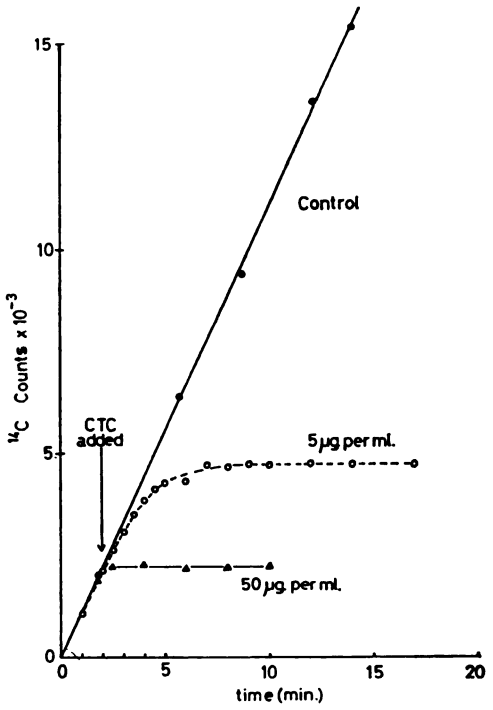

FIG. 10. *Effect of CTC on protein synthesis*
Protoplasts in the exponential phase of growth were incubated with leucine-^{14}C for 2 minutes prior to the addition of CTC at various concentrations. Samples of 0.5 ml were taken into trichloroacetic acid as indicated in the Materials and Methods section. (●) Control leucine-^{14}C added at time zero; (○) CTC (5 μg/ml) added at time 2 min; (▲) CTC (50 μg/ml) added at time 2 min.

support this hypothesis (see Fig. 10) especially since CTC at 5 μg/ml allows protein synthesis to continue at a continuously diminishing rate for about 5 min before complete inhibition is observed.

Whether or not this hypothesis is an oversimplification remains to be seen. If CTC binds reversibly to polyribosomes, hindering rather than preventing translocation, it may require slight modification.

The process referred to as "ribosome-aggregation" was favored by increased concentrations of CTC (Fig. 6) and did not seem to involve a CTC-induced clumping of ribosomes, since it seemed only to occur *in vivo* (Fig. 7a) and was dependent upon RNA synthesis (Fig. 9). The product was sensitive to degradation by RNase with release of 70 S ribosomes (Fig. 8) and therefore consisted, at least in part, of 70 S ribosomes held together by some species of RNA, possibly but not necessarily mRNA. It may be that ribosomal-RNA (perhaps present as incompleted ribosomal particles) is involved in maintaining the structure of the aggregates; since actinomycin D inhibits the process, newly synthesized RNA is presumably implicated. The extent to which other materials (such as proteins) may be present is not known, but it must be borne in mind that the aggregation process proceeds in the presence of CTC at 50 μg/ml—a concentration of drug sufficient to inhibit protein synthesis immediately and completely (i.e., greater than 99% within 10 sec). The evidence does not enable any definite conclusions to be drawn at present, except that the aggregates are, on average, heavier than normal polyribosomes since less material is visible in gradient profiles of aggregates as compared with normal polyribosomes (Fig. 7b). The missing material is recovered in the pellet (Table 1).

A complete understanding of the aggregation process must involve consideration of the state of nascent RNA (messenger and ribosomal) and of its rates of synthesis and degradation in the presence of CTC. The possible implication of preformed mRNA is also relevant.

Other antibiotics also modify polyribosome profiles. The extent to which the effects described in this paper are peculiar to the action of CTC will be discussed elsewhere.

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