THE KINETICS OF COLICIN E3 INDUCED FRAGMENTATION OF *ESCHERICHIA COLI* 16S RIBOSOMAL RNA *IN VIVO*

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(Received April 4, 1972)

Fixation of colicin E3 to sensitive bacteria is followed, after a lag of 2 to 6 min, by the rapid degradation of all the RNA of the 30S ribosomal subunits, yielding a large 15.5S fragment and a smaller fragment, containing the 3'-terminal end of the 16S RNA. The small RNA fragment which was estimated to consist of about 52 nucleotides, was retained within the 30S subunit *in vivo* and was subsequently recovered quantitatively without apparent further degradation. Kinetic studies of the cleavage of 16S RNA indicated that this is the primary and lethal effect of colicin E3 and the primary cause of the observed inhibition of protein synthesis *in vivo*. Small amounts of an RNA fragment, apparently identical in size to the small E3-fragment, were also isolated from 30S particles obtained from untreated bacteria.

1. INTRODUCTION

Colicins initially bind specifically to surface receptors of sensitive bacteria and subsequently promote changes in the synthesis or structure of macromolecules (*cf* 1). As such they are useful probes of the structural organization of the cell membrane and in the present study we have continued our examination of the intracellular consequences of E3 action, in the hope that ultimately this will aid the understanding of the colicin-membrane interaction which initially precedes these effects.

Adsorption of colicin E3 to the cell surface of sensitive bacteria is followed, after a short lag, by complete suppression of protein synthesis and cell death (2,3). If 30S ribosomal particles are subsequently extracted from treated cells they are found to be inactive in *in vitro* protein synthesis, although 50S subunits and cytoplasmic enzyme fractions are fully active (4). Examination of these E3-30S subunits reveals that the 16S RNA component is degraded by cleavage of the molecule close to the 3'-terminus (5,6). In addition, ribosomal reconstitution experiments have shown that the residual RNA and not the 30S subunit protein is associated with the incapacity of the E3-30S particles to function *in vitro* (6).

We have now examined the kinetics of the fragmentation of 16S RNA promoted

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by colicin E3 in an attempt to establish whether this event is a primary effect of E3 and therefore the cause of inhibition of protein synthesis *in vivo*.

2. METHODS

(a) *Organisms*

Escherichia coli CA38 was the source of colicin E3. *E. coli* A19 (*met*- RNase-1$^-$) was used as the colicin-sensitive strain. The strains and the general growth conditions have been described previously (3). Colicin E3 was used in the form of the crude sterilized supernatant fraction resulting from either heat shock or mitomycin-C induction of cultures of strain CA38. The production and assay of this material was described previously (3).

(b) *Growth conditions and treatment with colicin E3*

Strain A19 was grown with vigorous aeration in 2-liter flasks containing 1.7 liters of 1% tryptone broth (Oxoid) and 0.5% sodium chloride. Colicin E3 was added at a final concentration of 50 units per ml when the culture reached a cell density of 2 x 10^8 cells per ml. Under these conditions 99.9% of the cells are killed in 15 min and the molecular multiplicity of colicin E3 is approximately 2,500 molecules per cell (7).

(c) *Amino acid incorporation*

In order to determine amino acid incorporation in large-scale cultures, 15 ml volumes of colicin-treated or untreated cells were removed and added to flasks containing 8 µCi of [^{3}H]leucine (final specific activity 0.1 µCi per µg of cold leucine per ml). During subsequent incubation at 37°, 1 ml samples were removed at intervals and mixed with 1 ml of ice-cold 10% trichloroacetic acid (TCA). After storage for 60 min on ice the acid-precipitable material was collected by filtration and the radioactive content determined in a Packard tri-carb scintillation counter.

(d) *Isolation of ribosomal subunits*

Samples, 250 ml, were taken at intervals from control or E3-treated cultures of strain A19 growing at 37° and immediately poured onto 150 ml of crushed ice containing sodium azide (10^{-2} M) at -20°. During this procedure the mixture was agitated. Cooling by this method was extremely rapid, the mixture reaching the ice water temperature in 1 to 1.5 min. After harvesting the cells by centrifugation at 0° for 5 min at 10,000 x g, the cells were washed in 100 ml of TA buffer (0.01 M Tris·HCl, 0.05 M ammonium chloride, pH 7.2) containing 10^{-4} M magnesium sulfate. The cells were suspended in 1.5 ml of this buffer and sonicated at 4° for 2 min in bursts of 1 min with rests of 30 sec. The sonicated suspensions were clarified by centrifugation at 0° for 5 min at 10,000 x g and the supernatant fraction carefully removed and stored at -20° for 1 to 2 hr prior to layering on 36 ml, 5 to 30% linear sucrose gradients. Sucrose was prepared in TA buffer containing 10^{-4} M magnesium sulfate. The gradients were centrifuged in a Beckman L2-65B ultracentrifuge in an SW27 rotor at 3° for 16 hr and at 20,000 rpm. The gradients were analyzed with an Isco 180 UV analyzer at 254 nm and the fractions containing the 30S and 50S subunits were carefully collected avoiding contamination of either fraction with the other. Under these conditions no residual 70S particles were evident, and thus essentially all cellular ribosomes were converted to subunits. Frequently the collected subunits were then subjected to a further cycle of sucrose density gradient centrifugation to ensure purity. The fractions containing 30S subunits were collected in glass centrifuge tubes, to which were added 0.1 volume of

0.1 M magnesium acetate and 0.7 volume of absolute ethanol at -20°. After thorough mixing, the tubes were stored at -20° for at least 30 min. The precipitated ribosomal subunits were collected by centrifugation at -5° for 20 min at 20,000 x g, the pellet drained and RNA extracted.

(e) *RNA extraction*

The 30S ribosomal pellet was suspended in 0.9 ml of TA buffer containing 10^{-2} M magnesium sulfate and shaken for 5 min at room temperature with 0.1 ml 10% (w/v) sodium dodecyl sulfate. RNA was extracted at 4° by shaking with water-saturated phenol and precipitated from the aqueous fraction with 2 volumes of ethanol and potassium acetate, final concentration 0.2 M, for 30 min at -20°. The precipitate was collected by centrifugation at -5° for 20 min at 20,000 x g. RNA was similarly extracted from 50S ribosomal subunits in order to obtain a source of 5S RNA for molecular weight determinations.

(f) *Polyacrylamide gel electrophoresis*

Gels 10 cm in length composed of 3.5% (w/v) acrylamide, 0.09% (w/v) methylene bis acrylamide and 0.5% (w/v) agarose were made in the Tris·EDTA borate buffer (pH 8.3) of Peacock and Dingman (8). The RNA preparations were dissolved in a mixture of 2 mM disodium EDTA, 50 mM sodium chloride and 30% sucrose; 0.025 to 0.04 ml was then carefully applied to the gels. After 60 min electrophoresis at 4° at a constant voltage of 150 volts, the gels were removed from the tubes, rinsed in distilled water and scanned in a Gilford gel scanner at 260 nm. Control experiments established that the relationship between absorption and amount of RNA present on gels was essentially linear over the range of RNA concentrations encountered. The amount of the small fragment RNA species was then calculated from the areas under the peaks. For molecular weight determinations of small RNA species, 12.5 or 15% (w/v) gels were used and agarose omitted. These samples were electrophoresed at 4° for 3 to 4 hr at a constant voltage of 250 volts before scanning at 260 nm. When necessary, gels were then stained after a 10 min rinse in 6% (v/v) acetic acid at 37° by immersion for at least 60 min in 0.2% (w/v) methylene blue in 0.2 M sodium acetate and 0.2 M acetic acid. Excess stain was removed by thorough washing with several changes of distilled water.

3. RESULTS

(a) *Measurements of 16S RNA cleavage*

We have previously shown (5) that the large fragment RNA (ca 15.5S) obtained from E3-30S subunits is separable from normal 16S RNA on sucrose gradients. Resolution of the 2 species is difficult, however; and attempts were made to separate the 2 types of RNA on 3% polyacrylamide gels by the method of Peacock and Dingman (8). Separation by this technique also proved to be difficult and as shown in Fig. 1, resolution was poor even when approximately equal amounts of 16S and 15S RNA were present. Nevertheless Fig. 1 shows that when 30S ribosomal RNA is extracted at intervals from E3-treated cultures there is a rapid conversion of 16S to 15.5S RNA at some point between 3 and 10 min after addition of colicin. Since protein synthesis was completely inhibited by 9 to 12 min under these conditions, RNA degradation was evidently closely associated with cessation of protein synthesis.

Attention was then turned to the detection of the small 3'-terminal fragment produced in treated cultures from 16S RNA, as an alternative and perhaps a more precise measure of colicin action. Strain A19 was treated with E3 (2.5 units per 10^7 cells) for 10 min; the culture was rapidly cooled, and 30S particles extracted and purified as described in Methods. Finally the total RNA

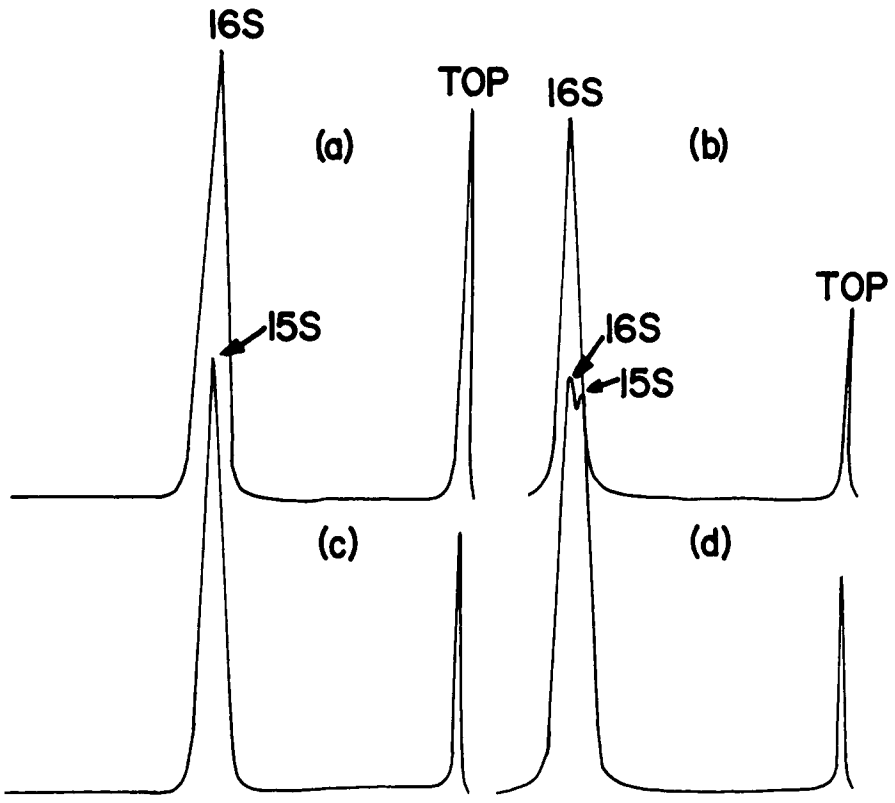
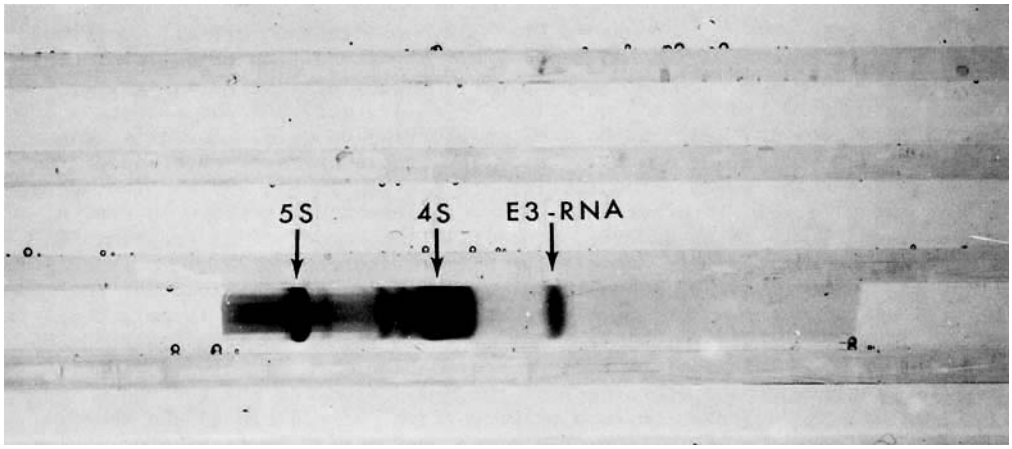


Fig. 1. Degradation of 16S RNA in E3-treated cultures. Strain A19 growing exponentially in tryptone broth was treated with colicin E3 (2.5 units per 10^7 cells) at time zero. Samples were removed at intervals and RNA extracted from isolated 30S ribosomes as described in Methods. Control 16S and E3-RNA were electrophoresed in 3% polyacrylamide gels containing 0.1% agarose and the gels scanned at 260 nm. Shown are tracings of the main peaks observed. (a) 20 µg 16S RNA from untreated cultures; (b) 20 µg RNA from cultures treated with E3 for 3 min; (c) 20 µg "16S" RNA from cultures treated with E3 for 10 min; (d) 10 µg control 16S RNA and 10 µg of E3-RNA as in (c).

was extracted and analyzed on 15 and 12.5% polyacrylamide gels together with 5S and unfractionated 4S RNA as markers. The results are shown in Fig. 2a where the RNA obtained from the E3-30S particles is seen as an apparently homogeneous band moving faster than 4S tRNA. From the electrophoretic mobilities of the marker RNA molecules (Fig. 2b) and assuming a linear relationship between log molecular weight and relative mobility, the molecular weight of the E3 fragment was calculated to be 1.74×10^4 daltons and therefore equivalent to about 52 nucleotides. This agrees well with the estimation of 50 nucleotides for the 3'-terminal fragment by Bowman *et al.* (6). Consequently, after cleavage of all cellular 16S RNA, the 3'-terminal fragment, assuming it is not further degraded, should constitute 3.3% of the total 30S subunit RNA.

(b) *Kinetics of small fragment RNA formation in E3-treated cells*

Exponentially growing cultures of strain A19 in tryptone broth medium were treated with E3 (2.5 units per 10^7 cells) and incorporation of [^{3}H]leucine de-



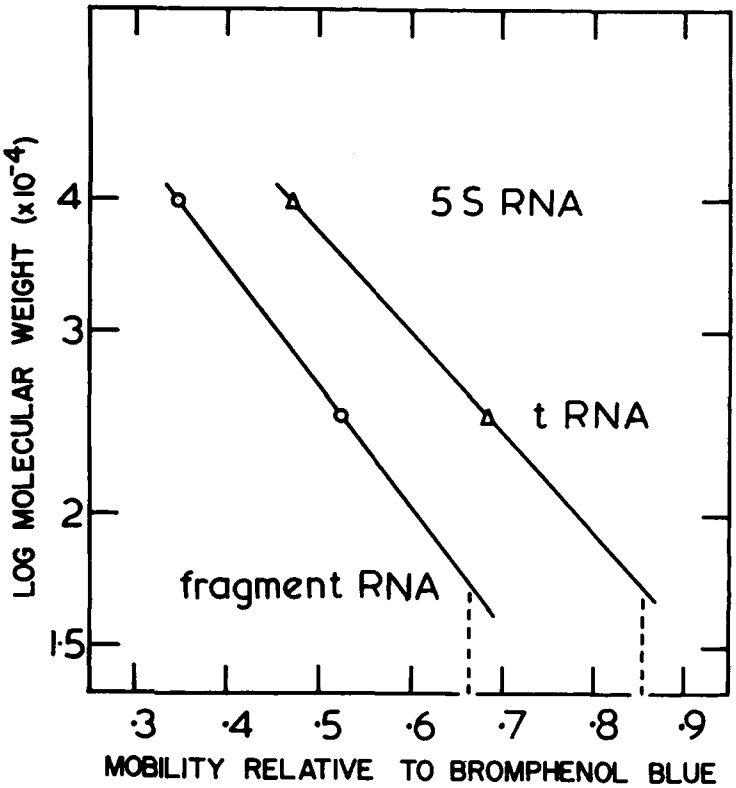


Fig. 2. Molecular weight determination of the small E3-RNA fragment. Cultures treated with E3 for 10 min as in Fig. 1. Approximately 300 μg total 30S ribosomal RNA from E3-treated cells was mixed with equal amounts of 50S ribosomal RNA (source of 5S RNA) and unfractionated 4S-RNA and applied to 15% and 12.5% polyacrylamide gels. (a) Appearance of a portion of a 15% gel after staining with methylene blue; electrophoresis was from left to right; (b) electrophoretic mobilities of RNA species relative to bomophenol blue was determined from unstained gels; 15% gels, O; 12.5% gels, Δ. The dashed lines indicate the position of the small E3-RNA fragment.

termined at intervals. As shown in Fig. 3a incorporation initially continued normally but then ceased between 4 and 7 min after addition of colicin. In the same experiment additional samples were removed at intervals, rapidly cooled and 30S subunits extracted and purified. Finally total RNA was extracted from the 30S particles and analyzed on 3.5% polyacrylamide gels. The gels were scanned at 260 nm and the amount of each RNA species present determined from the areas under the peaks. The results are plotted in Fig. 3b and the appearance of the same gels later stained with methylene blue is shown in Fig. 4. Between 2 and 3 min after addition of colicin the amount of E3 fragment RNA is seen to increase rapidly reaching a maximum after about 20 min at approximately 4% of the total 30S subunit RNA. This indicates, as expected, that the intracellular conversion of 16S RNA to the fragmented form is largely complete and that the small 3'-terminal fragment is being recovered from isolated 30S particles without much loss or further degradation.

The kinetics of the appearance of the small fragment RNA therefore indicate that 16S-RNA degradation is a primary effect of colicin E3 and that it closely parallels the inhibition of protein synthesis. In fact, Fig. 3b indicates that the initiation of 16S RNA cleavage may precede inhibition of protein synthesis by 1 to 2 min. This result has been obtained in repeated experiments and is also evident in Fig. 5. Possible interpretations of this finding will be considered in the Discussion. Finally, as shown in Fig. 3b and as found in the majority of similar experiments, significant levels of small molecular weight RNA was also found in 30S subunits from untreated cultures. The possible significance of this will also be discussed.

(c) *Kinetics of small fragment RNA formation induced by low concentrations of E3*

The timing of the onset of protein synthesis inhibition induced by E3 is a function of the colicin concentration (3). Thus, as shown in Fig. 5a, cultures treated with only 0.5 units E3 per 10^7 cells continue to incorporate [^{3}H]leucine for at least 7 min before the rate slows and inhibition is complete about 13 min after addition of colicin. Under these conditions the appearance of increased amounts of the small fragment in 30S subunits was also delayed until between 5 and 10 min after addition of colicin when the amount of the fragment abruptly increased (Fig. 5b). Thus the triggering of 16S RNA degradation like the inhibition of protein synthesis, a process which it closely parallels, is also E3-concentration dependent.

4. DISCUSSION

Both large and small RNA fragments can be extracted from 30S ribosomal particles isolated from E3-treated cells. The smaller fragment is about 52 nucleotides long and presumably contains the 3'-terminal end of normal 16S RNA as found previously by Bowman *et al.* (6). The kinetics of the formation of this fragment indicate that the specific cleavage of 16S RNA is a primary effect of colicin action on sensitive cells and therefore the probable cause of protein synthesis inhibition *in vivo*. Initiation of RNA degradation was observed approximately 2 min and 6 min after addition of 2.5 and 0.5 units E3 per 10^7 cells respectively; these lag period correspond very closely to the time during which treated cells are rescueable by trypsin digestion of the surface bound colicin (Senior and Holland, in preparation). Cleavage of the 16S RNA molecule is therefore probably a lethal event.

Fragmentation of 16S RNA was usually observed to precede by 1 to 2 min the inhibition of protein synthesis in the same culture. Although great care was taken to ensure very rapid cooling in the presence of sodium azide during breakage of treated cells, prior to isolation of 30S particles, we cannot exclude the possibility that the initiation of colicin action and the cleavage of 16S RNA continued 1 to 2 min after sampling. In this case the appearance

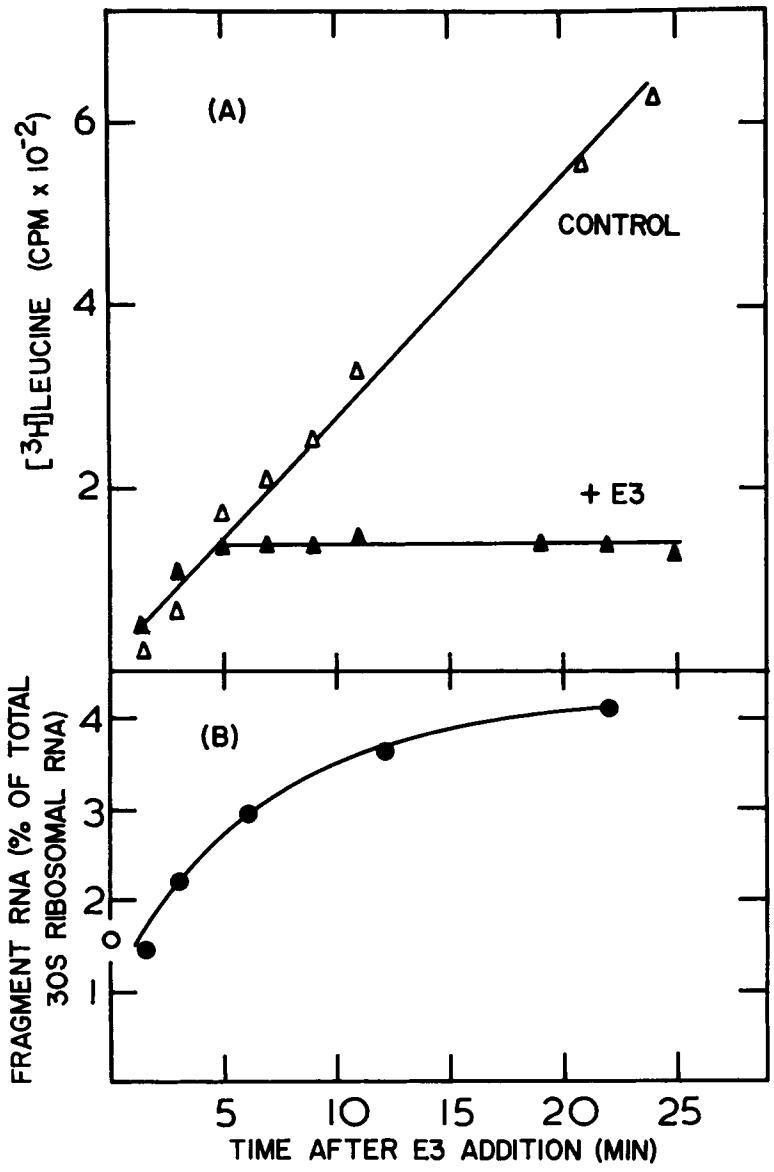


Fig. 3. Kinetics of inhibition of protein synthesis and the appearance of the small RNA fragment. (a) Incorporation of [^{3}H]-leucine (added at 1.5 min) in control cultures and cultures treated with E3 (2.5 units per 10^7 cells) at zero; (b) samples of treated and untreated cultures removed at intervals and total RNA extracted from purified 30S ribosomes. RNA was electrophoresed on 3.5% polyacrylamide gels containing 0.5% agarose, and the proportion of each species present determined from the areas under the peaks after scanning at 260 nm. The amount of the small E3-fragment present, expressed as a percent of total 30S subunit RNA applied to gel, is plotted against time, ●. Amount of small molecular weight RNA in untreated cultures is shown by O.

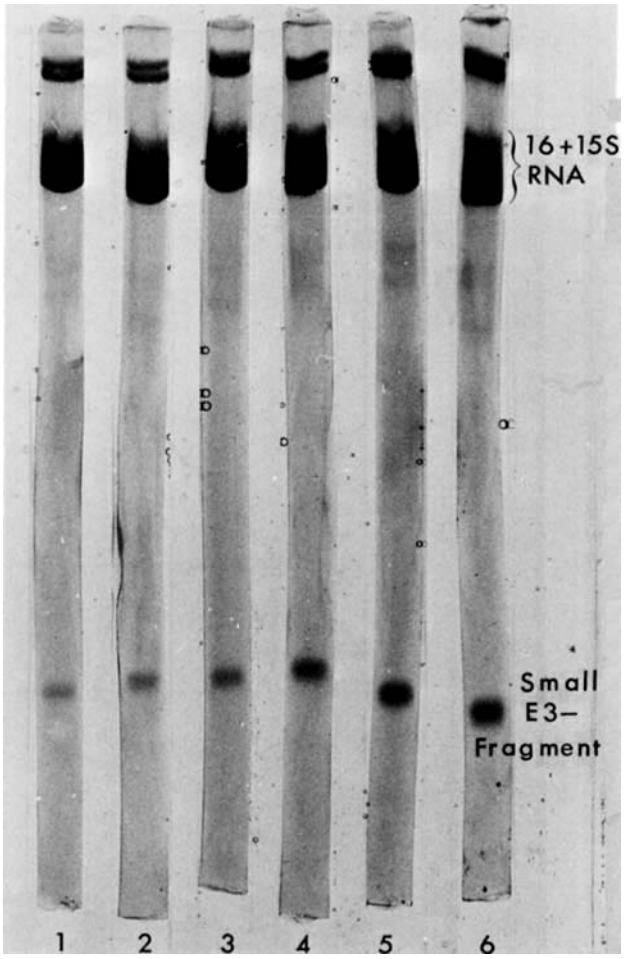


Fig. 4. Appearance of 3.5% acrylamide gels used in Fig. 3 after staining with methylene blue.
1: RNA from untreated control
2: E3 treatment for 1.5 min
3: E3 for 3 min
4: E3 for 6 min
5: E3 for 12 min
6: E3 for 22 min
Gels 1 and 2 loaded with 234 µg RNA; gels 3 and 4 with 165 µg RNA; gels 5 and 6 with 193 µg RNA. The nature of the stained bands at the top of the gel is unknown, but may represent precursor 16S RNA.

of degraded 16S RNA apparently prior to inhibition of protein synthesis may not be significant. Alternatively, 30S subunits receiving a lethal fracture to the RNA component may nevertheless be capable of completing nascent polypeptide chains and hence the inhibition of protein synthesis would be delayed. In relation to this we have previously shown that inhibition of protein synthesis in E3 treated cells is precisely paralleled by the development of physical instability of 50-30S couples. Furthermore nascent polypeptides become progressively more incompletely chased from polysome fractions (3). These results suggest that the 3'-terminal end of 16S RNA may be involved in correct coupling of 30 and 50S subunits and that fragmentation of 16S RNA in this region may block the normal polypeptide termination mechanism, if not the continued extension of polypeptide chains. This interpretation demands that the 3'-terminus of 16S RNA be located close to if not exposed on the surface of the 30S subunit. In support of this, Ehresmann *et al.* (9) have recently found that a 3'-terminal fragment (40 nucleotides) can be deleted from 16S RNA by incubation of 30S subunits with T1-RNase *in vitro*. In addition Bowman *et al.* (6) reported that E3-15S RNA plus 30S ribosomal proteins can be assembled into a virtually intact although non-functional 30S particle, indicating that the whole 16S RNA is not required for 30S particle assembly.

From the molecular size and proportion of the small RNA fragment recovered from E3-30S particles, it appears that essentially all 16S RNA is converted to

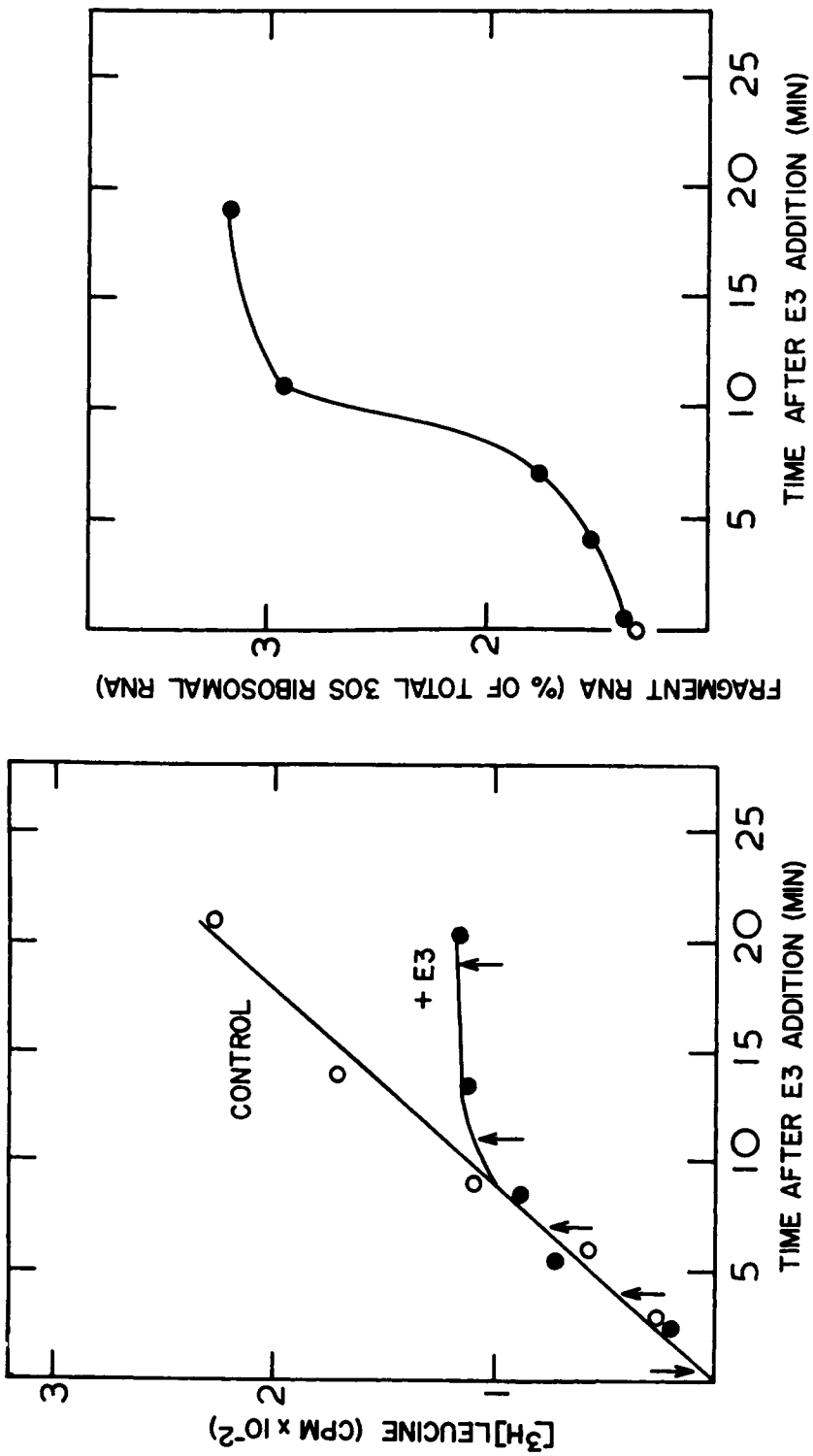


Fig. 5. Inhibition of protein synthesis and RNA degradation in E3-treated cultures. Experimental details as in Fig. 3 but cultures treated with 0.5 units E3 per 10^7 cells at zero. (a) [^{3}H]-leucine incorporation in control and treated cultures; arrows mark the times when samples were removed for determination of the proportion of the small E3-RNA fragment present; this data is plotted in (b).

the fragmented form in the cell and that the small 3'-terminal portion can be fully recovered from isolated 30S particles with little if any additional degradation. Bowman *et al.* (6) reported in contrast that most of the small fragment was lost from the 30S particle and was present in the cell cytoplasm. The reason for this discrepancy is not clear except that in the present study 30S particles were always isolated after relatively short incubation times in the presence of E3.

An intriguing feature of this study was the presence of small amounts of an RNA species, with an electrophoretic mobility equal to that of the E3 fragment, in 30S subunits from untreated cultures. Bowman *et al.* (6) also reported the presence of small, but significant, amounts of 15.5S RNA in 16S RNA preparations obtained from untreated cultures and thus it seems possible that at least some of the 30S particles isolated from normal cells contain fragmented 16S RNA identical to that found in E3-30S particles. Such degraded molecules may exist *in vivo*, or they may arise during isolation. Further study is required to determine this.

While this work was in preparation Boon (10) has reported that purified E3 will fragment the RNA of the 30S ribosomal subunit in a completely cell-free system. Similar results have been obtained by Bowman *et al.* (11) who have further shown that this *in vitro* reaction also cleaves 16S RNA close to the 3'-terminus. Both groups have found however that the cleavage reaction only proceeds in whole ribosomes and not with free 16S RNA. The possibility remains therefore that colicin E3 itself is not a nuclease but that the interaction of this molecule with 30S particles promotes the release of a ribosome-bound enzyme and that this enzyme is responsible for the degradation of a small fraction of the 16S RNA observed by us to be present in untreated cells. Further study of the E3 directed cleavage of 16S RNA *in vitro* should now reveal the precise mechanism of the degradative process.

ACKNOWLEDGMENTS

A.C.R. Samson and B.W. Senior wish to gratefully acknowledge the receipt of Beit Memorial Fellowships.

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