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Effect of Colicin E3 upon the 30S Ribosomal Subunit of *Escherichia coli*

B. W. SENIOR AND I. B. HOLLAND

Department of Genetics, University of Leicester, England

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ABSTRACT **The properties of 30S ribosomal subunits from untreated and from colicin E3-treated (E3-30S) bacteria have been compared. Polyacrylamide gel electrophoresis of ribosomal proteins revealed no differences, but several studies indicated that the 16S RNA from E3-30S particles was modified. E3-16S RNA showed slightly increased resistance to heat-induced degradation and had a reduced (15 S) sedimentation coefficient on sucrose gradients. Fingerprint analyses of E3-16S RNA revealed that the 3′-terminus of the molecule had been deleted. It was concluded that a primary effect of colicin E3 is the activation of a highly specific RNase that degrades 30S ribosomal RNA *in situ*, and that the resulting fragment(s) are probably retained within the 30S particle.**

The adsorption of colicin molecules to surface binding sites of sensitive bacteria is normally lethal and is accompanied by specific intracellular changes characteristic for the colicin. Evidence presented by Nomura and his coworkers (1–3) has indicated that colicins do not penetrate the cell surface. It has also been demonstrated that colicins do not induce detectable permeability changes in the plasma membrane of sensitive bacteria (4, 5). Colicins E2 and E3 can act upon nongrowing cells and upon those in which macromolecular synthesis has been inhibited (6, 7), but colicin action is blocked by inhibitors of energy metabolism (6, 8–10).

It is most likely, therefore, that colicins act by inducing specific conformational changes in cell membranes with a resultant disturbance of essential cell functions associated with the membrane. In support of this hypothesis, mutants have been isolated that are refractory to colicin E2, a colicin that induces rapid degradation of bacterial DNA; these mutants show disturbed DNA metabolism (11). Furthermore, such mutants produce increased amounts of a specific envelope polypeptide (12). Mutants specifically refractory to colicin E3 have been isolated by us, but have been found to be unstable (unpublished results); therefore, investigations with this colicin have been restricted to a study of the intracellular changes promoted by E3.

Nomura first showed that adsorption of colicin E3 led to a rapid inhibition of protein synthesis without any apparent effect upon nucleic acid metabolism (13). Subsequently, it was demonstrated (14) that 70S ribosomes isolated from E3-treated bacteria had reduced activity in *in vitro* protein synthesis, the defect being in the 30S subunit. More recently, Senior *et al.* (7) have shown that the inhibition of protein synthesis in E3-treated cells is precisely paralleled by a physical change in polysomes that renders 50–30S couples unstable at low concentrations of magnesium ion. This strongly suggests that the primary effect of E3 action is the modification of a polysomal component, presumably the 30S subunit. In order to confirm this hypothesis, and ultimately to facilitate the study of E3 action at the membrane level, we examined the precise E3-induced change in the 30S ribosomal subunit.

MATERIALS AND METHODS

Bacterial strains

E. coli CA38 was the source of colicin E3; *E. coli* A19 (*met*$^-$ *RNase* I$^-$) was the colicin-sensitive strain used. These strains

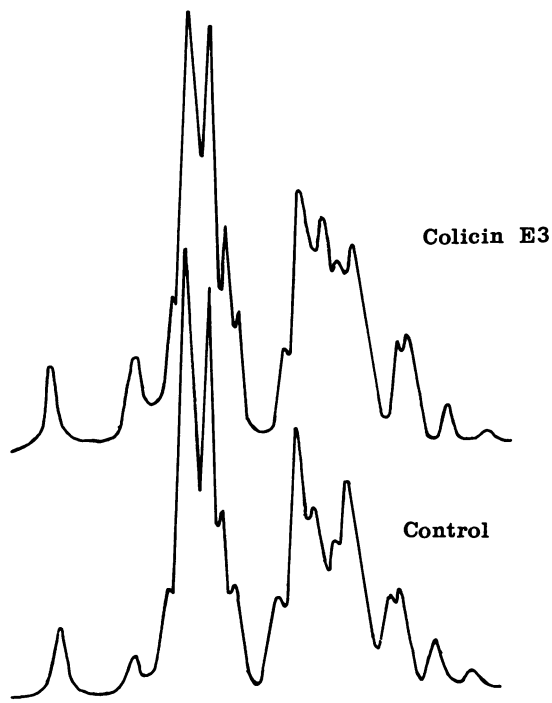

FIG. 1. Joyce-Loebl microdensitometer profiles of proteins from control and E3-30S ribosomal subunits fractionated on polyacrylamide gels at pH 4.5. Electrophoresis is from left to right. The bottom portion of the gels contained 10% acrylamide and 0.15% methylenebisacrylamide in 8 M urea and 2% acetic acid. The upper portion of the gels contained 2.5% acrylamide and 0.625% methylenebisacrylamide in 8 M urea and 0.35% acetic acid; 0.1 ml of this gel was used as a spacer and up to 50 μg of protein, in up to 0.2 ml of urea–phosphate buffer, was mixed with 0.1 ml of the upper gel for use as the sample. Both the spacer and upper gels were polymerized by exposure to fluorescent light in the presence of riboflavin.

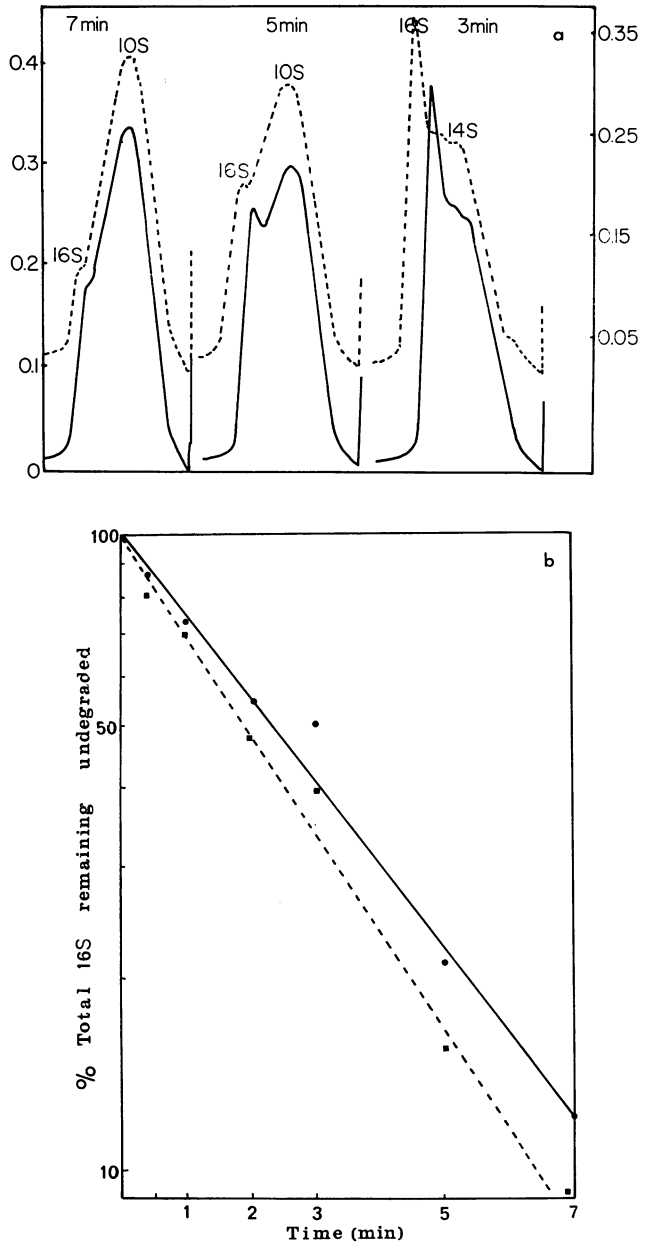

FIG. 2. (*a*) Examination for "hidden breaks" in 16S RNA extracted from control and E3-treated cultures. Control and E3-treated cells were lysed and bulk RNA was extracted and purified by centrifugation on 5–20% sucrose gradients. Material sedimenting at 16 S was collected, subjected to a further cycle of sucrose gradient centrifugation to ensure purity, and finally precipitated with ethanol. 30-μg portions of this material were dissolved in 0.2 ml of sodium acetate–NaCl buffer and heated in glass tubes for 3, 5, or 7 min at 90°C as indicated, then rapidly cooled on ice. The RNA was then centrifuged on sucrose gradients and analyzed as before. Sedimentation is from *right* to *left*. Absorbance at 260 nm is plotted for - - - control RNA and —— E3 RNA.

(*b*) Comparison of the extent of breakdown of "16"S RNA from control (- - -) and from E3 (——)-treated cultures heated to 90°C for different times. Amounts of RNA were calculated from the areas under the respective peaks, and the fraction of RNA apparently remaining undegraded is plotted against time of heating.

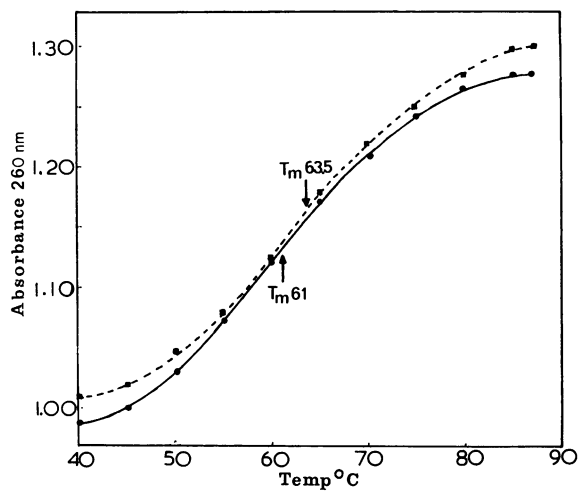

FIG. 3. Melting curves for "16"S RNA from control (- - -) and E3(——)-treated cultures. Equal amounts (50 μg) of control and E3-16S RNA, extracted and purified as described in *Methods*, were dissolved in freshly autoclaved, cool buffer (0.2 M NaCl–0.01 M sodium acetate, pH 4.6). Using an SP 800 Unicam recording spectrophotometer fitted with an automatic heating device, we heated the samples at a rate of 1°C/min; the increase in absorbance at 260 nm was simultaneously recorded.

and general growth conditions have been described (8). Colicin E3 was used in the form of a crude cell-free lysate, obtained from a culture of *E. coli* CA38 treated with mitomycin C to induce colicin formation. The production and assay of this material were described previously (7).

Preparation of 30S ribosomal subunits and 16S RNA

For the preparation of 16S RNA, E3-treated and untreated cultures were first lysed with lysozyme–EDTA and Brij 35, as described by Godson and Sinsheimer (15). Bulk RNA was prepared by phenol extraction and fractionated on 5–20% sucrose gradients (17 ml) containing 10^{-2} M sodium acetate–10^{-1} M NaCl (pH 7.6). Gradients were centrifuged at 2°C in an SW27 rotor at 24,000 rpm in a Beckman L-265B ultracentrifuge for 20 hr. Gradients were scanned at 254 nm in an ISCO model 180 density-gradient analyzer; material sedimenting in the 16S region was collected and purified by a further cycle of sucrose gradient sedimentation.

For the preparation of 30S ribosomal subunits, cells were sonicated and 70S ribosomes were isolated (7). The 70S ribosomes were pelleted by centrifugation at 150,000 × *g* for 45 min, resuspended, and dialyzed against 10^{-2} M Tris buffer (pH 7.8)–10^{-4} M magnesium acetate. The 30S subunits produced were then purified by two cycles of centrifugation on 5–20% sucrose gradients containing 10 mM Tris–0.1 mM magnesium acetate–0.2 M KCl–1 M NH_4Cl (pH 7.8). Gradients were centrifuged for 16 hr at 20,000 rpm in an SW27 rotor at 2°C and analyzed.

Analysis of 30S ribosomal proteins

The 30S ribosomal subunits were isolated from sonicated E3-treated and untreated cultures as described above. The 30S subunits were purified by two cycles of centrifugation on sucrose gradients; then ribosomal proteins were isolated by the method of Hardy *et al.* (16). Final samples, containing up to 50 μg of protein in 0.2 ml of urea–phosphate buffer [6 M urea–

50 mM NaH_2PO_4–1.2 mM methylamine (pH 6.5)] plus 10 mM mercaptoethanol, were applied to polyacrylamide gels. Electrophoresis was in β-alanine–acetate buffer (pH 4.5; 0.6% β-alanine–0.16% acetic acid) at 3 mA/gel with pyronine B as tracker dye. The gels were stained in 1% Napthalene Black (in 10% acetic acid) overnight and destained electrophoretically in 7% acetic acid at 8 mA/gel. Gels were then analyzed immediately with a Joyce-Loebl microdensitometer.

Preparation and fingerprint analysis of ^{32}P-labeled 16S RNA

Bacteria were grown for three generations in the low-phosphate medium described by Kjeldgaard (17) in the presence of [^{32}P]orthophosphate (Radiochemical Center, Amersham). Control and E3-treated cells were lysed with lysozyme–EDTA and Brij, and bulk RNA was obtained by the phenol method. The 16S RNA was purified as described above; the final product had a specific activity of 5×10^6 cpm/μg of RNA. Each sample of RNA was then partially digested with T1 ribonuclease and alkaline phosphatase for 60 min at 37°C in 10 mM Tris buffer (pH 8.0). Enzyme to substrate ratios were 1:10 and 1:5 for T1 ribonuclease and alkaline phosphatase, respectively. The digests were then analyzed by two-dimensional ionophoresis according to the method of Sanger *et al.* (18) and Brownlee and Sanger (19), or by homochromatography (20).

RESULTS

Comparison of ribosomal proteins from E3-30S subunits and normal 30S subunits

The treatment of exponentially growing cultures of the sensitive strain, *E. coli* A19, in M9 medium supplemented with colicin E3 (50 units/10^8 cells/ml) results in detectable inhibition of protein synthesis after a lag of about 5 min, inhibition usually being complete after a further 6 min (7). After 20 min of treatment with colicin E3, or in the case of the control culture with heat-inactivated E3, cells were harvested, thoroughly washed in buffer, and disrupted by sonication. The 30S ribosomal subunits were isolated and purified by two cycles of centrifugation on sucrose density gradients; ribosomal proteins were extracted by the "self digestion" method of Hardy *et al.* (16) and analyzed on polyacrylamide gels.

As shown in Fig. 1, the profiles of normal and E3-30S proteins were indistinguishable; in particular, there was neither loss nor addition of specific proteins that might have reflected E3-induced perturbation of the normal cyclic association of initiation and dissociation factors with 30S subunits. Furthermore, the results did not reveal any chemical modification of any of the 30S proteins, although this possibility was not excluded by the data. Nevertheless, we turned our attention to the 16S RNA component of the 30S ribosomal subunit since any changes in properties of this material might be more readily demonstrated.

Comparison of 16S RNA from control and E3-treated bacteria

Exponentially growing cultures of *E. coli* A19 were treated with colicin E3 for 20 min. The 16S RNA was isolated, purified, and compared with 16S RNA obtained from untreated bacteria.

As reported previously (14), both 16S and 23S RNA from E3-treated bacteria sedimented in sucrose density gradients as single, apparently normal, peaks. We therefore searched for small numbers of hidden breaks in the 16S RNA from E3-

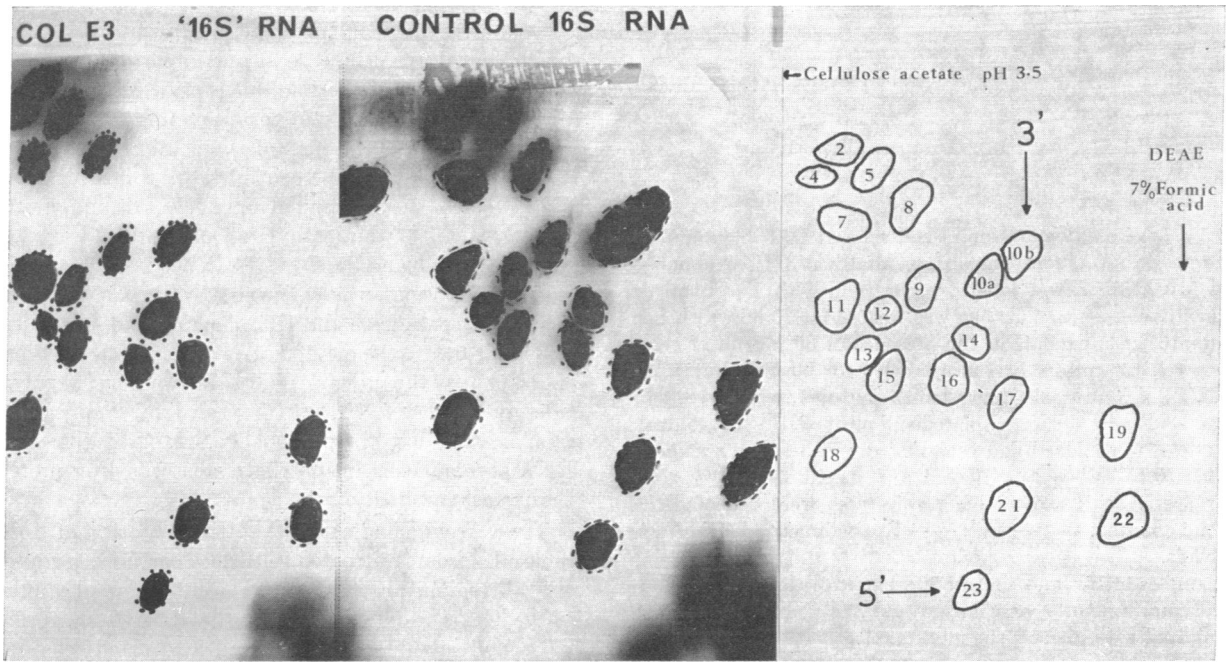

FIG. 4. Autoradiographs of fingerprints of "16"S RNA from control and E3-treated cultures. Purified ^{32}P-labeled 16S RNA was partially digested with T1 RNase and alkaline phosphatase. The digest was first fractionated by high-voltage ionophoresis on a cellulose acetate strip in 7 M urea and 5% acetic acid buffer, pH 3.5; after "blotting through" onto DEAE-paper, samples were further ionophoresed at right angles to the first dimension on DEAE-paper in 7% formic acid. An enlargement of part of the fingerprints of "16"S RNA from control and E3-treated cultures is shown. The spots have been numbered according to the scheme of Fellner *et al.* (23); the 3′- and 5′-terminal oligonucleotides are indicated by arrows.

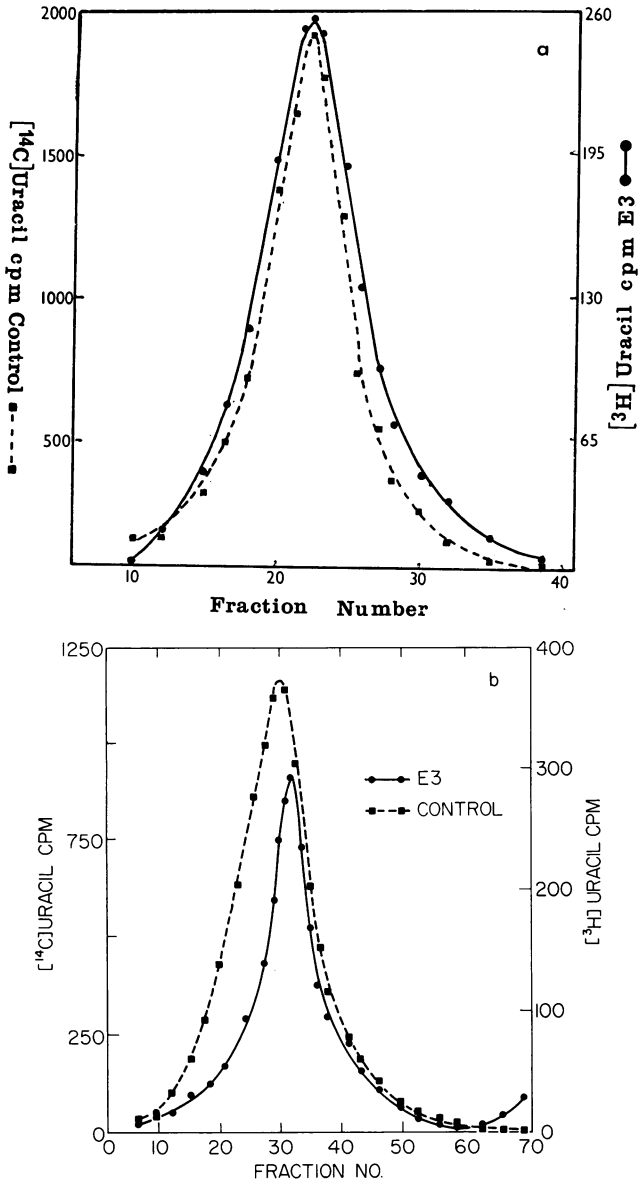

FIG. 5. (*a*) Cosedimentation of control and E3-30S subunits. A culture of strain A19 growing exponentially at 37°C in supplemented M9 medium was labeled for 60 min with 1 μCi/ml of [^{3}H]uracil (Amersham) and then was treated for 30 min with E3 (50 units/10^8 cells per ml) in the presence of 30 mg/ml of cold uracil. A control culture was also labeled for 60 min, but with 0.25 μCi/ml of [^{14}C]uracil (Amersham). Cultures were harvested, lysed, and 30S subunits were isolated and purified. E3- and control 30S particles were mixed and centrifuged on a sucrose gradient. Fractions (0.15 ml) were collected and mixed with 10% cold trichloroacetic acid; radioactive precipitates were collected on filters and counted. - - - control; —— E3. Sedimentation is from right to left.

(*b*) Samples of E3- and control 30S particles were obtained as above. Equal amounts were mixed and the whole sample was deproteinized with phenol; the mixture of 16S RNAs was centrifuged on a sucrose gradient as in Fig. 2. Fractions (0.15 ml) were collected and acid-precipitable radioactivity was determined.

treated cells. Ehresmann and Ebel (21) have reported that such damage in ribosomal RNA can be detected by heating it for different times at 90°C, rapidly cooling it, and then resedimenting the RNA on sucrose density gradients. When this practice was applied to control and E3 RNA, the principal breakdown product in both was 10S RNA. Moreover, the E3 RNA was not more readily degraded than the control 16S RNA by this treatment, i.e., E3-induced hidden breaks were not detected. Indeed, E3-16S RNA appeared to be less susceptible to heat-induced degradation than was the control RNA (see Fig. 2*b*).

Melting characteristics of both kinds of 16S RNA were also compared in the hope that any substantial chemical modification of E3-16S RNA might be revealed. The T_m's of E3- and control 16S RNA were 61°C and 63.5°C, respectively, but the profiles were extremely similar and no evidence of extensive changes in E3 RNA was apparent (Fig. 3).

Finally, comparisons were made of fingerprints of E3- and normal 16S RNA in the hope that any chemical modification of the RNA induced by E3 would be revealed by this technique. Control experiments first established that the kinetics of inhibition of protein synthesis induced by E3 and the sensitivity of A19 cells to this colicin were identical whether they were cultured in the low-phosphate medium (25 μg of Na_2HPO_4/ml) of Kjelgaard (17) or in the usual minimal-salts M9 medium (22). Accordingly, A19 cells growing exponentially in low-phosphate medium were labeled for three generations with [^{32}P]orthophosphate before treatment with colicin E3 (50 units/10^8 cells/ml) for 30 min. The cells were then harvested and lysed with detergent, and the RNA was isolated and fractionated on sucrose gradients. The 16S component was further purified by centrifugation; samples from both control and E3-treated cultures were partially digested with T1 ribonuclease and phosphatase and subjected to either high-voltage ionophoresis in two dimensions upon cellulose acetate strips and DEAE-paper (18, 19) or to "homochromatography" (20).

Autoradiographs of the fingerprints obtained were very similar, but differed in at least one important respect. Thus, from that portion of the total fingerprint presented in Fig. 4, it can be seen that the spot corresponding to the 3′-terminal polynucleotide [see the map of the fingerprint of *E. coli* 16S RNA (23)] present in the control RNA is missing in the E3 RNA, whereas the 5′-terminal polynucleotide and, in addition, apparently all of the methylated nucleotides were found in both the control and the E3 RNA. The presence of the methylated nucleotides was confirmed by elution of appropriate nucleotide spots from the DEAE-paper and their identification according to the method of Sanger *et al.* (18). Other differences, namely the presence of at least two unusual polynucleotides in the E3 RNA, were revealed by "homochromatography" (22), but these probably arise from precursor 16S RNA accumulating in the E3-treated cells after protein synthesis was inhibited.

These results indicated that the majority of 16S RNA molecules from E3-treated cells were partially degraded from the 3′-terminus, but otherwise were apparently intact. In view of this result, the size of the RNA from purified 30S ribosomal subunits of control and E3-treated cells was carefully reexamined. As shown in Fig. 5*a*, E3-30S ribosomal subunits sedimented in an identical position as control 30S subunits on sucrose gradients. However, when the differentially labeled RNA was extracted from a mixture of these subunits and centrifuged on sucrose gradients, it was repeatedly found that the E3-RNA sedimented significantly slower than the

control 16S RNA (Fig. 5*b*). The sedimentation coefficient of E3 RNA was estimated to be 15 S (evident also in Fig. 2*a*), indicating that perhaps as much as 6% of the total 16S molecule had been deleted. Furthermore, the E3, but not the control, RNA preparation showed a small amount of labeled material sedimenting at the top of the gradient. This material may be the 3′-terminal portion deleted from the E3-16S RNA. If so, its presence under these conditions indicates that the fragment or fragments are only released from the 30S subunit by the phenol extraction procedure and are not shed from the inactive 30S particle *in vivo*.

DISCUSSION

The results obtained in this study clearly show that the RNA in the 30S ribosomal subunit from E3-treated bacteria has been modified. The major change in the RNA molecule appears to be the deletion of an extensive portion of the 3′-end. The 5′-end and the major methylated bases appear to be present normally, but other, more subtle, changes in the molecule have not been eliminated. These results do not rule out the possibility that modification of 30S proteins also occurs. This seems unlikely, however, since in the following paper (24) Nomura and coworkers clearly show that the RNA moiety, and not the proteins, of the 30S ribosomal subunit are defective in *in vitro* protein synthesis. In agreement with our findings, Bowman *et al.* also show that E3-16S RNA is degraded from the 3′-terminus.

These results indicate that the action of colicin E3, like that of the closely related colicin E2, involves the activation of a highly specific nuclease. We have not yet established, however, that this nucleolytic activity promoted by colicin E3 is necessarily its primary effect or the primary cause of the inactivation of the 30S ribosomal subunit. Further experiments will be required to rigorously establish cause and effect.

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