

ON THE RIBONUCLEIC ACID SYNTHESIZED IN A CELL-FREE SYSTEM  
OF *ESCHERICHIA COLI*\*

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*Communicated by A. E. Mirsky, January 8, 1962*

In a previous communication,<sup>1</sup> we described a method for the preparation of "undegraded" messenger-RNAs from bacterial cells (*E. coli*). Messenger-RNAs have been described before<sup>5</sup> and the selective synthesis of this type of RNA under special culture conditions has been reported by Hayashi and Spiegelman.<sup>3</sup> These RNA fractions are rapidly labeled in isotope experiments and have base compositions resembling that of the bacterial DNA.<sup>2</sup> In our experiments the "undegraded" messenger-RNA fraction was prepared by the phenol method after first breaking the cells in the presence of Duponol in order to assure the immediate inhibition of ribonuclease activity. Messenger-RNA could be distinguished from ribosomal RNA by chromatography on columns of methylated serum albumin or by centrifu-

gation in a sucrose density gradient. The messenger-RNA was shown to be heterogeneous, with sedimentation constants of 23–28 S, 19 S, and 12 S respectively, in contrast to the 23 S and 16 S of *E. coli* ribosomal RNA.<sup>4</sup> This method of preparation yields RNAs with sedimentation coefficients considerably larger than the 8 S value previously reported for messenger-RNA.<sup>5</sup>

The experiments to be described in this paper are concerned with similar RNA fractions synthesized in an *in vitro* system derived from *E. coli*. Care has been taken to assure that the RNA is synthesized under conditions where degradation of the product is adequately prevented. A net synthesis of RNA *in vitro* has been achieved, and the behavior of the newly synthesized RNA on methylated serum albumin column chromatography or in sedimentation analysis is in essential agreement with that of the messenger-RNA fraction synthesized *in vivo*. The *in vitro* synthetic system is a continuation of work begun in other laboratories<sup>6–12</sup> in which it was shown that RNA synthesis from the nucleoside triphosphates of adenine, guanine, cytosine, and uracil, requires DNA as a “primer” and yields RNA fractions of base composition complementary to that of the DNA used. The correspondence between DNA and RNA base ratios in both messenger-RNAs prepared from living cells,<sup>1, 3</sup> and in RNA synthesized *in vitro*<sup>7, 8, 11</sup> suggests their identity. This viewpoint is supported by observations that both types of RNA can form hybrids with DNA under appropriate conditions.<sup>3, 12</sup> The work to be described below offers further evidence for the identity of the RNA synthesized *in vitro* with the messenger-RNA of the cell. Under conditions in which degradation of the product is prevented, the *in vitro* system is found to produce RNA molecules considerably larger than the earlier estimates of Geiduschek *et al.*<sup>12</sup> and Tissières and Hopkins<sup>13</sup> who reported the RNA synthesized in their cell-free systems to be comprised of 5.5 S and 10 S components.

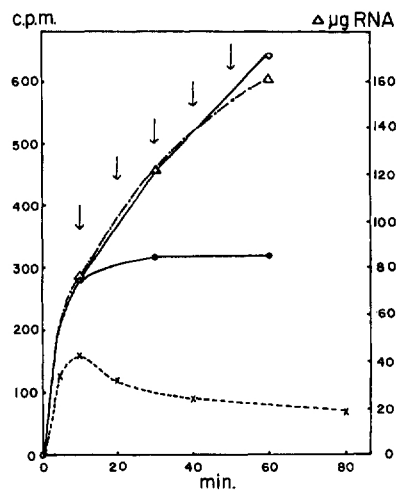
*Materials and Methods.*—Exponentially growing *E. coli* B (H) cells in a modified medium of Cowie *et al.*<sup>14</sup> were collected, washed twice with cold 0.025 M tris buffer (pH 7.5) containing 0.01 M Mg acetate, 0.001 M Mn sulfate, and 0.05 M KCl, ground with quartz sand, extracted with 3 volumes of the above buffer, and centrifuged 2 hr at 105,000 × *g*. The resultant supernatant which contains, in 1 ml, about 2 mg protein, 120 μg DNA, and 200 μg RNA (mainly RNAs) was used as the enzyme preparation. The synthesis of the RNA was estimated by the incorporation of radioactivity from 8-C<sup>14</sup>-ATP into RNA in the presence of the additions described in the legend to Figure 1. Incubation was carried out at 37°C. After cooling, cold 10% trichloroacetic acid (TCA) was added to the reaction mixture, and the acid insoluble precipitate was washed with 5% TCA twice. The radioactivity left in the residue was measured. For the direct measurement of RNA, the TCA precipitate was treated with 10% perchloric acid at 70°C for 20 min. The RNA content was estimated on the acid supernatant by the orcinol reaction.

Nucleic acids which contain the newly synthesized C<sup>14</sup>-RNA were isolated in the cold by the duponol-phenol method<sup>4</sup> from the reaction mixture which had been incubated for 60 min under the same conditions described in the legend of Figure 1. At the beginning of the isolation several volumes of Duponol treated, nonlabeled 105,000 × *g* supernatant were added as carrier. Under the conditions the yield of the radioactive RNA was about 70%.

The nucleic acids prepared were fractionated by a methylated serum albumin column chromatography<sup>17, 18</sup> with or without added ribosomal RNA as “reference.” No difference was found between them. The RNA contained in fractions No. 72–120 of Figure 2a was precipitated with two volumes of ethanol and dialyzed 18 hr. The sample was examined by sucrose density gradient centrifugation according to the method of Britten and Roberts.<sup>19</sup> The centrifugation was carried out at 2°C either at 25,000 rpm for 10 hr with No. SW 25 rotor or at 39,000 rpm for 4 hr with No. SW 39 rotor.

*RNA synthesis in the 105,000 × g supernatant from E. coli:* It was observed

FIG. 1.—Time course of RNA synthesis in the  $105,000 \times g$  supernatant of *Escherichia coli*. Reaction vessel contained in 0.5 ml: Mg acetate ( $5 \mu\text{M}$ ), Mn sulfate ( $1 \mu\text{M}$ ), KCl ( $25 \mu\text{M}$ ), tris, pH 7.5 ( $12.5 \mu\text{M}$ ),  $8\text{-C}^{14}\text{-ATP}$  ( $0.5 \mu\text{M} = 84.4 \text{ m}\mu\text{c}$ ), GTP, CTP, and UTP (each  $0.25 \mu\text{M}$ ), pyruvate kinase ( $20 \mu\text{g}$ ), phosphoenolpyruvate ( $2.5 \mu\text{M}$ ), salmine sulfate ( $15 \mu\text{g}$ ), and 0.2 ml of the  $105,000 \times g$  supernatant of *E. coli*. —●—: Complete mixture without further addition of GTP, CTP and UTP. —○—: Complete mixture to which GTP, CTP, and UTP (each  $0.5 \mu\text{M}$ ) were supplied at every 10 min as indicated by arrow. —△—: Same, net increase of RNA measured by orcinol reaction. —×—: Complete mixture minus salmine, without further addition of GTP, CTP, and UTP.



that the RNA synthesis stops after 10–15 min, followed by degradation of the product if salmine is *not* present. One of the reasons for this may be due to the action of some kind of ribonuclease. It was found that salmine sulfate, at the optimum concentration of  $30 \mu\text{g}/\text{ml}$ , markedly stimulated the initial reaction rate and also effectively protected the product from degradation. Even in the presence of salmine, the RNA synthesis was terminated after about 10 min. It was then supposed that hydrolysis of the ribonucleoside triphosphates might be occurring in our crude system; shortage of the substrates would then result in the termination of the reaction. When three nucleoside triphosphates (guanine, cytosine, and uracil) were supplied at 10-min intervals, the RNA synthesis continued for a further 10–15 min. Simultaneous addition of  $\text{C}^{14}\text{-ATP}$  was not necessary. As shown in

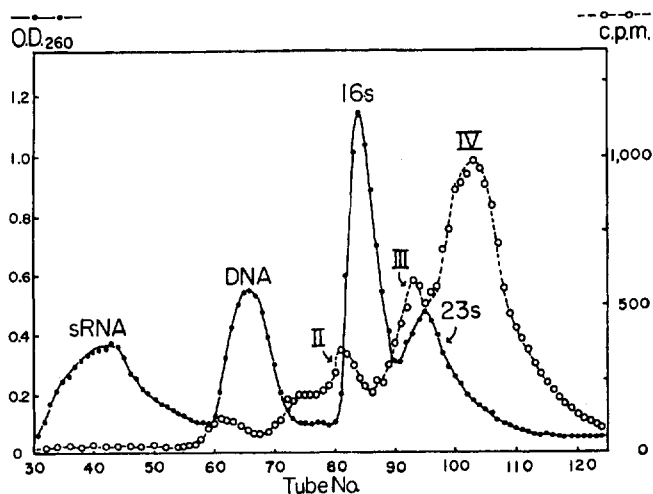


FIG. 2a.—Fractionation of the RNA synthesized in the  $105,000 \times g$  supernatant of *E. coli* with methylated serum albumin column chromatography. 4 ml/tube/10 min were collected at room temperature.

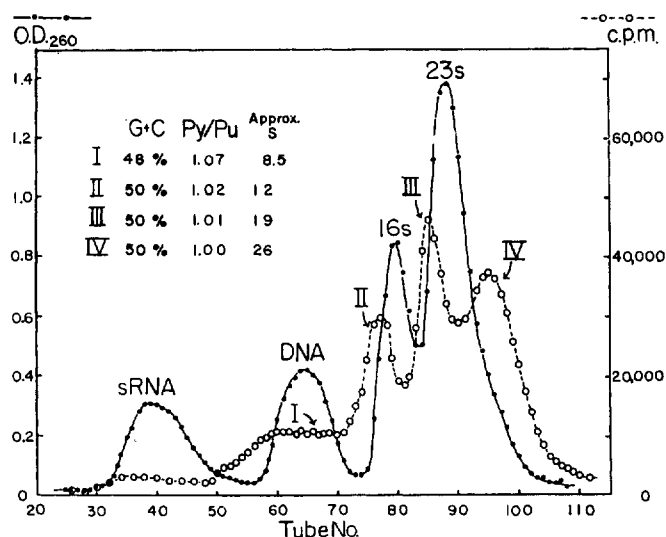


FIG. 2b.—Fractionation of the RNA labelled with  $P^{32}$  for 30 sec in growing *E. coli* cells with the same chromatographic method as in Fig. 2a. (From M. Takai, N. Kondo and S. Osawa.<sup>1</sup>)

Figure 1, addition of these three triphosphates at every 10 min supported a continuous synthesis of the RNA at least for 60 min. Further synthesis of the RNA may be possible if the incubation is continued under the same conditions. Omission of any one of the triphosphates from the reaction mixture reduced the incorporation over 85 per cent. Addition of 5  $\mu\text{g}/\text{ml}$  deoxyribonuclease completely inhibited the reaction. These results show that the RNA synthesis in this system, as in others,<sup>6-12, 15</sup> proceeds using four ribonucleoside triphosphates as precursors and is dependent on the presence of DNA. Usually 160–200  $\mu\text{g}$  of RNA were synthesized after 60 min in 0.5 ml reaction mixture. The ratio of the newly synthesized RNA to DNA is between 1.3 and 1.6. The results suggests that a considerable part of the RNA formed is liberated from the DNA template.<sup>16</sup>

*Characterization of the RNA synthesized in vitro with methylated serum albumin column and sucrose density gradient centrifugation:* A typical chromatographic pattern of the RNA on a methylated serum albumin column is reproduced in Figure 2a. For the purpose of comparison, the pattern of the messenger-RNA as demonstrated by  $P^{32}$  pulse labelling of growing *E. coli* cells is also shown (Fig. 2b). The radioactive RNA synthesized *in vitro* can be fractionated on the column into at least 3 fractions. The first one is eluted just before the 16s ribosomal RNA. The second is located a little earlier than that of the 23s ribosomal RNA. The third one predominates in amount and is eluted after the 23s RNA. From the figure it is seen that no appreciable amount of sRNA or of ribosomal RNA is formed in this system. The chromatographic positions of these three radioactive RNAs corresponds well to Peak II, Peak III, and Peak IV of the pulse labeled messenger-RNA respectively as may be seen in Figures 2a and 2b. Evidence for the presence of Peak I RNA is not clear.

Examination of the RNA in sucrose density gradient centrifugation demonstrated the presence of three RNA peaks with approximate sedimentation coefficients of

10s-12s, 17s-19s, and 26s-30s, respectively. The s-values are in essential agreement with those found for the messenger-RNA. Considering the data on the messenger-RNA in which each radioactive peak isolated after methylated serum albumin column chromatography was separately examined in density gradient centrifugation, it appears that the first peak in the sedimentation analyses corresponds to Peak II in the chromatogram, the second one to Peak III, and the third one to Peak IV respectively. The third peak was found to be very unstable and is easily converted to the smaller units. It is of considerable interest to suppose that the third one is the functional messenger-RNA. In this connection we have recently found<sup>20</sup> that, in *E. coli* normally infected with T<sub>2</sub>-phage, three main messenger-RNA peaks which are similar to those of noninfected *E. coli* can be detected on the methylated serum albumin column chromatography; when protein synthesis is prevented by chloramphenicol virtually no Peak IV was detectable.

*Conclusion.*—RNA can be continuously synthesized in the 105,000 × *g* supernatant of *E. coli* in the presence of four ribonucleoside triphosphates, DNA, an energy generation system, and salmine sulfate. The RNA synthesized is well protected from degradation by salmine sulfate. The addition of salmine to the reaction mixture therefore makes it possible to prepare the “undegraded” RNA by the phenol method. Examination of the RNA using methylated serum albumin column chromatography or sucrose density gradient centrifugation indicated that the synthesized RNA behaves essentially like the “undegraded” messenger-RNA which is detectable by pulse labelling of growing *E. coli* cells. These observations, together with the identity of the base ratios (which are equivalent to those of DNA) of these RNAs support the view that the RNA synthesized in the cell free system is the messenger-RNA. Neither sRNA nor ribosomal RNA seems to be synthesized in the system described in this paper.

The authors are much indebted to Dr. A. E. Mirsky and Dr. V. G. Allfrey for their interest and constant encouragement. They also acknowledge the helpful advices of Dr. M. Takai of our laboratory.

\* Supported by grants from the Rockefeller Foundation, the Ministry of Education of Japan, and the Asahi Press.

<sup>1</sup> Takai, M., N. Kondo, and S. Osawa, *Biochim. Biophys. Acta*, submitted for publication.

<sup>2</sup> We adapt the term “messenger-RNA” to this type of RNA (see ref. 5).

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<sup>11</sup> Stevens, A., *J. Biol. Chem.*, 236, PC 43 (1961).

<sup>12</sup> Geiduschek, E. P., T. Nakamoto, and S. B. Weiss, these PROCEEDINGS, 47, 1405 (1961).

<sup>13</sup> Tissières, A., and J. W. Hopkins, to be published.

<sup>14</sup> Cowie, D. B., G. N. Cohen, E. T. Bolton, and H. Robichon-Szulmajster, *Biochim. Biophys. Acta*, 34, 39 (1959).

<sup>15</sup> Huang, R. C., N. Maheshwari, and J. Bonner, *Biochem. and Biophys. Res. Comm.*, 3, 689 (1960).

<sup>16</sup> In a current experiment, we have found that a protein fraction isolated from the 105,000 × *g* supernatant can catalyze a similar RNA synthesis in the presence of four nucleoside triphosphates and the *E. coli* DNA prepared by the method of Marmur (*J. Mol. Biol.*, **3**, 208, 1961). In this system also, the addition of salmine enhances the initial rate of the synthesis and prevents the degradation of the product. The rate is about comparable to that of the crude system. However the reaction practically ceases after ten minutes even if the nucleoside triphosphates are further added at 10-min intervals. The ratio of the synthesized RNA to the DNA added is around 0.5. The result might be explained by assuming a factor necessary for the liberation of the synthesized RNA from the DNA. In the crude system, the RNA would be continuously liberated from DNA in the presence of the "factor;" in the "purified" system, the RNA would not be liberated because of the absence of the "factor."

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<sup>18</sup> Philipson, L., *J. Gen. Physiol.*, **44**, 899 (1961).

<sup>19</sup> Britten, R. J., and R. B. Roberts, *Science*, **131**, 32 (1960).

<sup>20</sup> Ishihama, A., unpublished experiment (1961).