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THE EFFECT OF UNIVALENT CATIONS ON THE BINDING
OF sRNA TO THE TEMPLATE-RIBOSOME COMPLEX*

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Synthetic polynucleotides such as polyuridylic acid (poly U)¹ have been shown to bind to ribosome complexes by connecting them into more or less organized units. Such assemblies of ribosomes, the so-called polyribosomes, have now frequently been identified as units on which the synthesis of polypeptides proceeds.²⁻⁴ We wish to report here on a template-requiring reaction between amino acyl sRNA and ribosomes that appears to precede amino acid polymerization. Ammonium or potassium ions are essential for this reaction.⁵⁻⁷ It has been observed, furthermore, that the polynucleotide templates must be specific for the particular amino acid to attach firmly amino acyl sRNA's to ribosomes.^{4,8} Similar observations have recently been made by Kaji and Kaji.⁹ This effect of potassium and ammonium ions appears to furnish an explanation for the requirement of these ions in polypeptide synthesis.

Materials and Methods.—*Sucrose gradient fractionation:* The exponential density gradients were prepared essentially as described in an earlier communication.¹ The mixing flask contained 2.3 ml of 5% sucrose and the reservoir 4.8 ml of 22% sucrose. Both solutions contained 0.01 M Tris buffer pH 7.4, and 0.017 M magnesium acetate. The sample to be fractionated was applied to the top of the sucrose gradients in a volume of 0.25 ml. It was centrifuged in a Spinco Model L ultracentrifuge as described below, and generally two-drop fractions were collected after piercing the bottom of the cello tube with a needle.

Conditions for the binding of sRNA: The standard incubation mixture was composed of 0.015 M Tris-HCl buffer pH 7.4, 0.013 M magnesium acetate, 0.5 mg of washed ribosomes, 0.25 mg of C¹⁴-L-phenylalanyl-sRNA containing 13,500 cpm, and additions made up to a final volume of 0.25 ml. The specific activity of the amino acid was 45.3 $\mu\text{C}/\mu\text{mole}$. The sRNA was prepared by phenol treatment of frozen *E. coli* cells; it was stripped of amino acids and charged with C¹⁴-phenylalanine.⁵

The reagents were mixed at 0° and generally incubated for 5 min at either 25° or 37°. The incubation was terminated by chilling the mixture in an ice bath; the contents were then layered on top of sucrose gradients and centrifuged in the SW39 rotor of the Spinco ultracentrifuge. The centrifugation was carried out for 1 hr at 37,000 rpm unless otherwise indicated. In most cases, 3 gradient tubes were centrifuged simultaneously. Approximately 5 min elapsed from the end of the incubation to the time centrifugation was begun.

The amount of sRNA associated with the ribosomes was assayed by collecting alternate two-drop fractions on 1 $\frac{1}{4}$ -in. stainless steel planchets, and measuring their radioactivity after addition of acetone and drying in an oven. The counts obtained were corrected for self-absorption due to the presence of sucrose. A windowless counter with an efficiency of 35% was used.

Preparation of ribosomes: Ribosomes from a frozen paste of *E. coli* cells were prepared and washed three times according to a procedure described previously.⁷

Polyuridylic acid (poly U) and polyadenylic acid (poly A) were purchased from Miles Chemical Company. C^{14} -L-phenylalanine was obtained from Nuclear-Chicago Corporation, and C^{14} -L-lysine ($114 \mu\text{C}/\mu\text{mole}$) from Schwarz BioResearch, Inc. GTP was a product of Pabst Laboratories, and puromycin a gift of Dr. L. Goldman of Lederle Laboratories.

The Effect of Ammonium Ions on the Binding of sRNA.—To hold the phenylalanyl sRNA on polyribosome complexes with poly U, the presence of ammonium ions is essential, as is depicted in Figure 1. In the absence of poly U, very little

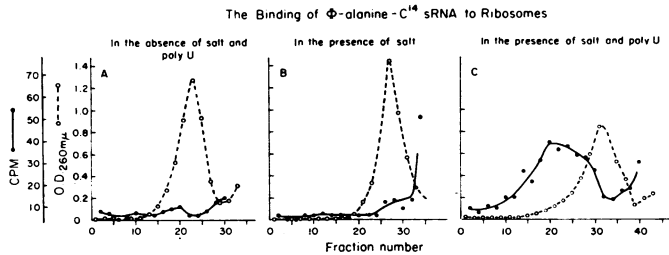


FIG. 1.—The effect of ammonium chloride and poly U on the binding of phenylalanyl sRNA to ribosomes. Experimental conditions as described in *Methods*. Incubation was for 5 min at 25° .

binding takes place nor do ammonium ions without poly U have a significant effect (Fig. 1A and B). However, when both poly U and ammonium ions are added simultaneously, the association of C^{14} -phenylalanyl sRNA with poly U-charged ribosomes takes place (Fig. 1C) as shown by the appearance of radioactivity in the region of polyribosomes. Analogous experiments, not included here, have shown that poly U does not promote the binding of phenylalanyl sRNA if ammonium ion is omitted. The data in Figure 1 indicate salt + poly U to bring about a ninefold increase in the binding of sRNA.

Further Characterization of the Binding of sRNA.—To test whether ammonium ions were required for maintenance of the complex or whether, once formed, the complex could be maintained in its absence, phenylalanyl sRNA, ammonium chloride, poly U, and ribosomes were incubated and then exposed to gradient centrifugation through a solution not supplied with ammonium salt. In this way, the sRNA-ribosome-poly U complex would be depleted of ammonium chloride by its sedimentation through the salt-free medium.

It can be seen in Figure 2A that during a 1-hr centrifugation, about 40 per cent phenylalanyl sRNA was removed as indicated by the loss of radioactivity compared with control gradient 2B. It appears, therefore, that on passage through the salt-free medium a loss of amino acyl sRNA from the poly U-ribosome complex occurs. This experiment also serves indirectly as a control for the experiments in Figure

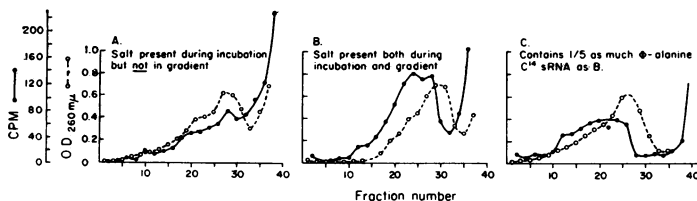


FIG. 2.—The effect of removing ammonium chloride on the binding.

1C; that is, the presence only of poly U which, as we know, does not dissociate from the ribosomes, is not sufficient to retain the sRNA on the complex.

In all experiments described thus far, 0.25 mg of phenylalanyl sRNA was added containing 3 μ moles of phenylalanine per mg, corresponding to 13,400 cpm. Of this, only about 1,000 cpm, or less than 10 per cent, were found in the area of the polyribosomes. By reducing the amount of amino acyl sRNA to 0.05 mg, or 20 per cent of the above, the binding diagram seen in Figure 2C was obtained; the quantity bound is reduced, but to only 60 per cent of that observed in B, indicating that now approximately 30 per cent was fixed, or three times more than previously.

The Binding of sRNA as a Function of Temperature.—Figure 3 shows that the binding is increased by incubating above 0°. Some sRNA is bound at 0° (Fig. 3A),

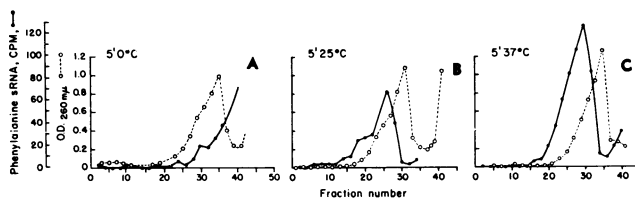


FIG. 3.—The effect of incubation on the binding. The radioactivity in the experiment performed at 25° is 426 cpm from fraction 1 to 28; for the experiment at 37° it is 1,056 cpm from fraction 1 to 32.

but by raising the temperature from 25° to 37°, the amount of sRNA associated with the ribosomes is doubled (Fig. 3B and C). These samples were incubated for 5 min at the indicated temperatures and were chilled in an ice bath before application to the gradients. Thus, the results obtained in Figure 3 reflect on the events that took place during the period of incubation, which were not reversed by lowering the temperature during centrifugation.

The fact that the binding of sRNA is increased at higher temperatures may imply it to be promoted by an enzymatic reaction. Most soluble components such as the supernatant factors are largely removed by repeated washing. Ribosomes as used here were washed many times and incorporated amino acids at less than 3 per cent the rate obtained when they were supplemented with supernatant. However, the participation of an enzyme tightly bound to or part of the ribosomes remains to be considered.

Binding of sRNA as a Function of Salt Concentration.—As shown in the preceding paper,⁵ amino acid incorporation is proportional to ammonium ion concentration up to saturation somewhat above 0.1 M. It seemed important, therefore, to determine whether the binding of phenylalanyl sRNA exhibited a similar concentration dependence. Three different concentrations of ammonium chloride were used: 0.03 M, 0.1 M, and 0.2 M. The concentration of salt in the gradients was kept identical to that in the incubation mixture. The results are shown in Figure 4. The amount of sRNA bound at 0.1 M, gradient B, is higher than that at 0.03 M, gradient A; there is no over-all further increase at 0.2 M, gradient C. Therefore, the polymerization brought about by addition of the supernatant fraction appears to be proportional to the initial binding of amino acyl sRNA.

The Effect of Puromycin on the Binding.—It should be emphasized that known cofactors of protein biosynthesis were absent from the incubation mixtures tested

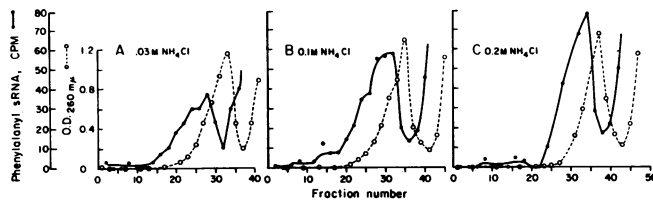


FIG. 4.—Concentration series of ammonium chloride. Incubation was at 37° for 5 min. Radioactivity under peaks integrates as follows: A, 378 cpm from fraction 1 to 30; B, 656 cpm from 1 to 34; C, 626 cpm from 1 to 36.

for binding of sRNA; neither GTP nor supernatant factors were present. The ribosomes had been washed three times, and polypeptide synthesis with such a preparation had been found to be very low. Although it seemed unlikely that the radioactivity seen in the gradients was due to phenylalanine polymerization, experiments were performed to test for the degree of polymerization. Duplicate reaction mixtures were incubated, one of which was applied to a gradient; the other was terminated by addition of trichloroacetic acid (TCA), and heated. As shown in Table 1, radioactivity recovered after heating with TCA was 5–7 per cent of the total amount bound to the ribosomes in the sucrose gradients, indicating little, if any, polymerization to have taken place.

TABLE 1
HOT TCA INSOLUBLE PEPTIDES FORMED DURING THE BINDING

	Cpm	Per cent control
Phenylalanyl sRNA bound	338	100
After heating 15 min 90° in TCA	18	5.3

Heating of the sample is performed at 90° for 15 min. The coagulated protein is subsequently transferred to a Millipore filter and washed with 20 ml of 5% TCA. It is then dried, glued onto a planchet, and counted.

To explore a possible effect of puromycin on the binding step, the inhibitor was added to the incubation mixture and also to the gradient fluid. It can be seen from the results in Figure 5 that a concentration of puromycin, which results in 85 per cent inhibition of polyphenylalanine synthesis, did not detectably decrease the amount of sRNA bound to the ribosomes.

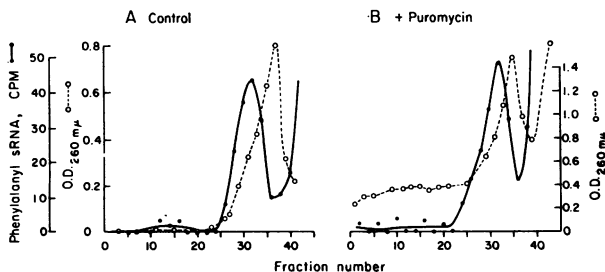


FIG. 5.—The effect of puromycin on the binding. The higher background in the optical density profile of gradient B is due to the presence of puromycin in the gradients. Puromycin was used at $5 \times 10^{-4} M$ in the incubation and $1 \times 10^{-4} M$ in the gradient. Incubation was at 37° for 5 min. The radioactivity in A is 302 cpm from fraction 1 to 34; in B, 353 cpm from 1 to 34.

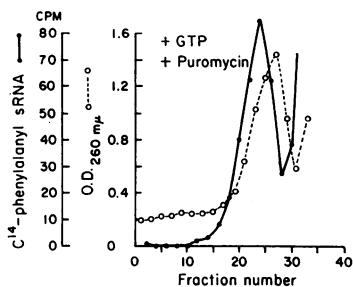


FIG. 6.—The effect of GTP on the binding. Radioactivity is 508 cpm from fraction 1 to 26. GTP was used at a concentration of 0.002 *M*, puromycin at 10^{-4} *M*.

The nonessentiality of GTP is shown in Figure 6. Puromycin was also added to both the incubation and the gradient in order to minimize any increase in the background incorporation due to the addition of GTP. When the amount of radioactivity in Figure 6 is compared with its control, Figure 5*B*, no great difference can be observed between the two. Thus, exposure to GTP does not lead to a permanent increase in the binding of sRNA.

The Effect of Other Monovalent Cations.—The chloride salts of ammonium, sodium, and lithium were used, each at a final concentration of 0.1 *M* both in the incubation mixture and in the gradient fluid. The results appear in Figure 7. It can be seen that ammonium ion was the most effective in inducing association of phenylalanyl sRNA with the ribosomes. Potassium ion, which has been widely used in protein biosynthesis studies, was found here to be about half as active as ammonium. Sodium and lithium ions were without effect. The latter observations should be correlated to the recent findings of Lubin⁷ that lithium ions inhibit amino acid incorporation in extracts of *E. coli*.

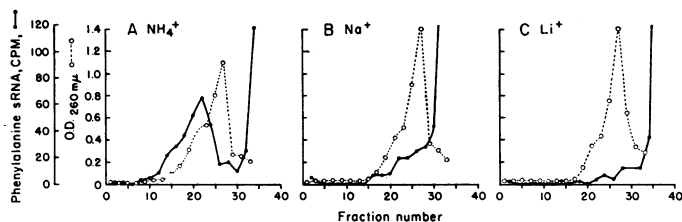


FIG. 7.—Binding of sRNA in the presence of various monovalent cations.

Specificity of the Binding.—In order to study the question of specificity, the binding of radioactive lysyl RNA to the poly U-ribosome complex was measured, as shown in Figure 8*A*. The amount of radioactivity is to be compared with the control experiment using phenylalanyl sRNA (Figure 8*B*). It should be noted that

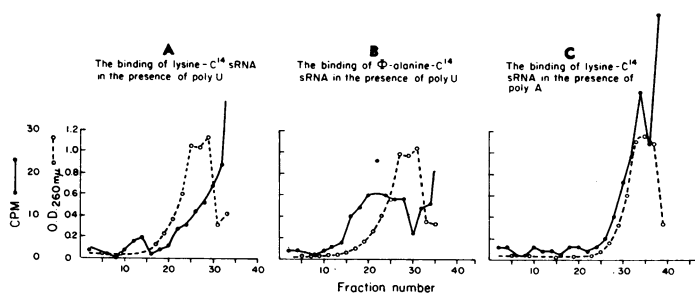


FIG. 8.—Specificity of sRNA-template-ribosome interaction. 10 μ g of poly A and 0.1 mg of lysyl sRNA were added to the other components of the reaction mixture. Incubation was at 25° for 5 min.

lysine of a threefold greater specific activity than phenylalanine was used and very little of the former is bound. Poly U, therefore, does not induce attachment of lysyl sRNA. In Figure 8C, however, lysyl sRNA is shown to attach to ribosomes charged with poly A, a template coding for lysine. In gradient 8C, the radioactivity in lysyl sRNA follows the 70 S peak; it does not appear in the area of the heavy ribosomes as has been the case whenever poly U was employed. This suggested that heavy ribosomes were not formed by the addition of poly A under our conditions, and that polylysine was mainly synthesized on the 70 S monomers. Preliminary experiments have shown this to be correct; that is, the nascent polylysine chains appear predominantly on the 70 S ribosomes rather than on the polyribosomes.

Comments.—A binding of sRNA to ribosomes has recently been reported by Gilbert³ and Cannon *et al.*¹⁰ Such a binding needs to be compared with that reported here. These workers largely followed the P³²-label of sRNA through gradient centrifugation. In their experiments all sRNA's were labeled *in vivo*, and a preparation of high specific activity was used. They found a binding which is diminished by addition of salt in the cold and in the absence of template. Their experimental setup differs considerably from the one we used. In our experiments, the label of C¹⁴-phenylalanine was attached to one of the sRNA's and followed specifically through gradient centrifugation. Our relatively small binding at 0°, or in the absence of salt, shown by Figures 1A and 3, is similar in its characteristics to those reported.^{9, 10} The much smaller amount of label found on the ribosome is explained by our use of the label on the phenylalanine-accepting sRNA species of relatively low abundance against their following the P³²-label present in all sRNA. The nonspecific and apparently looser attachment of an average of one sRNA per ribosome observed by Gilbert's group, therefore, contrasts with the specific binding of a sRNA to its complementary template that appears to be stabilized by a high concentration of NH₄⁺ or K⁺, as is reported here. Gilbert³ observed, furthermore, that polypeptidyl sRNA remains bound on ribosomes or, rather, on the 50 S fragment and does not require poly U to stay on. Experiments, not included here, have shown that this polypeptidyl sRNA-ribosome complex connection is also independent of the presence of monovalent ions.

Under the conditions of our experiments, as shown in the preceding paper by Conway,⁵ the preincubation of an sRNA charged with phenylalanine, poly U, and salt eliminates a lag in the onset of polymerization caused by addition of polymerizing enzymes (supernatant fractions), and GTP. In this experimental setup, the binding seems to be a slow reaction as shown by Figure 3, and, in Conway's experiments, to be rate-determining. As shown more recently by Nakamoto,⁶ the template-bound amino acyl sRNA initiates the formation of the peptide chain which would explain the binding being a rate-determining step.

Summary.—The specific binding of amino acyl sRNA to ribosomes is described. It requires the presence of a complementary template and of rather high concentrations of ammonium ions. Mostly, the attachment of C¹⁴-phenylalanyl sRNA requiring poly U, but also the attachment of C¹⁴-lysyl sRNA requiring poly A, were followed by gradient centrifugation. The optimum concentration of ammonium salt is 0.16–0.2 M. On raising the temperature, increasing amounts of label were found on the ribosome using C¹⁴-phenylalanyl sRNA + poly U. Once the attach-

ment had taken place, the centrifugation carried out at 0° did not reverse. This specific binding appears to be the initial and sometimes rate-limiting step in amino acid polymerization.

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ACTION SPECTRUM FOR FERRICYANIDE PHOTOREDUCTION AND REDOX POTENTIAL OF CHLOROPHYLL 683*

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Nearly thirty years ago, Hill¹⁻⁴ discovered that chloroplasts isolated from green leaves liberate molecular oxygen upon illumination when an appropriate hydrogen acceptor (Hill oxidant) is provided. It is now generally accepted that this reaction represents the photochemical step in photosynthesis.

Meanwhile, the possible photochemical function of the pigments associated with chloroplasts, namely, the chlorophylls and carotenoids, has been examined by a number of investigators.⁵⁻¹⁰ Chen measured the action spectrum for photoreduction of 2,6-dichlorophenol indophenol by chloroplasts from Swiss chard.¹¹ The action spectrum for this reaction coincided well with the action spectrum for photosynthetic oxygen evolution as well as with the absorption spectrum of the chloroplasts. Action spectra for NADP photoreduction and the coupled phosphorylation, measured by San Pietro *et al.*,¹² Black *et al.*,¹³ and Jagendorf *et al.*¹⁴ coincided with the absorption spectrum of the chloroplasts.

We have studied the action spectra for the photoreduction of ferricyanide and the coupled phosphorylation and found that the resultant action spectra differ from the absorption spectrum of the chloroplasts. In addition, we have determined the redox potential of the chlorophyll component with an absorption maximum at 683 m μ .