*BINDING OF SPECIFIC SRNA TO 30S RIBOSOMAL SUBUNITS: EFFECT OF 50S RIBOSOMAL SUBUNITS**

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As a continuation of our preceding work on the binding of specific sRNA to ribosomes,[1-4] and the binding of aminoacyl sRNA to the 30*S* ribosomal subunits,[5, 6] we have investigated the effect of 50*S* subunits on the binding capacity of 30*S* subunits. The binding of aminoacyl sRNA to 30*S* subunits is specific in that only the aminoacyl sRNA coded for in the messenger RNA was induced to bind.[5, 6] In view of the current notion that the 50*S* subunit has the binding site for sRNA,[7] the effect of 50*S* subunits on the binding of specific sRNA to the 30*S* subunit was of interest. It was found that the addition of 50*S* subunits to a 30*S* subunit preparation resulted in about a twofold stimulation of aminoacyl sRNA binding capacity of the 30*S* subunit preparation.

Materials and Methods.—E. coli extract and other materials: Preparation of ribosomes, 30*S* and 50*S* subunits of ribosomes, sRNA from *E. coli*, and aminoacyl sRNA, have been described in the preceding communications.[1-6] The ribosomes had been washed three times as described,[2, 3] and were found to be free from the aminoacyl sRNA transfer activity. Aminoacyl sRNA synthetase from *E. coli* was prepared as described previously.[8] Specific radioactivities of materials used in this paper were as follows: C^{14}-poly U, 0.18 μc/mg; C^{14}-phenylalanine, 200 μc/μmole; C^{14}-arginine, 250 μc/μmole; C^{14}-leucine, 246 μc/μmole, and H^3-phenylalanine, 2450 μc/μmole. Counting efficiency was 10^6 cpm/μc for C^{14} and 3×10^5 cpm/μc for H^3.

Reaction mixture for the binding of aminoacyl sRNA: For the assay of binding of specific aminoacyl sRNA, the method devised by Nirenberg and Leder was used.[9] A typical reaction mixture for phenylalanyl sRNA binding contained the following in μmoles per 0.2 ml: 10 Tris-HCl (pH 7.1), 4 magnesium acetate, 2 KCl, 0.2 β-mercaptoethanol, 0.125 puromycin. In addition it contained 40 μg of poly U, 6900 cpm of phenylalanyl sRNA, and ribosomal subunits or 70*S* ribosomes. The binding reaction was carried out for 15 min at 24°C. Although the ribosomes used were free from aminoacyl sRNA transfer factor, to nullify the possibility of forming polyphenylalanine in the binding mixture, puromycin was added most of the time. Identical results were obtained in the absence of puromycin. Under these conditions, the amount of bound sRNA was proportional to the amount of ribosomes or 30*S* subunits up to 100 μg of 70*S* ribosomes or 30*S* subunits per the reaction mixture.

Reaction mixture for the binding of sRNA and C^{14}-poly U: For binding of free (deacylated) sRNA, the reaction mixture contained the following in μmole/0.7 ml: 6 Tris-HCl (pH 7.8), 30 KCl, and 20 magnesium acetate. In addition, it contained 0.375 mg of *E. coli* sRNA, 0.2 mg of C^{14}-poly U, 75 mg of sucrose, and 0.54 mg of the 30*S* subunit fraction or 0.90 mg of 50*S* subunit fraction prepared as described previously.[5, 6] The mixture was made at 0°C, and in some cases it was incubated at 25°C for 15 min. A 0.5-ml portion of the mixture was layered on top of 4.7 ml of 12.5–20% sucrose gradient in a buffer which contained 0.05 *M* KCl, 2×10^{-2} *M* magnesium acetate, and 0.01 *M* Tris-HCl (pH 7.8). The tube was centrifuged in a Spinco SW-39 rotor at 37,000 rpm for 2.5 hr at 5°C. After the centrifugation, 2-drop fractions were collected from the bottom of the tube. The amount of phenylalanine sRNA in each fraction was determined by incubating each fraction with the aminoacyl sRNA synthetase and measuring the incorporation of H^3-phenylalanine into sRNA as described previously.[1, 2]

Preparation of trypsin-treated 50S subunits: The 50*S* subunit preparations (640 μg) were incubated for 5 min at 37°C with 6 μg of trypsin in 12% sucrose, 10^{-4} *M* magnesium acetate, 0.01 *M* Tris-HCl (pH 7.4), 0.006 *M* β-mercaptoethanol, and 0.06 *M* KCl in a total volume of 0.4 ml. At the end of incubation, 40 μg of trypsin inhibitor (soybean) were added. In a control experi-

ment it was found that the binding of specific aminoacyl sRNA to 70*S* ribosomes was not influenced by the presence of the trypsin inhibitor.

Results.—Effect of the addition of 50S subunits to the various amounts of 30S subunits: In the experiment shown in Figure 1, the binding of C^{14}-phenylalanyl sRNA to the 30*S* subunits in the presence of poly U was studied with various amounts of 30*S* subunits. When 50*S* subunits were added to each of the reaction mixtures containing various amounts of 30*S* subunits, the binding capacity of each of the

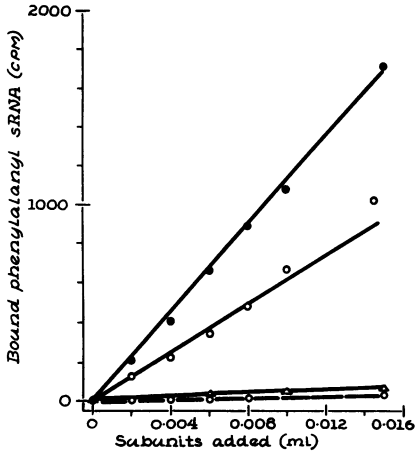

FIG. 1.—Comparison of the specific sRNA binding capacity of the 30*S* subunits and the reconstituted 70*S* ribosomes. The amount of bound C^{14}-phenylalanyl sRNA was plotted against the amount of 30*S* subunits or 50*S* subunits in 0.2 ml of the reaction mixture. The stock solution of 30*S* and 50*S* subunits preparation contained 7 μg and 16 μg of subunits, respectively, in 0.01 ml. ○——○, 30*S* subunit preparation; ●——●, 30*S* and 50*S* subunits were added (for example, "0.01 ml subunits" means that 0.01 ml of the 30*S* subunit preparation and 0.01 ml of the 50*S* subunit preparation were added to the mixture); △——△, 50*S* subunits; ○- - ○, 30*S* subunits, but poly U was omitted from the reaction mixture.

mixtures was approximately doubled. On the other hand, the binding of C^{14}-phenylalanyl sRNA to the 50*S* subunit was negligible, confirming preceding reports.[5, 6] The binding was dependent on the presence of poly U indicating its specific nature.

In the experiment shown in Figure 2, limited amounts of 50*S* subunits were added to various amounts of 30*S* subunits, and the binding activity of the mixture was plotted against the amount of 30*S* subunits in the reaction mixture. If the observed stimulatory effect of 50*S* subunits is due to the formation of 70*S* ribosomes, one would expect that the slope of the curve would become less at the point where excess 30*S* subunits were present in the reaction mixture. In the presence of 15.1 μg of 50*S* subunits, the slope became about half at the point where approximately 7 μg of 30*S* subunits were present. In the presence of 23 μg of 50*S* subunits, the slope decreased to half at the point where approximately 13 μg of 30*S* subunits were present. It is noted that the initial slope, in the presence of excess 50*S* subunits, was approximately twice as much as the slope observed with 30*S* subunits only.

Effect of the addition of various amounts of 50S subunits to a limited amount of 30S subunits: The effect of the addition of increasing amounts of 50*S* subunits to the

binding capacity of constant amount of 30S subunits is shown in Figure 3. Since 50S subunits alone do not bind phenylalanyl sRNA appreciably, the binding capacity of the mixture should become constant when all the available 30S subunits are saturated with the 50S subunits. As shown in Figure 3, the binding capacity of the mixture containing 7 μg of 30S subunits reached plateau when approximately 23 μg of 50S subunits were added to the mixture. The level of the plateau was approximately twice as much as the binding capacity of 30S subunits alone, indicating that when all 30S subunits in the mixture were saturated with 50S subunits, the binding capacity of the mixture became twofold.

Addition of 50S subunits after completion of binding of phenylalanyl sRNA to 30S subunits: In the experiment shown in Figure 4, the binding of phenylalanyl sRNA was carried out in two steps. In the first step, the binding of phenylalanyl sRNA to the 30S subunit was completed, and in the second step, 50S subunits were added and the binding was allowed to continue. It is noted that in this case also the

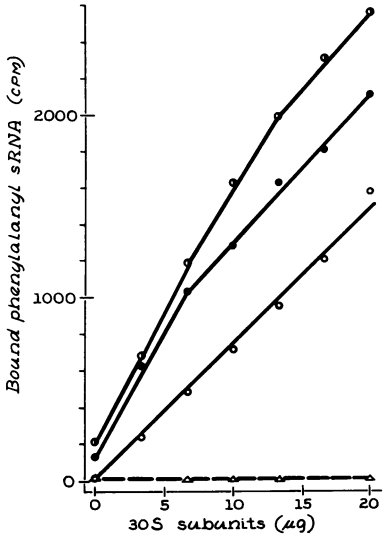

FIG. 2.—Addition of limited amounts of the 50S subunit preparation to various amounts of 30S subunits. O——O, 30S subunits only; ●——●, 30S subunits and 15.1 μg of 50S subunits; ◑——◑, 30S subunits and 22.7 μg of 50S subunits; △ - - △, 30S subunits and 22.7 μg of 50S subunits, but poly U was omitted from the reaction mixture.

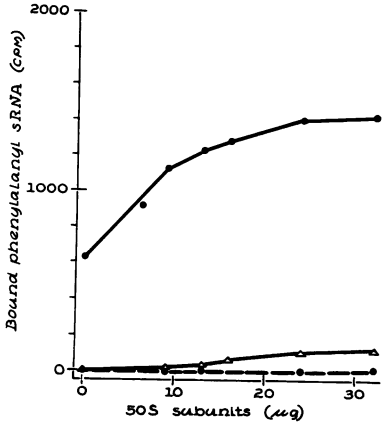

FIG. 3.—Addition of 50S subunits to a fixed amount of 30S subunits. The reaction mixture contained, in addition to other components, 7 μg of 30S subunits, and various amounts of 50S subunits as indicated in the figure. ●——●, 30S subunits and 50S subunits; △——△, 50S subunits only; ● - - ●, 30S and 50S subunits, but poly U was omitted.

addition of 50S subunits resulted in approximately twofold increase of the bound phenylalanyl sRNA. These results indicate that 50S subunits can still bind to 30S subunits and exert their stimulatory effect even after poly U and sRNA are bound to the 30S subunit.

It has been found that mild trypsin treatment of 70S ribosomes resulted in loss of sRNA binding capacity of the ribosomes.[10] This observation suggests that ribosomal protein plays an important role for the interaction of sRNA and messenger RNA. As shown in Figure 4, the 50S subunits which had been treated with tryp-

sin did not stimulate the binding of phenylalanyl sRNA. In a separate experiment, it was found that the sedimentation velocity of trypsin-treated 50*S* subunit was not appreciably different from that of the control 50*S* subunit. It was also found that the trypsin treatment, under the experimental conditions, caused the 50*S* subunit to lose its capacity to associate with the 30*S* subunit.

Effect of magnesium ion concentration on the binding activity of reconstituted ribsomes: It has been found that the response of the activity of 30*S* subunits to the change of magnesium ion concentration was quite different from that of the original unfractionated 70*S* ribosomes.[6] It is noted in Figure 5 that the behavior of the reconstituted system toward the change of Mg^{++} was qualitatively similar to those of the 30*S* subunits. At lower Mg^{++} concentration, the reconstituted system behaved more like the 30*S* subunit system. The addition of 50*S* subunits resulted in approximately twofold stimulation on the phenylalanyl sRNA binding capacity of the mixture in the presence of 10^{-2} *M* or higher Mg ion concentration.

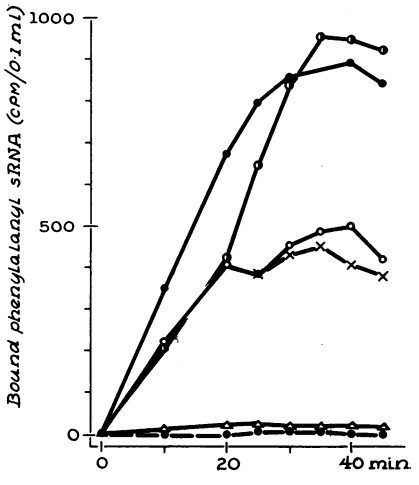

FIG. 4.—Addition of 50*S* subunits after binding of phenylalanyl sRNA to 30*S* subunits was completed. Total volume of the reaction mixture was 0.85 ml and contained 32.6 μg of 30*S* subunits. At the various time intervals, 0.1-ml aliquot was taken and the bound phenylalanyl sRNA was measured. O——O, 30*S* subunits; ●——●, 30*S* subunits and 80 μg of 50*S* subunits were added at zero time; ● - - ●, 30*S* subunits and 80 μg of 50*S* subunits were added at zero time, but poly U was omitted from the mixture; △——△, 80 μg of 50*S* subunits, but no 30*S* subunits were added; ◑——◑, 30*S* subunits were added at zero time, but 80 μg of 50*S* subunits were added at 20 min after the onset of reaction; x——x, as above, but trypsin-treated 50*S* subunits were added at 20 min.

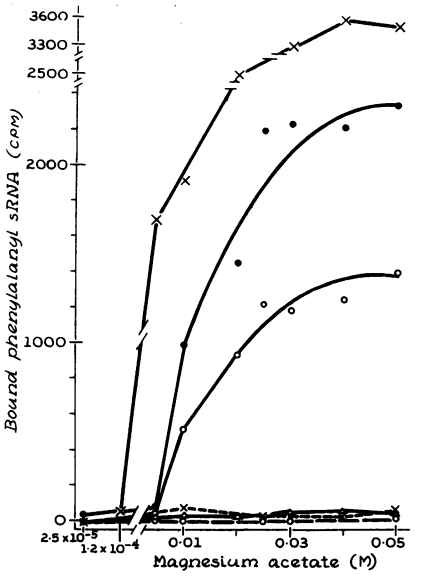

FIG. 5.—Comparative studies of Mg^{++} effect on the binding of phenylalanyl sRNA. The reaction mixture contained, in addition to other components, 14 μg of 30*S* subunits, and 32 μg of 50*S* subunits, or each of subunits alone, or 60 μg of 70*S* ribosomes. The bound phenylalanyl sRNA was plotted against Mg^{++} concentration. O——O, 30*S* subunits; ●——●, 30*S* subunits and 50*S* subunits; △——△, 50*S* subunits; x——x, 70*S* ribosomes; O - - O, 30*S* subunits but poly U was omitted; x - - x, 70*S* ribosomes but poly U was omitted from the reaction mixture.

Calculation of the effect of 50S subunits: The behavior of the reconstituted system to Mg^{++} ions suggested that not all of the 30*S* subunits were bound with 50*S* subunits under the experimental conditions. In support of this view, sedimenta-

tion analysis with a Spinco model E ultracentrifuge revealed that only about 77 per cent of the 30*S* subunits in the reconstituted system associated with the added 50*S* subunits. The data shown in Table 1 summarize the effect of 50*S* subunits on

TABLE 1

EFFECT OF 70*S* RIBOSOME FORMATION ON THE QUANTITY OF BOUND PHENYLALANYL sRNA

Experiment	Bound C^{14}-Phenylalanyl sRNA (cpm) 30*S* subunits (1)	50*S* subunits and 30*S* subunits (2)	Ratio (2)/(1)	Ratio Corrected value
1	612	1043	1.7	1.9
2	624	1191	1.9	2.2
3	508	898	1.8	2.0
4	508	944	1.9	2.1
5	1193	2168	1.8	2.1
6	420	748	1.8	2.0
Average	—	—	1.8	2.1

Corrected value was obtained by determining the amount of 30*S* and 70*S* components in the reconstituted system and subtracting the binding due to the residual 30*S* subunits.

the binding capacity of 30*S* subunits after the correction based on the actual amount of 30*S* subunits converted to 70*S* ribosomes was applied. The degree of stimulation, as well as degree of association of 30*S* subunits with 50*S* subunits, varied. Therefore, each set of data shown in Table 1 was corrected according to the degree of association of 30*S* subunits in each reaction mixture which was analyzed by the Spinco model E ultracentrifuge. As shown in this table, an approximately twofold stimulation of binding capacity for phenylalanyl sRNA was observed when association of 30*S* subunits with 50*S* subunits took place.

Effect of tetracycline: In preceding reports,[11, 12] tetracyclines were reported to inhibit the binding of aminoacyl sRNA to 70*S* ribosomes. The data shown in

TABLE 2

EFFECT OF TETRACYCLINE

	Bound Phenylalanyl sRNA (cpm) Experiment I Tube 1	Tube 2	Tube 3	Tube 4	Experiment II Tube 1	Tube 2	Tube 3	Tube 4
Tetracycline (μg)	0	40	0	40	0	40 (at 20′)	0	40 (at 20′)
50*S* subunits (μg)	0	0	17.5	17.5	0	0	17.5 (at 20′)	17.5 (at 20′)
30*S* subunits (μg)	7	7	7	7	7	7	7	7
Bound phenylalanyl sRNA (cpm)	781	226	1288	237	851	674	1599	959
Inhibition (%)	—	71	—	82	—	21	—	40
Effect of 50*S* (cpm)	—	—	+507	+11	—	—	+748	+285
Inhibition on the 50*S* effect (%)	—	—	—	98	—	—	—	62

In expt. I, all components of the reaction mixture were the same throughout the incubation period of 20 min. In expt. II, some components were added at 20 min as indicated in the table after the onset of the binding reaction. Total incubation time was 40 min.

Table 2 indicate that tetracycline inhibits the binding of phenylalanyl sRNA to the reconstituted ribosomes as well as to the 30*S* subunits. In experiment II of Table 2, the binding reaction was carried out for 20 min, then tetracycline and 50*S* subunits were added, and incubation was carried out for another 20 min. As shown in the table, once the phenylalanyl sRNA is bound to the ribosomes or to the 30*S* subunits, tetracycline apparently cannot dissociate the bound phenylalanyl

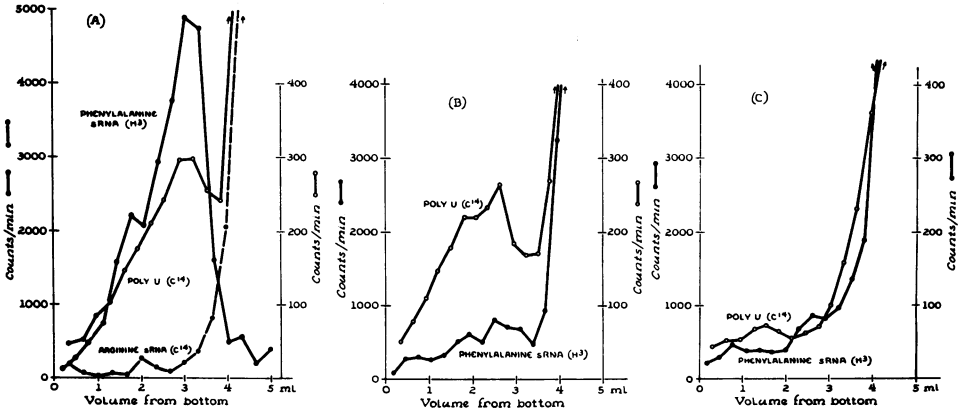

FIG. 6.—Binding of deacylated sRNA and C^{14}-poly U. (*A*) Binding of C^{14}-poly U and sRNA specific for phenylalanine to isolated 30*S* subunits. Two separate mixtures of 30*S* subunits and other components were incubated for 15 min at 25°C and separately centrifuged as described in *Methods*. Mixture 1 contained, in addition to other components, C^{14}-poly U. Mixture 2 was the same as mixture 1, except that nonlabeled poly U was used. After the sucrose gradient centrifugation, odd-numbered fractions from mixture 1 were used to measure the amount of phenylalanine sRNA, and 0.1 ml of each of the even-numbered fractions were used for measurement of C^{14}-poly U as described.[2] The fractions from mixture 2 were used to determine the amount of arginine sRNA. ●——●, Phenylalanine sRNA distribution assayed with H^3-phenylalanine; O——O, C^{14}-poly U distribution; ● - - ●, arginine sRNA distribution assayed with C^{14}-arginine. (*B*) Binding of C^{14}-poly U at 0–5°C to isolated 30*S* subunits and absence of binding of phenylalanine sRNA. Mixture 1 of (*A*) was made at 0°C and immediately subjected to the sucrose gradient centrifugation at 0–5°C. O——O, Distribution of C^{14}-poly U; ●——●, distribution of phenylalanine sRNA assayed with H^3-phenylalanine. (*C*) Absence of binding of C^{14}-poly U and phenylalanine sRNA to 50*S* subunits. The reaction mixture for the binding of C^{14}-poly U and sRNA was the same as mixture 1 of (*A*), except that it contained 50*S* subunits instead of 30*S* subunits.

sRNA. Thus, the inhibitory effect of tetracycline was much less when tetracycline was added 20 min after the onset of the binding reaction. It is also noted in this table that the binding of specific phenylalanyl sRNA to the reconstituted ribosomes is almost equal in sensitivity to the binding to 30*S* subunits, indicating that the presence of 50*S* subunits does not alter the sensitivity of the system to tetracycline.

In experiment II of this table, the effect of tetracycline on the stimulatory effect of 50*S* subunits was studied under conditions where most of the active 30*S* subunits had phenylalanyl sRNA. It is seen that there was 60 per cent inhibition of the stimulatory effect of 50*S* subunits under these conditions. On the other hand, in experiment I, the effect of tetracycline on the stimulatory effect of 50*S* subunits was studied under conditions where none of the ribosomes had bound phenylalanyl sRNA. It is noted that under these conditions, tetracycline abolished almost completely the stimulatory effect of 50*S* subunits.

Binding of free (discharged) sRNA to 30S subunits: It is known that binding of aminoacyl sRNA,[1, 2, 13, 14] as well as sRNA,[1–4] takes place to 70*S* ribosomes or their aggregates. In the experiment shown in Figure 6*A*, the isolated 30*S* subunits were mixed with C^{14}-poly U and sRNA. The sRNA used had been pretreated so that any possible aminoacyl sRNA present in the preparation was deacylated.[15] After sucrose density gradient centrifugation of the mixture, the bound sRNA specific for phenylalanine in each fraction was assayed using H^3-phenylalanine and aminoacyl sRNA synthetase. The distribution of bound phenylalanine sRNA in the centrifuge tube followed closely the distribution of bound poly U. It is noted

that there was no appreciable binding of arginine sRNA to the 30*S* subunits in the presence of poly U, indicating the specific nature of the binding.

It has been found that binding of specific phenylalanyl sRNA to 30*S* subunits did not take place at 0°C,[5] whereas the binding to 70*S* ribosomes took place even at 0°C. As shown in Figure 6*B*, the binding of discharged sRNA to the 30*S* subunits is dependent on the incubation of the mixture at higher temperature, and no appreciable binding was observed at 0–5°C. On the other hand, it is noted that the binding of C^{14}-poly U to the 30*S* subunits took place under the same conditions (at 0–5°C). Figure 6*C* shows that neither C^{14}-poly U nor discharged sRNA bound to the 50*S* subunits appreciably.

Discussion.—It is tempting to explain the stimulatory effect of 50*S* subunits on the assumption that two sRNA molecules are bound to one 70*S* ribosome particle.[2, 13, 16] One can explain the twofold stimulation by the addition of 50*S* subunits on the hypothesis that the first sRNA molecule can be bound to the 30*S* subunit and the binding of the second sRNA is dependent on the 50*S* subunit. The twofold stimulation of binding of aminoacyl sRNA by the 50*S* subunit preparation was also observed with leucyl sRNA in the presence of poly UC (6:1) and 2×10^{-2} *M* Mg^{++}. In this case, since there would be few codons for leucine in the poly UC (6:1), reassociated ribosomes having sRNA would mostly contain deacylated sRNA specific for phenylalanine and few would have C^{14}-leucyl sRNA adjacent to sRNA specific for phenylalanine. The fewest of all would have bound C^{14}-leucyl sRNA's adjacent to each other.

Calculations of the data presented in this paper show that only 25–30 per cent of 30*S* subunits present in the reaction mixture have bound phenylalanyl sRNA. It is therefore possible that 50*S* subunits, by reassociation, activate those inactive subunits. This possibility seems unlikely, however, because one would have to assume that the 70*S* ribosomes bind only one sRNA and the activation by 50*S* subunits is limited to twofold and not more.

Summary.—When 50*S* subunits were added to 30*S* subunits, the binding capacity of the mixture for specific aminoacyl sRNA was found to be approximately twice the binding capacity of the original 30*S* subunit preparation. The 50*S* subunit alone did not bind specific aminoacyl sRNA. These findings are consistent with the hypothesis that one molecule of sRNA can bind to the 30*S* subunit and that the second binding site is generated by the formation of 70*S* ribosomes. Free (deacylated) specific sRNA also binds to the 30*S* subunit. Tetracycline inhibits the binding of phenylalanyl sRNA to the 30*S* subunits-poly U complex.

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*ISOLATION OF THE NUCLEIC ACID OF MOUSE MAMMARY TUMOR VIRUS (MTV)**

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The mouse mammary tumor virus (MTV) was one of the first tumorigenic viruses found in mammals.[1, 2] Nevertheless, little progress has been made on the elucidation of the molecular structure of the virus.[3] The absence of a rapid bioassay has hindered progress in virus purification, and the difficulty in growing the virus in tissue culture has made use of radioisotopes impractical.[4] MTV is normally transmitted through the mother's milk. This establishes a lifelong infection, and virus can be recovered from tissue or milk at any time. The ultimate development of a mammary tumor in an infected female is dependent not only upon the presence of the virus but also upon genetic and hormonal factors.

Previous chemical studies have shown that MTV contains 30 per cent lipid and its nucleic acid is RNA.[5] Electron micrographs indicate[2, 6, 7] that its size and structure are similar to the other large, lipid-containing RNA viruses such as the avian tumor viruses, the mouse leukemia viruses, and the myxoviruses. The purification of virus and isolation of high-molecular-weight RNA from other RNA tumor viruses such as Rous sarcoma and Rous-associated virus (RSV + RAV),[8] avian myeloblastosis virus (AMV),[9] and the Rauscher mouse leukemia virus (MLV),[10, 11] as well as the recent development of a fast serological assay for MTV,[12] suggested that virus purification and RNA isolation could be accomplished with MTV. This report describes such a study. A high-molecular-weight single-stranded RNA similar to that obtained from other RNA tumor viruses was found and its properties were studied.

Materials and Methods.—Virus: P^{32}- or H^3-labeled virus was used in all experiments. The virus was obtained from mouse milk which is a rich source of virus.[13] Milk was collected from lactating female A/Crgl or BALB/cfC$_3$HCrgl mice approximately 1 week post partum; this milk contains infective MTV.[14] In a control study of milk from mice not infected with MTV, milk was obtained from BALB/cCrgl females. Twenty-four hr before milking, each mouse was injected with 1.5–2.5 mc carrier-free $P^{32}O_4$ or 1.5 mc H^3-orotic acid (0.23 mc/μM) in 0.2–0.4 ml Tris buffer pH 7.4 containing 0.1 *M* NaCl. The females were not permitted to suckle their young for 12 hr prior to milking. Each mouse received 0.1 ml of Pitocin (Parke-Davis Co.) intraperitoneally