Polypeptide Synthesis with Methyl-Deficient Soluble Ribonucleic Acid*

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ABSTRACT: The biological function of the enzymatic methylation of soluble ribonucleic acid (sRNA) has been examined with particular attention to the ribosome-mediated transfer of amino acids into polypeptide in an *Escherichia coli* system. In the kinetics of f2 viral coat protein synthesis from free amino acids, methyl-deficient sRNA behaved normally and elicited the production of a normal protein product, as determined by tryptic digestion and fingerprinting. Methylation of the sRNA with heterologous (yeast) sRNA methylases did not alter the kinetics of f2 coat protein synthesis. If the artifical messengers polyuridylic acid (poly U) and poly UC were used in place of f2 RNA, the levels of coding errors with methyl-deficient sRNA were no higher than those normally observed. Submethylated species of phenylalanine-specific sRNA were separated from their normal counterparts by countercurrent distribution. Submethylation of these species was proved by *in vitro* methylation of the sRNAs followed by binding to the poly U–ribosome complex as demonstrated in sucrose gradients. In the initial kinetics of the transfer reaction, submethylated phenylalanyl-sRNA performed somewhat more slowly than normal phenylalanyl-sRNA. This effect was more marked at magnesium concentrations below the optimum for the *in vitro* system. Submethylated phenylalanyl-sRNA was also less efficient than the normal in binding to poly U, poly UC, and poly UA, but no evidence was found for increased ambiguity on the part of submethylated sRNA in the binding reaction. The conclusion is reached that in the transfer reaction methylation has a relatively small effect on the efficiency of sRNA function. This effect may be due to an ability for submethylated sRNA to adopt routinely certain conformations which are accessible to normal sRNA only under special conditions.

Since the discovery of methyl-deficient sRNA[1] by Mandel and Borek (1963), and the recognition of the specific sRNA methylases capable of methylating such sRNA (Fleissner and Borek, 1962, 1963; Svensson *et al.*, 1963a; Hurwitz *et al.*, 1964), the question of how methylation affects the function of sRNA has lacked a definitive answer. (This subject has been recently reviewed by Borek and Srinivasan, 1966.) Any approach to testing the function of submethylated sRNA[1] encounters the obstacle that in the usual source of such sRNA, methyl-deficient sRNA from *Escherichia coli* K-12 W-6, the submethylated sRNAs are mixed with an equal amount of normal, fully methylated sRNA which had been present in the bacterial cell prior to the removal of methionine (Mandel and Borek, 1963). In such a mixture of normal and submethylated sRNA, only a markedly abnormal behavior on the part of the latter component would escape being obscured by the normal functioning of the former. Negative results in experiments designed to detect such abnormal behavior only enable one to set limits to the degree of abnormality that submethylation may induce in the sRNA. The results of the preliminary experiments described in this paper and results from other laboratories (Svensson *et al.*, 1963b; Starr, 1963; Neidhardt and Eidlic, 1963; Littauer *et al.*, 1963; Peterkofsky *et al.*, 1964) made it seem likely that the degree of abnormality would be small. In the present work CCD[2] of methyl-deficient sRNA was used to separate several species of phenylalanine-specific sRNA, two of which are not present in normal sRNA. The separation achieved is similar to that reported by Littauer and his collaborators (Revel and Littauer, 1965; Littauer *et al.*, 1966), who used MAK. The submethylation of the novel species has been verified, and these species have been pitted against normal phenylalanine-specific sRNA in experiments designed to test efficiency of transfer and possible miscoding. The abnormal

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[1] In this paper the term "methyl-deficient sRNA" is used to refer to the total sRNA extractable from methionine-starved cells of *E. coli* K-12 W-6. As stated in the text, this preparation contains approximately 50% normally methylated and 50% unmethylated sRNA. The term "submethylated sRNA" is used for the latter component or for individual amino acid acceptor species within that component.

[2] The following abbreviations are used: CCD, countercurrent distribution; MAK, methylated albumin kieselguhr; TCA, trichloroacetic acid; CCA, the cytidylylcytidylyladenosine terminus of sRNA; GSH, glutathione; ATP and GTP, adenosine and guanosine triphosphates.

phenylalanine-specific sRNA is somewhat less efficient than the normal in transferring phenylalanine into polypeptide linkage, but it does not appear to be more subject to coding errors than the normal species.

Experimental Procedures

Materials. *E. coli* K-12s, provided by Dr. Ernest Borek, was grown in a biogen (American Sterilizer Co.) in a complex medium (Nathans and Lipmann, 1961). Cells for isolation of enzymes or ribosomes were harvested in the logarithmic phase of growth, washed in isotonic KCl, and frozen. For isolation of normal sRNA, the cells were allowed to reach the end of log phase. *E. coli* K-12 W-6, starved of methionine for 3 hr according to Borek *et al.* (1955), was generously provided in large batches by Merck Sharp and Dohme, Rahway, N. J., and was used for preparing methyl-deficient sRNA. f2 virus used for isolation of viral RNA was a gift of Dr. Norton Zinder.

Four-times-washed ribosomes, prepared by the procedure of Allende *et al.* (1964), were washed in 0.01 M Tris–HCl, pH 7.4, containing 0.01 M $MgCl_2$ and 0.2 M NH_4Cl. Five-times-washed ribosomes received an additional wash in 0.01 M Tris–HCl, pH 7.4, 0.001 M $MgCl_2$, and 0.5 M NH_4Cl (Salas *et al.*, 1965). For experiments with f2 RNA, ribosomes were isolated from a preincubated S-30 (Nirenberg and Matthaei, 1961) and washed in 0.01 M Tris–HCl, pH 7.4, with 0.008 M $MgCl_2$, and 0.1 M NH_4Cl. All ribosomes were stored frozen at 80 mg/ml in 0.01 M Tris–HCl, pH 7.4, with 0.008 M $MgCl_2$. The 0.5 M NH_4Cl-washed ribosomes were activated by incubation for 20 min at 30° (T. Nakamoto, personal communication).

Supernatant factors for *in vitro* protein synthesis from free amino acids ("two-step" system) were prepared from *E. coli* by a procedure developed with the assistance of R. Krug and T. Nakamoto. An S-100 (Nirenberg and Matthaei, 1961) was treated with protamine sulfate (1.5 mg/ml), the precipitate was removed by centrifugation, and to the supernatant fluid was added four volumes of a 2% suspension of DEAE-cellulose in 0.01 M Tris–HCl, pH 7.8, and 0.005 M β-mercaptoethanol. The DEAE-cellulose was washed in a sintered-glass funnel with 0.04 M Tris–HCl, pH 7.8, and 0.005 M β-mercaptoethanol, and was then eluted with the same buffer containing 0.3 M KCl. The eluate was concentrated by negative-pressure dialysis *vs.* 0.01 M Tris–HCl, pH 7.8, containing 0.06 M KCl and 0.004 M GSH, to yield a final protein concentration of 8 mg/ml.

Transfer factors from *E. coli* used for polypeptide synthesis from phenylalanyl-sRNA ("one-step" system) were partly purified from an S-100 fraction. After addition of one volume of 0.08 M potassium phosphate, pH 7.4, and treatment with protamine sulfate (3 mg/ml), the activity was precipitated from the supernatant fluid with ammonium sulfate between 55 and 63% saturation. The ammonium sulfate precipitate dissolved in a small volume of 0.01 M Tris–HCl, pH 7.4, was passed twice through Sephadex G-25 (Pharmacia) equilibrated with 0.05 M Tris–HCl, pH 7.4, containing 0.01 M GSH. The enzyme preparation was free of detectable sRNA. The residual sRNA methylase and phenylalanyl-sRNA synthetase activities were insufficient to affect the bulk of the susceptible sRNA in incubation mixtures under the conditions used for the transfer reaction.

Phenylalanyl-sRNA synthetase was partially purified from an S-30 of *E. coli* by pH fractionation between pH 4.5 and 4.1, ammonium sulfate fractionation between 44 and 55% saturation (Conway *et al.*, 1962), and chromatography on Sephadex G-25 in 0.05 M Tris–acetate, pH 7.2, with 0.01 M GSH. The preparation was free of detectable sRNA, and there was no significant contamination with sRNA methylase activity.

E. coli sRNA methylases were prepared as previously described (Fleissner and Borek, 1963), except that pH precipitation was omitted, protamine sulfate was added directly to the S-100 to a final concentration of 4 mg/ml, and the supernatant fluid was used for ammonium sulfate fractionation. The 35–55%-saturated ammonium sulfate fraction was further purified by a stepwise elution from DEAE-cellulose (0.1–0.3 M KCl in 0.02 M Tris–HCl, pH 7.4, with 0.005 M β-mercaptoethanol) and reprecipitation with ammonium sulfate at 70% saturation. The protein precipitate was dissolved in 0.025 M Tris–HCl, pH 7.8, and passed through Sephadex G-25 in the same buffer. The milieu of the enzyme preparation was then adjusted to 0.05 M Tris–HCl, pH 7.8, 0.01 M $MgCl_2$, and 0.006 M β-mercaptoethanol. After incubation with 1 mg/ml of Macaloid for 10 min at 30° and centrifugation at 30,000*g* for 20 min, the supernatant fluid was used as a source of methylating enzymes. This preparation displayed methylating activity toward uracil, adenine, and guanine in sRNA, and also contained an active phenylalanyl-sRNA synthetase that was useful in an experimental procedure outlined below. The preparation was free of sRNA.

Yeast sRNA methylases were prepared according to Svensson *et al.* (1963b) through the ethanol precipitation step. The activity was then further purified by stepwise elution from DEAE-cellulose, as described above for the *E. coli* sRNA methylases, and precipitation with ammonium sulfate at 75% saturation.

sRNA of *E. coli* was prepared by the method of von Ehrenstein and Lipmann (1961) with the following modifications. The cells were extracted with phenol for only 5 min. After 1 M NaCl fractionation, the sRNA was reprecipitated with ethanol, treated with 0.1 M Tris–HCl, pH 9, for 45 min at 37° to remove amino acids, and then, after pH adjustment to 7 and addition of $MgCl_2$ to 0.01 M, exposed to 2 μg/ml of DNase (Worthington, electrophoretically pure) for 30 min at 25°. The sRNA was adsorbed to a short column of DEAE-cellulose equilibrated with 0.02 M Tris–HCl, pH 7.8. After washing with the same buffer containing 0.3 M KCl, the sRNA was eluted with buffer containing 0.6 M KCl. The sRNA was precipitated with ethanol, washed with 80 and 95% ethanol, and dried.

f2 RNA was prepared by phenol extraction of whole

virus, ethanol precipitation, and dialysis. Poly U and poly UC (1:1) were from Miles Chemical Co.; poly UA (2:1) was obtained from Dr. M. C. Ganoza. *S*-Adenosylmethionine was obtained from Sigma and the same compound, labeled in the methyl group with ^{14}C (40 mc/mmole), from New England Nuclear. L-[^{14}C]Phenylalanine (333 mc/mmole) was from the latter source; L-phenylalanine (ring-4-^{3}H, 9.6 c/mmole) was from Nuclear-Chicago. MAK, prepared according to Mandell and Hershey (1960), was a gift of Dr. Max Gottesman. DEAE-cellulose was from Calbiochem. Macaloid (Baroid Division, National Lead Co., Houston) was washed several times in 10^{-3} M Tris–HCl, pH 7.5.

Methods

Incubation mixtures for polypeptide synthesis from free amino acids (two-step system) in the presence of poly U or poly UC contained: 0.05 M Tris–HCl, from 0.01 to 0.03 M $MgCl_2$, 0.04 M β-mercaptoethanol, 0.16 M NH_4Cl, 0.004 M ATP, 0.2 mM GTP, 10 μg of poly U or 50 μg of poly UC, 0.004 mM [^{14}C]phenylalanine or other [^{14}C]amino acids, 800 μg of four-times-washed ribosomes, 50 μg of either normal or methyl-deficient sRNA, and 400 μg of supernatant factors, in a total volume of 0.25 ml at pH 7.5. Incubations were performed at 25°. Radioactivity incorporated into protein was determined after depositing the hot TCA-insoluble material on Millipore filters (Conway, 1964). For the experiments portrayed in Table I, 80 μg of supernatant factors, 50 μg of ribosomes, and purified sRNA fractions (*cf.* text) were used, and the temperature of incubation was 30 instead of 25°.

With f2 RNA as a messenger, the same supernatant factors were used with preincubated ribosomes, but the other components were adjusted to the conditions of Nathans *et al.* (1962). A small quantity of [^{12}C]-methionyl-sRNA was included in place of methionine to minimize methylation of the added sRNA.

Assays of phenylalanine transfer from charged sRNA into polypeptide in the presence of poly U (one-step system) were carried out in a volume of 0.25 ml with the following components (Conway, 1964): $MgCl_2$ as indicated, 0.05 M Tris–HCl, pH 7.4, 0.006 M β-mercaptoethanol, 0.16 M NH_4Cl, 0.001 M GTP, 400 μg of ribosomes (four or five times washed), 5–10 μg of transfer factors, 5–10 μg of poly U, and the radioactively labeled phenylalanyl-sRNAs being tested. For some experiments a higher mercaptoethanol concentration was used (0.04 M). Incubations were performed at 25°. Radioactivity incorporated into polypeptide was determined as above.

Charging of sRNA fractions from CCD with phenylalanine for subsequent use in transfer and binding experiments was carried out using the partially purified phenylalanyl-sRNA synthetase described above. The charging reactions contained 0.05 M cacodylate buffer, pH 6.8, 0.016 M $MgCl_2$, 0.008 M β-mercaptoethanol, 0.008 M ATP, 3 mμmoles of [^{3}H]- or [^{14}C]phenylalanine, 400 μg of serum albumin, 200 μg of synthetase, and 0.5 mμmole of sRNA (in terms of phenylalanine-acceptor sRNA), in a volume of 2 ml. Incubations were performed at 30°. When sampling (*cf.* below) indicated that the sRNA was fully charged (at 30 min), an equal volume of water-saturated phenol was added to the main reaction mixture. After shaking in the cold for 10 min and centrifuging to separate the phases, the aqueous phase was extracted several times with ether. Extensive dialysis was then carried out against deionized water, and the samples were finally stored frozen.

Routine assays for phenylalanine activation were carried out in a system buffered by 0.2 M Tris–HCl, pH 7.4, with the same additional components listed above, except that serum albumin was omitted and the enzyme source was the preparation of supernatant factors for *in vitro* protein synthesis from free amino acids. This system was used for determining the phenylalanine acceptance profile in the CCD analysis of methyl-deficient sRNA. Incorporation of [^{14}C]phenylalanine in the various samples was determined after stopping the reaction with a large excess of 5% TCA and collecting and washing the precipitates on Millipore filters.

Methylation of sRNA by *E. coli* enzymes and subsequent charging with phenylalanine was done with the *E. coli* sRNA methylase preparation, using the milieu of the enzyme preparation itself for the methylation

TABLE I: Specific Stimulation by sRNA Fractions of Phenylalanine Incorporation Coded for by Poly U or Poly UC.[a]

	Phe-sRNA I (cpm)	Phe-sRNA II (cpm)	Phe-sRNA III (cpm)	Phe-sRNA IV (cpm)
Poly U (45 min)	745	830	880	970
Poly UC (45 min)	118	101	114	127
Poly U (10 min)	745	750	810	860
Poly UC (10 min)	45	44	38	46

[a] The incorporations stimulated by addition of individual phenylalanine-specific sRNA fractions to an *in vitro* protein-synthesizing system dependent on either poly U or poly UC. The incorporation of [^{14}C]-phenylalanine into peptide linkage is expressed as counts per minute per micromicromole of phenylalanine-specific sRNA added to the reaction mixture in the range (0–3 $\mu\mu$moles) where the total incorporation is a linear function of the specific sRNA concentration. The magnesium concentration was 20 mM. Incorporation without added sRNA at 45 min was 590 cpm for poly U and 210 cpm for poly UC; at 10 min the values were 495 and 100 cpm, respectively. In the absence of added messenger and in the presence of 3 $\mu\mu$moles of phenylalanine-specific sRNA (normal), the system incorporated 45 and 101 cpm in 10 and 45 min.

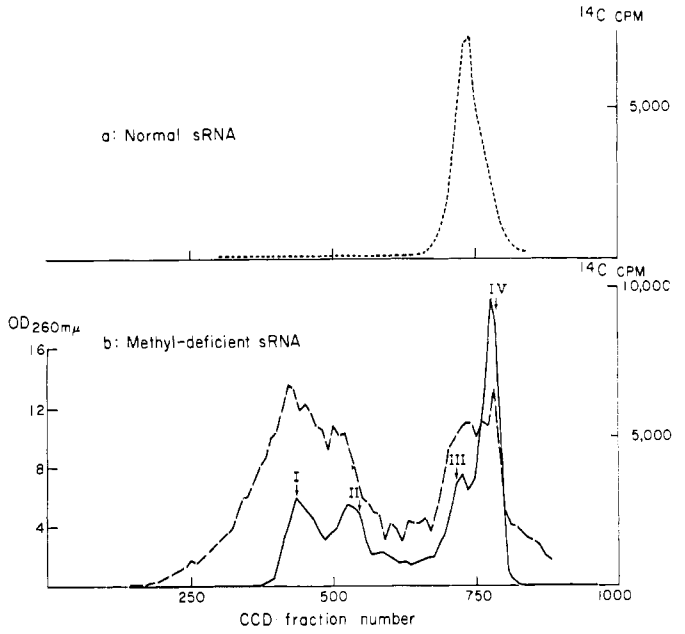

FIGURE 1: CCD separation of novel phenylalanine-acceptor species in methyl-deficient sRNA. In a is shown the profile of phenylalanine acceptance in a distribution of normal sRNA (J. Goldstein and E. Fleissner, unpublished data). In b, the optical density profile is indicated for the dialyzed fractions of methyl-deficient sRNA by the dashed line and the phenylalanine acceptance by the solid line. The roman numerals indicate fractions used for subsequent experiments.

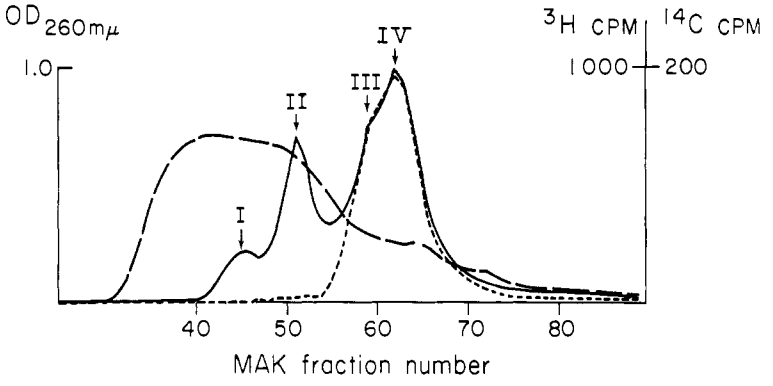

FIGURE 2: MAK chromatography of [^{3}H]phenylalanyl-sRNA (methyl-deficient) and [^{14}C]phenylalanyl-sRNA (normal). The solid line represents ^{3}H and the dotted line ^{14}C. The optical density profile (dashed line) is due largely to 2 mg of carrier methyl-deficient sRNA. The Roman numerals denote positions in MAK chromatography of the CCD peak fractions of Figure 1.

step. [^{14}C]Methyl-labeled *S*-adenosylmethionine was added in a small volume to a concentration of 0.075 mM. Of the appropriate sRNA fraction, 100 μg was added in a small volume to make a final reaction volume of 1 ml. After 60 min at 30°, when the plateau in the methylation reaction was reached, 8 μmoles of ATP, 10 μmoles of $MgCl_2$, and 30 μc of [^{3}H]phenylalanine in a volume of 0.12 ml were introduced, and the incubation was continued for 17 min until the sRNA was fully charged. The sRNAs were reisolated by phenol extraction, ether extraction, and dialysis, as described above.

The methylation of methyl-deficient sRNA by yeast enzymes was carried out under conditions similar to those described above for the initial reaction with the *E. coli* methylases: 0.05 M Tris–HCl, pH 7.8, 0.01 M $MgCl_2$, 0.006 M β-mercaptoethanol, 0.1 mM *S*-adenosylmethionine, 500 μg of methyl-deficient sRNA, and yeast enzymes (dialyzed ammonium sulfate fraction), 5 mg, in a volume of 1 ml. After 85 min at 30°, the methylation had reached a plateau unaffected by addition of extra enzyme or *S*-adenosylmethionine. The sRNA was reisolated by phenol extraction, alcohol

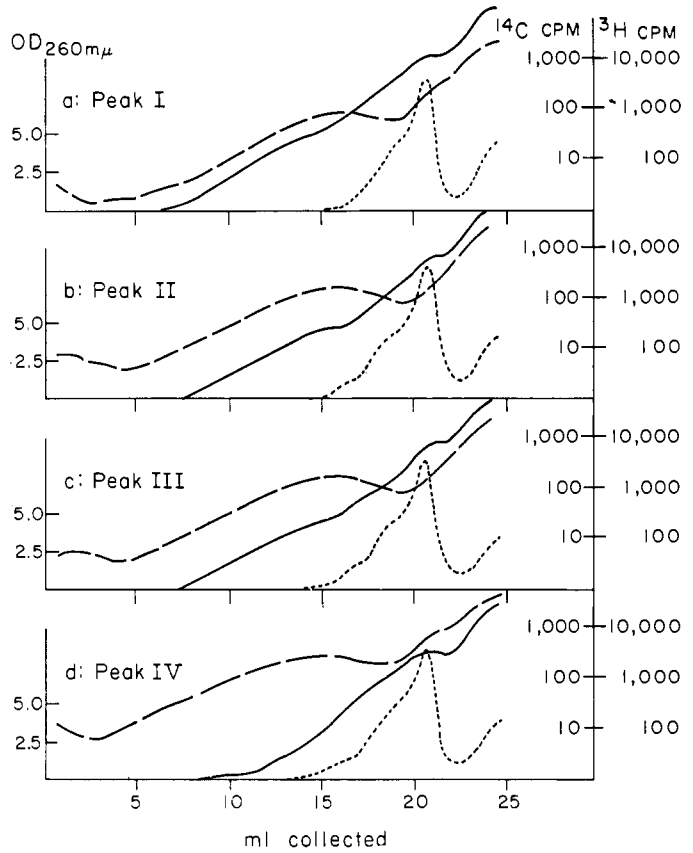

FIGURE 3: Sucrose gradient patterns obtained with ribosomes, poly U, and ^{14}C-methylated [^{3}H]phenylalanyl-sRNAs. The panels a–d are given roman numeral designations, according to the CCD peaks (I–IV) used for preparation of the labeled sRNA. Note that the radioactivity scales are logarithmic but the optical density scales are linear. The solid lines represent ^{14}C, the dashed lines ^{3}H, and the dotted lines optical density. Counts per minute added to the gradients: for peak I, 74,000 for ^{3}H and 44,000 for ^{14}C; for II, 92,500 for ^{3}H and 36,000 for ^{14}C; for III, 106,000 for ^{3}H and 38,500 for ^{14}C; and for IV, 176,000 for ^{3}H and 12,700 for ^{14}C. These values are for sRNA samples which have been saturated with [^{3}H]phenylalanine and [^{14}C]methyl groups; thus the material in peak IV is much less methyl-deficient originally than the other peak materials.

precipitation, and dialysis.

CCD (952 transfers) was performed according to Goldstein *et al.* (1964) with 1.2 g of methyl-deficient sRNA as starting material. The temperature during the run was 24°. Since methyl-deficient sRNA displayed a somewhat higher over-all partition coefficient at this temperature than normal sRNA (J. Goldstein and E. Fleissner, unpublished data), the volume of upper phase in each chamber was reduced to 2 ml, with 3 ml of lower phase. After the distribution was completed, the contents of the chambers were pooled in sets of five. The pooled fractions were extracted twice with 10 ml of ether and dialyzed for 3 days against frequent changes of deionized water. The samples were then lyophilized and dissolved in 2 ml of water. MAK chromatography as carried out as described by Sueoka and Yamane (1962).

The procedure for sucrose gradient centrifugation was essentially that of Spyrides (1964). *In vitro* methylated phenylalanyl sRNAs were incubated with 3.2 mg of five-times-washed ribosomes (preincubated at 30°) and 10 μg of poly U in 1-ml volumes containing 0.01 M Tris–HCl, pH 7.4, 0.014 M $MgCl_2$, and 0.16 M NH_4Cl. After a 20-min incubation at 25° the reaction mixture was layered on to a preformed linear gradient of 5–22% sucrose (volume 24 ml) in the same buffered salts medium. Centrifugation was carried out for 2 hr at 24,000 rpm in the SW-25 rotor. Samples were dissolved in Bray's solution (Bray, 1960) for radioactivity determinations. The sucrose solutions used for making the gradients were pretreated with Macaloid at a concentration of 100 μg/ml to remove possible nuclease contamination; the Macaloid was subsequently removed by centrifugation.

Binding experiments were carried out according to Nirenberg and Leder (1964), except that a magnesium concentration of 0.014 M was used so as to approach as closely as possible to the optimum for the transfer reaction. (Five-times-washed ribosomes used in the binding experiments were found to have a higher

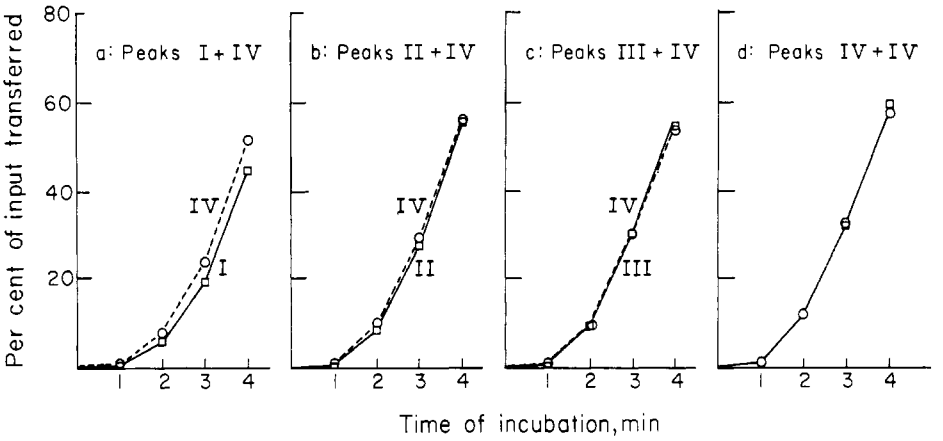

FIGURE 4: Transfer of phenylalanine from CCD sRNA fractions into polyphenylalanine. Peak IV sRNA charged with phenylalanine was mixed in successive experiments (a–d) with peaks I–IV similarly charged, as indicated in the heading for each panel. The different sRNAs carried differently labeled (^{3}H or ^{14}C) phenylalanine in each case, so that panel d represents an internal control on the double-counting procedure. The curves in a–c represent the combined results of experiments in which the reciprocal combinations of isotopes were used with the same pair of sRNA fractions. Dotted lines and circles represent peak IV values, and solid lines and squares the values for peaks being compared with IV. Counts per minute of ^{3}H added were: 9489 for peak I, 8575 for II, 9624 for III, and 11,207 for IV. Counts per minute of ^{14}C added were: 1028 for I, 999 for II, 994 for III, and 1320 for IV. Five-times-washed ribosomes at 0.011 M $MgCl_2$.

magnesium optimum (from 0.012 to 0.014 M) in the transfer reaction than ribosomes that had not been washed in 0.5 M NH_4Cl.

Radioactivity measurements were made in a scintillation counter.

Results

Preliminary Experiments. The principal results discussed in this paper are derived from experiments with RNA fractionated by CCD so as to separate submethylated and normal components. Nevertheless, at the start of this investigation it was necessary to examine the possibility that the absence of methylation, or improper methylation by heterologous enzymes, might produce frequent and radical alterations in sRNA function, detectable in experiments with crude sRNA. This possibility was virtually excluded by three sets of results.

(1) In an *in vitro* system with f2 RNA as messenger, the rate of protein synthesis (when dependent on sRNA concentration) was insensitive to the replacement of normal sRNA by methyl-deficient sRNA. In the latter instance, the product was normal viral coat protein, as judged by the fingerprint technique (Nathans *et al.*, 1962).

(2) A second approach was suggested by the well-documented species specificity of sRNA methylating enzymes (Svensson *et al.*, 1963a; Srinivasan and Borek, 1963; Gold *et al.*, 1963). It was found that the rate of f2-RNA-directed protein synthesis *in vitro* showed the same dependence on sRNA concentration for two samples of sRNA: (a) methyl-deficient sRNA, which had accepted an average of 0.7 methyl group/molecule from a preparation of yeast sRNA methylases, and (b) methyl-deficient sRNA that had been exposed to the same enzymes in the absence of the methyl donor, *S*-adenosylmethionine. Thus, interspecies methylation produced no kinetically discernible effect on the ability of *E. coli* sRNA to function with a natural messenger in a system of *E. coli* activating enzymes, transfer enzymes, and ribosomes.

(3) Normal and methyl-deficient sRNAs were used to stimulate the rate of *in vitro* polypeptide synthesis directed by poly U and poly UC (1:1) in the presence of labeled amino acids for which these polymers would code only ambiguously (leucine, serine, valine, and tyrosine in the case of poly U, valine and threonine in the case of poly UC). In order to prejudice the system toward possible miscoding, low temperature (25°) and a variety of magnesium concentrations (10–30 mM) were used (Szer and Ochoa, 1964; Davies *et al.*, 1964; Friedman and Weinstein, 1964), and the concentrations of correctly coded amino acids were reduced to as low a level as possible (Nirenberg and Jones, 1963). No significant enhancement in ambiguity was observed with the use of methyl-deficient sRNA in place of normal sRNA.

Physical Separation of Submethylated and Normal Phenylalanine-Specific sRNAs. These preliminary findings pointed to the need for more subtle comparisons of normal and submethylated sRNA. Such comparisons became possible through CCD separation of a specific amino acid acceptor species of submethylated sRNA from its normal counterpart in the methyl-deficient sRNA preparation. The CCD experiments were initiated in collaboration with Dr. Jack Goldstein. It was found that though most of the submethylated species of methyl-deficient sRNA have distribution patterns very

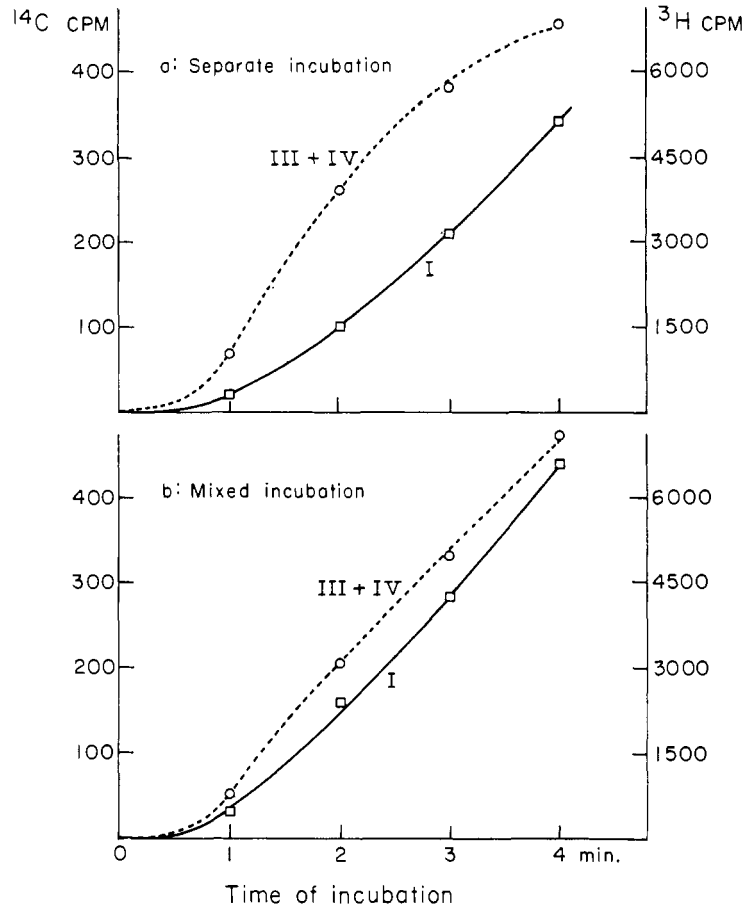

FIGURE 5: Transfer of phenylalanine: the effect of mixing phenylalanyl-sRNA fractions. (a) Kinetic curves for [^{14}C]-phenylalanyl-sRNA (peak I) and [^{3}H]phenylalanyl-sRNA (peaks III and IV) in separate incubations. (b) The same determined in incubations with the phenylalanyl-sRNA fractions mixed. The solid lines represent ^{14}C and the dotted lines ^{3}H. The ordinates are adjusted to represent equimolar amounts of [^{14}C]- and [^{3}H]phenylalanine. Counts per minute added were: 770 for ^{14}C and 11,800 for ^{3}H. Four-times-washed ribosomes at 0.008 M $MgCl_2$.

similar to or identical with those of their normal sRNA counterparts in CCD, new peaks of phenylalanine acceptor sRNA appear which are absent in the normal sRNA distribution (J. Goldstein and E. Fleissner, unpublished data). This observation was initially made in the laboratory of Dr. E. B. Keller (personal communication).

Figure 1b shows the profiles of optical density and phenylalanine acceptance in a CCD experiment with methyl-deficient sRNA. The position of the phenylalanine species in a similar distribution of normal sRNA is also indicated (Figure 1a). Peaks I and II of phenylalanine acceptor activity in Figure 1b are unique to methyl-deficient sRNA. Peaks III and IV are in the position of the single, somewhat asymmetric peak for phenylalanine acceptance in the normal sRNA preparation. Since it is difficult to duplicate precisely the conditions in two separate CCD experiments, it cannot be stated from this evidence whether both peak III and peak IV materials are or are not present in normal sRNA. The evidence from MAK chromatography (*cf.* below) is that they are. Moreover, it has been observed that under some conditions in CCD, peaks III and IV of methyl-deficient sRNA appear to coalesce into a single peak such as that of Figure 1a (J. Goldstein and E. Fleissner, unpublished data).

When methyl-deficient sRNA charged with [^{3}H]-phenylalanine is subjected to chromatography on MAK together with normal sRNA charged with [^{14}C]-phenylalanine, the elution pattern shown in Figure 2 is observed. Again there are additional peaks of phenylalanine-specific sRNA in the methyl-deficient material, and if allowance is made for the crowding of the pattern in MAK chromatography as compared with CCD, the patterns with the two methods of separation appear very similar. In fact, when materials from the four peak regions in CCD were charged with phenylalanine and analyzed on MAK, a one-to-one correspondence of CCD and MAK peaks was established, as indicated by the numbering of the peaks in Figure 2. The asymmetry of the normal phenylalanyl-sRNA peak in Figure 2 demonstrates that material

corresponding to peak III (a shoulder in this case) is present in normal sRNA. These results confirm the reports of Littauer and his collaborators (Revel and Littauer, 1965; Littauer *et al.*, 1966), who have reported the separation of multiple peaks of phenylalanyl-sRNA by chromatography of methyl-deficient sRNA on MAK and more recently on methylated albumin–silicic acid. In their studies too, two peaks were assigned to normal phenylalanyl-sRNAs.

Evidence for Submethylation of Phenylalanine-Specific sRNAs. If the four peak fractions from CCD are methylated *in vitro* with *S*-adenosyl[methyl-^{14}C]-methionine and then charged with [^{3}H]phenylalanine, it is possible to carry out binding experiments with ribosomes and poly U in order to find out which fractions were originally submethylated. The results of such experiments analyzed by the sucrose gradient procedure (Nakamoto *et al.*, 1963) are given in Figure 3. (Binding experiments with the technique of Nirenberg and Leder (1964) were inconclusive owing to high background binding of ^{14}C-methylated sRNA in the absence of poly U.) Note that the radioactivity scales are both logarithmic, whereas the optical density scale is linear. Differences in the amounts of the two isotopes added to the different incubation mixtures before sucrose gradient centrifugation were necessitated by the different specific incorporations of [^{14}C]methyl groups and [^{3}H]-phenylalanine into the CCD fractions (*cf.* legend to Figure 3 and Methods). On the logarithmic scale a constant distance between the ^{3}H and ^{14}C curves for a given sample demonstrates a constant ratio of the two isotopes over that part of the gradient, and the distance is a measure of the ratio. The polysome regions show ^{14}C binding, which is in a constant ratio to ^{3}H for sRNAs of peaks I, II, and III, but not for peak IV sRNA. Peak I sRNA appears to have the most $^{14}CH_3$, *i.e.*, to have been originally the most submethylated. From the absolute efficiencies of counting the two isotopes it can be calculated that each molecule of peak I phenylalanyl-sRNA has accepted two methyl groups. The same calculation yields an average of 0.55 group/molecule for peak II sRNA, 0.45 group for peak III, and 0.05 or less for peak IV. The CCD profile of Figure 1 shows an apparent equilibrium between peak II and peak III sRNAs, since the spread of peak II to the right is broader than would be theoretically predicted for a peak in that position. Thus, the methyl acceptance on the part of peak III material may represent the appearance in the position of peak III of partially submethylated molecules which also appear (with a different conformation) in peak II.

Lower Efficiency of Submethylated Phenylalanyl-sRNA Compared with Normal Phenylalanyl-sRNA in the Transfer Reaction. Preliminary experiments with unfractionated sRNA showed that both methyl-deficient sRNA and normal sRNA could transfer into polyphenylalanine 95–100% of the phenylalanine introduced into the transfer system as phenylalanyl-sRNA. Apparently all the sRNA molecules in the methyl-deficient preparation that could activate phenylalanine could also transfer the activated amino acid in the presence of poly U. The unfractionated methyl-deficient sRNA did not display any detectable abnormality in the initial kinetics of the transfer reaction under optimal conditions. Elimination of the kinetic lag by preincubation of nonenzymic components (Conway, 1964) proceeded normally with the unfractionated methyl-deficient sRNA. Owing to the normal sRNA component in methyl-deficient sRNA these results were not considered conclusive, and following the CCD fractionation of this sRNA a new investigation of its role in the transfer reaction was undertaken.

The transfer reaction was studied using the material of the four CCD peaks charged with ^{3}H- or ^{14}C-labeled phenylalanine. The peak IV sRNA was used as a reference against which to compare the other peaks. The reference sRNA and the sRNA being tested, both charged with phenylalanine, were placed in the same incubation mixture with washed ribosomes, transfer factors, and poly U, plus other requirements of the transfer reaction (see Methods). In each case, one of the sRNAs was charged with [^{3}H]phenylalanine and the other with [^{14}C]phenylalanine. The reciprocal isotope combination with the same sRNA fractions was always tested at the same time. Good agreement between results of the reciprocal incubations was obtained in every case, ruling out artifacts due to double-isotope counting. The incorporations in Figure 4 are presented as percentages of the inputs of each isotope found in the polyphenylalanine product, thus normalizing for any differences in the ratio of counts added. At this sRNA concentration the polymerization rate is dependent on the concentration of phenylalanyl-sRNA. The counts added were such as to make each phenylalanyl-sRNA species approximately equimolar. In Figure 4a–c, the curve for each sRNA species is the average of the pair of curves obtained from the incubations with reciprocal patterns of isotopic labeling. The last panel (Figure 4d) shows the degree of agreement obtained with one such pair, that for peak IV sRNA charged with [^{3}H]phenylalanine and the same sRNA charged with [^{14}C]phenylalanine.

The results in Figure 4 indicate a small, but significant, difference between peak I and peak IV sRNAs in the transfer reaction. There is some indication of a difference between peak II and peak IV sRNAs also, but the difference in this case is at the limit of resolution possible with this experimental procedure. The difference between peak I and peak IV sRNAs is unequivocal, and since it is obtained by mixing both phenylalanyl-sRNA fractions, differentially labeled, in the same incubation, there can be no question of a fortuitous inhibitor in the peak I fraction, unless it is strongly bound to the phenylalanyl-sRNA.

In Figure 5 is shown a comparison of peak I sRNA with peaks III and IV. The peak sRNA fractions were obtained from a CCD of methyl-deficient sRNA in which there was no separation of peaks III and IV. Figure 5a shows the results of a kinetic experiment with either peak I sRNA or peaks III and IV as exclusive phenylalanine donors. Figure 5b shows the results of mixing both phenylalanyl-sRNA preparations, dif-

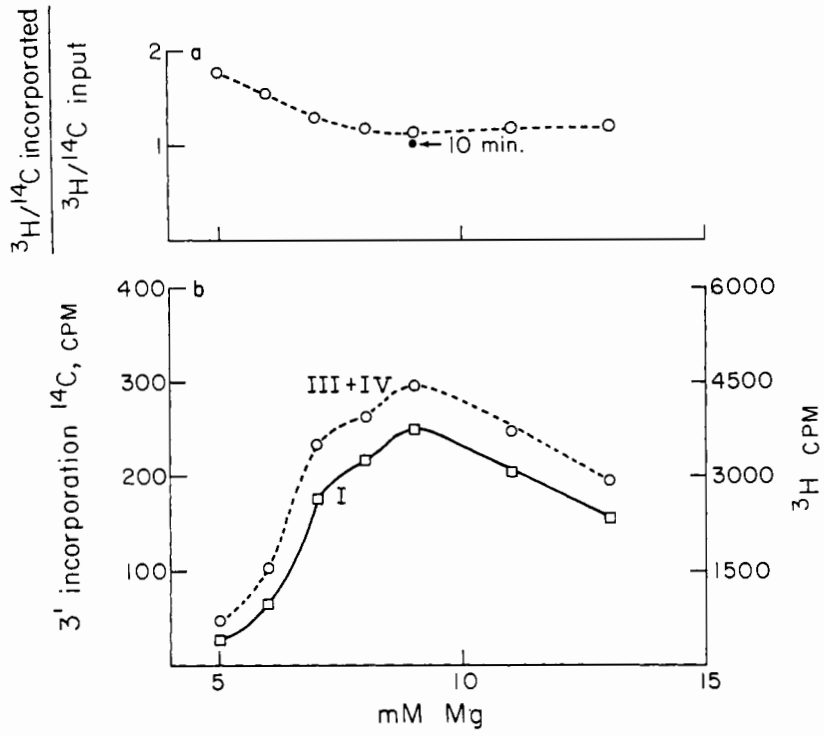

FIGURE 6: The magnesium concentration dependence of phenylalanine transfer from a mixture of [^{14}C]phenylalanyl-sRNA (peak I), represented by the solid line in part b, and [^{3}H]phenylalanyl-sRNA (peaks III and IV), represented by the dotted line in b. Conditions as in Figure 5, with an incubation time of 3 min. In part a is plotted the relative incorporation of the two isotopes corrected for their input ratio. The closed circle represents the ratio obtained if the incubation at that magnesium concentration is permitted to proceed for 10 min.

ferentially labeled, in the same tube, as was the case for the experiments of Figure 4. The total incorporation of phenylalanine into polypeptide is doubled, since the concentration of phenylalanyl-sRNA is rate limiting in the reaction. Notice that peak I sRNA by itself is a somewhat poorer donor in the transfer reaction than when mixed with peaks III and IV. This is not surprising if we imagine that, in these nonpreincubated reactions, chain starting through binding of phenylalanyl-sRNA is a slow step. When the normal sRNAs are present, they can start chains for peak I sRNA.

Figure 6 demonstrates that the difference in functioning of peak I sRNA compared with peaks III and IV can be increased if the magnesium concentration is lowered below the optimum for the poly U system. As over-all incorporation decreases with decreasing magnesium concentration, the ability of peak I sRNA to function, even in the presence of the normal peaks III and IV, is reduced in comparison with the normal peaks, as shown in Figure 6a. The same effect of magnesium concentration can be demonstrated in a "two-step" *in vitro* system incorporating free phenylalanine into polyphenylalanine.

Similarity in Coding Properties between Submethylated and Normal Phenylalanine-Specific sRNAs. The system developed for *in vitro* protein synthesis from free amino acids (two-step system) was used to test the peak CCD fractions for efficiency of transfer with poly UC as compared with poly U. With poly U the kinetics were linear for about 10 min; with poly UC, linear kinetics were obtained for 30 min. As shown in Table I, stimulation of the rates of incorporation by the added sRNA fractions did not reveal evidence for extra ambiguity (response to poly UC) specifically introduced by the submethylated peaks. It should be noted that the error level in this type of experiment is as great as 10%. There are some differences in the facility with which the peaks support phenylalanine incorporation in the presence of poly U. These differences reflect the differences in transfer efficiency which have already been discussed above in connection with the "one-step" system.

A more stringent test for ambiguity was possible using the binding assay of Nirenberg and Leder (1964). Reciprocal combinations of [^{3}H]phenylalanyl- and [^{14}C]-phenylalanyl-sRNAs prepared from the CCD fractions were used as in the transfer experiments of Figure 4. In Figure 7 each curve except the one in the fourth (d) panel of each set is really the average of two curves, which, in general, agree as closely as the two sets of points in the fourth panels indicate for the peak IV sRNA plus peak IV combination. Evidently peak I phenylalanyl-sRNA binds far less well to the three polymers tested than the other three peaks, but the

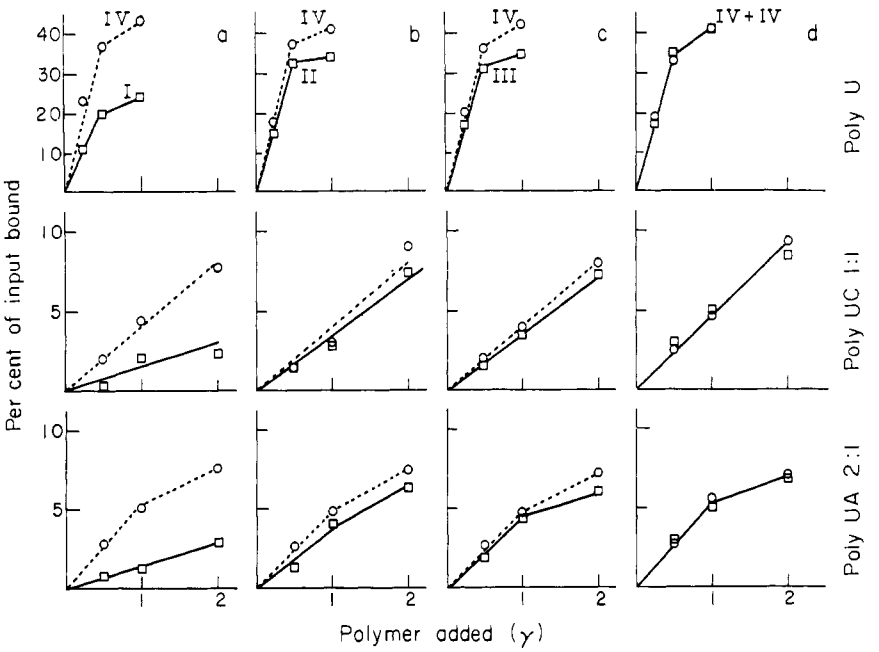

FIGURE 7: Millipore filter binding assays with phenylalanine-specific sRNA fractions. Results are plotted as a function of polymer concentration for three different polymers, which are designated on the right of each set of experiments. Pairs of charged peak sRNAs designated by roman numerals in the first row of figures were mixed in the assays as in the transfer experiment of Figure 4. Dotted lines and circles represent values for peak IV sRNA, and solid lines and squares the values for fractions being compared with IV. As in Figure 4, the results plotted in a–c are averages of two experiments in which reciprocal patterns of isotopic labeling were used. The results in the fourth panel (d) of each set show the agreement obtained using peak IV sRNA charged with both [^{3}H]- and [^{14}C]phenylalanine. Inputs of each isotope were one-fifth of the values reported in the legend to Figure 4.

deficiency in binding is the same with the three polymers. Thus, there is no evidence for more ambiguous codon recognition by this submethylated sRNA. sRNAs of peaks II and III are also somewhat less well bound than peak IV sRNA, in agreement with their partial submethylation as indicated by the sucrose-gradient experiment of Figure 3. With peak II and peak III sRNAs, the decreased binding efficiency is also identical with the three polymers tested; again, there is no evidence for increased ambiguity.

In summary, there is a decreased efficiency of transfer correlated with a decreased binding to messenger on the part of the submethylated species of phenylalanine-acceptor sRNA compared with the normal species. There do not seem to be changes in the coding properties of these sRNA species as a consequence of submethylation.

Discussion

In discussing the possible changes in sRNA function as a result of methylation, it is useful to distinguish two orders of effects. (1) There may be large-scale effects, such as inability of an sRNA molecule to function in protein synthesis without proper methylation, or radical changes in coding properties. (2) There may be marginal effects on either efficiency or fidelity. The first alternative has been investigated by Littauer *et al.* (1963), Peterkofsky *et al.* (1964), and Starr (1963) for the case of poly U stimulated incorporation of phenylalanine. These workers reached the conclusion that gross differences due to methylation are not found. The same workers found, in agreement with Svensson *et al.* (1963b) and Neidhardt and Eidlic (1963), that large effects on amino acid activation were not discernible. The preliminary experiments reported here with natural and artificial messengers are also inconsistent with the first alternative cited above.

The principal evidence presented in this paper supports the second alternative: an improved efficiency in the transfer reaction is correlated with normal methylation of phenylalanine-specific sRNA, especially at lower magnesium concentrations (Figures 4–6). Littauer *et al.* (1963) also concluded that submethylated phenylalanine-specific sRNA was somewhat less efficient in protein synthesis than normal sRNA, but this conclusion was later questioned by Littauer and Milbauer (1965). No evidence was found in the present work for an effect of methylation on the coding properties of the phenylalanine sRNA species (Table I and Figure 7). The latter result differs from the results of Revel and Littauer (1966), and the reason for this difference is not clear. However, these workers used sRNA fractions reisolated after MAK chromatog-

raphy, whereas in the present study sRNA fractions from CCD were used for biological assays. Peterkofsky *et al.* (1966) have reported that the degree of methylation of leucine-specific sRNA of *E. coli* determines the reactivity of the sRNA with normal leucine codons. In the case of sRNAs specific for leucine and serine, for which the number of corresponding codons is relatively large, methylation may play a special role in fixing codon recognition.

The differences in efficiency between submethylated and normally methylated sRNAs specific for phenylalanine reported here are not large, but *in vivo* they could provide an extra margin of efficiency that would justify the retention of the methylation system in the evolutionary process. Moreover, it is possible that the relatively larger differences, approaching a factor of 2, in the efficiencies of the two types of sRNA at lower magnesium concentrations (Figure 6) are more representative of the *in vivo* situation. There are indications that the conditions that are optimal for the poly U directed synthesis of polyphenylalanine are not the optimal conditions found *in vivo*. Under optimal conditions in the poly U system, chain starting appears to be the rate-limiting step. If *N*-formylmethionine is the natural chain starter in *E. coli*, then the initiation of peptide chains *in vitro* with other amino acids could require special conditions such as high magnesium. Sundararajan and Thach (1966) have reported that the specificity for *N*-formylmethionyl-sRNA as a "phase selector" is markedly reduced at higher magnesium concentrations, and is greatest at concentrations below 10 mM. Nakamoto and Kolakofsky (1966) have found that the requirement for *N*-formylmethionine as a chain initiator *in vitro* is most stringent at magnesium concentrations below the optimum for the poly U system. These considerations point to an *in vivo* situation where the free magnesium concentration is less than the optimum value for polyphenylalanine synthesis *in vitro*.

A difficulty in the present investigation is the lack of any proof that the abnormalities exhibited by submethylated phenylalanine-specific sRNA, both in its physical properties and in its biological functioning, are due to submethylation alone. One possible basis of the separations observed in CCD can be ruled out: that any one of the phenylalanine species is lacking part or all of a CCA end. The rates of phenylalanine activation with the isolated peak materials were similar, and charging proceeded rapidly under conditions where the activity of the CCA-addition enzyme was negligible in the preparations used for charging. Yet the RNA may possess some other, unrecognized defect. The failure to alter markedly either the physical or the biological properties of the submethylated phenylalanine-specific sRNA by *in vitro* methylation (E. Fleissner, unpublished data) would appear to support such a contention. Two alternative explanations exist, however. (1) The *in vitro* methylation might not be complete, since it is known that some methylated bases found in normal sRNA are not formed by the *in vitro* enzyme system (Fleissner and Borek, 1963; Hurwitz *et al.*, 1964). Saturation methylation of the submethylated phenylalanine-specific sRNA is not necessary if the objective is merely to use acceptance of radioactive methyl groups as a criterion of prior submethylation, as was done in the sucrose gradient experiments. (2) Even if the sRNA were completely methylated, specific renaturing conditions could be required to establish the "native" conformation of the molecule (Lindahl *et al.*, 1966).

In fact, the unusual behavior of certain submethylated sRNA molecules as reported in this paper may be evidence that these molecules are partially "denatured." Multiple chromatographic species of normal phenylalanine-specific sRNA have been reported (Littauer *et al.*, 1966; Muench and Berg, 1966). Such behavior is probably due to conformational changes. Thus, if normal sRNA can undergo such changes, it is possible that the absence of methylation confers on sRNA increased propensities to adopt unusual conformations. The hypochromic shift in absorbancy associated with methylation of sRNA as reported by Borek and Christman (1965) is consistent with this hypothesis. These unusual conformations available to submethylated sRNA might then explain its abnormal behavior in protein synthetic systems.

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