THE CHARACTERIZATION OF A NEW SPECIES OF LEUCYL-sRNA FORMED DURING METHIONINE DEPRIVATION OF ESCHERICHIA COLI WITH RELAXED CONTROL

By Robert A. Lazzarini and Alan Peterkofsky

NATIONAL INSTITUTE OF DENTAL RESEARCH, NATIONAL INSTITUTES OF HEALTH

Communicated by Fritz Lipmann, January 28, 1965

Most amino acid auxotrophs of *Escherichia coli* synthesize RNA at a significant rate only when the essential amino acid is supplied and, therefore, when the cells are capable of net protein synthesis.[1] However, Borek *et al.*[2] have shown that a particular methionine auxotroph of *E. coli* (58-161) is capable of net RNA synthesis during periods of methionine deprivation and differs from other auxotrophs in that the control of RNA synthesis is "relaxed" rather than "stringent." Subsequent genetic analysis of this mutant[3, 4] has shown that it contains at least two genetic lesions, one related to the methionine requirement, the other to the control of RNA synthesis.

Investigations of the nature of the RNA formed during methionine deprivation of the relaxed control mutant have indicated that the sRNA does not contain the full complement of methylated purines and pyrimidines,[5, 6] which are primarily localized in sRNA. The evidence accumulated to date indicates that despite this deficiency the sRNA formed during methionine starvation can be acylated with amino acids in reactions catalyzed by the homologous amino acid activating enzymes.[7–9] Furthermore, methyl-deficient sRNA species active as a substrate for one amino acid activating enzyme are not substrates for other activating enzymes,[9] indicating that the amino acid activating enzymes exhibit the same high degree of substrate specificity with methyl-deficient sRNA that they exhibit with normal sRNA.

In a previous publication[10] it was shown that sRNA obtained from *E. coli* after methionine deprivation was a poor substrate for the leucine activating enzyme from yeast as compared to sRNA from normal *E. coli* cells. This change was attributed to the formation during methionine starvation of a species of sRNA which was a competent acceptor for leucine with the *E. coli* enzyme but not with the yeast enzyme. This proposal is supported by the fact that the elution profiles from methylated albumin columns (MAK) of leucyl-sRNA from normal and methyl-deficient sRNA were strikingly different and indicated that a new species of leucine acceptor sRNA appeared during methionine deprivation. In the present communication, evidence is presented indicating that the new chromatographic component is a methyl-deficient species of leucyl-sRNA. This new component accumulates during methionine deprivation but not during leucine deprivation of a relaxed control mutant auxotrophic for both of these amino acids. Under conditions identical to those leading to the accumulation of the new component in the relaxed control mutant, a methionine auxotroph of *E. coli* with stringent control does not accumulate it. Furthermore, its accumulation during methionine deprivation parallels the increased ability of the sRNA to accept methyl groups from S-adenosylmethionine in the RNA methylase reaction.

Materials and Methods.—Organisms: Cultures of the relaxed control methionine auxotroph

58-161 (RC^{rel} Met^-), a methionine-leucine double auxotroph W-1305 (RC^{rel} Met^-Leu^-), and the stringent control methionine auxotroph 58-161 archtype (RC^{str} $Met^-Biotin^-$) were kindly supplied by Dr. Gunther Stent.

Cells of *E. coli* B were used for the preparation of leucyl-sRNA synthetase and RNA methylase.[11]

Culture conditions: *E. coli* was cultured aerobically at 37° in minimal medium containing glucose (0.3%) and the necessary amino acids (25 μg/ml). Cells were harvested at mid-log phase and washed with minimal medium. Aliquots of the washed cells to be used in the preparation of normal sRNA were frozen, and the balance of the cells was dispensed into fresh medium lacking either methionine or leucine to yield the same cell density as the original culture. Incubations in the deficient media routinely were carried out aerobically for 4 hr. The starved cells were harvested, washed as before, and stored frozen.

Preparation of sRNA: Soluble RNA was prepared from washed frozen cells by the method of Fleissner and Borek[11] except that the buffer used to extract the alumina-ground cells contained 0.01 *M* $MgCl_2$.

Purification of leucyl-sRNA synthetase: The initial steps in the purification of leucyl-sRNA synthetase were identical to those described in a previous publication[12] for the purification of glutamyl-sRNA synthetase (method B). Frozen *E. coli* cells (100 gm) were carried through the stage of the calcium phosphate gel supernatant and then applied to a DEAE-cellulose column (3 × 20 cm) previously equilibrated with 0.01 *M* potassium phosphate, pH 7.5, containing 0.003 *M* mercaptoethanol. The enzyme was eluted from the column with a linear gradient between 750 ml of 0.01 *M* potassium phosphate, pH 7.5, containing 0.003 *M* mercaptoethanol and 750 ml of 0.25 *M* potassium phosphate, pH 6.5, containing 0.003 *M* mercaptoethanol. The fractions containing significant leucyl-sRNA synthetase activity (1350–1500 ml effluent volume) were combined, dialyzed against 0.002 *M* Tris, pH 7.5 (vol: 120 ml), and concentrated by lyophilization to 12 ml. The enzyme was stored in small aliquots under liquid nitrogen. This procedure yielded a preparation of leucyl-sRNA synthetase that was approximately 50–100-fold purified, and was sufficiently free of nuclease activity so that prolonged incubation during the enzymatic incorporation of C^{14}-leucine into sRNA did not lead to a decrease in the TCA-precipitable radioactivity.

Preparation of C^{14}-leucyl-sRNA: Radioactive leucine was incorporated into the sRNA enzymatically using *E. coli* leucyl-sRNA synthetase. The incubation mixture contained in 2.5 ml: potassium cacodylate, pH 7.0, 80 μmoles; $MgCl_2$, 50 μmoles; ATP (sodium salt), 25 μmoles; glutathione (sodium salt), 50 μmoles; C^{14}-leucine (1.5×10^7 cpm/μmole), 0.03 μmoles; sRNA, 2–4 mg; and an amount of enzyme calculated to yield maximal incorporation of the amino acid in 30 min at 37°. The course of the reaction was followed by withdrawing 0.1-ml aliquots of the reaction mixture at 15-min intervals and measuring the radioactivity precipitated with trichloroacetic acid. When maximal incorporation was reached, the sRNA was precipitated from the balance of the reaction mixture by adding 0.1 vol of 5 *M* NaCl and 2 vol of cold ethanol, separated by centrifugation and dissolved in 1–2 ml of 0.5 *M* potassium succinate, pH 6.0. The resulting C^{14}-leucyl-sRNA solution was passed through a column of Sephadex G-25 equilibrated with 0.005 *M* potassium succinate pH 6.0.

Chromatography on methylated albumin-kieselguhr (MAK) columns: MAK columns were prepared according to the method of Mandell and Hershey,[13] with the exception that celite 545 was employed as the supporting material and the buffers were adjusted to pH 6.0. The columns were equilibrated with 0.2 *M* NaCl, 0.05 *M* potassium phosphate, pH 6.0. After passage through Sephadex, the salt concentration of the leucyl-sRNA solution was adjusted approximately to that of the column by adding 0.1 vol of 2 *M* NaCl, 0.5 *M* potassium phosphate, pH 6.0. The leucyl-sRNA solution was then applied at a flow rate of 0.5–1 ml per minute. Elution of the RNA was carried out at a flow rate of 4 ml per minute with a linear gradient formed between 200 ml of 0.2 *M* NaCl, 0.05 *M* potassium phosphate, pH 6.0, and 200 ml of 2.0 *M* NaCl, 0.05 *M* potassium phosphate, pH 6.0. Two-ml fractions were collected. After the elution was complete, the column was regenerated by washing it with 250 ml of the 0.2 *M* NaCl containing 0.05 *M* potassium phosphate, pH 6.0. Using these conditions it is possible to use one column as many as 10 times without significant loss in resolution or capacity.

The absorbance at 260 mμ of each fraction was measured in a Beckman DU spectrophotometer. The radioactivity of the fraction was then determined by mixing it with 10 ml of the liquid scintilla-

tion solution described by Bray,[14] and counting in a Packard Tricarb liquid scintillation spectrometer. The scintillations of these samples as measured in the spectrometer decreased with time but reached a stable value 8 hr after the samples had been placed in the spectrometer. Control experiments indicated that the large aqueous-salt component in the scintillation mixture caused a 63% quenching. When corrected for this quenching, the recovery of counts from the MAK columns was approximately the same as the recovery of absorbance at 260 mμ, 80–105%.

Results.—The changes in total sRNA and leucyl-sRNA that accompany methionine deprivation of the relaxed control auxotroph are shown in Figure 1 and Table 1. Equal aliquots of a culture of *E. coli* 58-161 were harvested just before a period of methionine deprivation and at 2-hr intervals thereafter for the preparation of

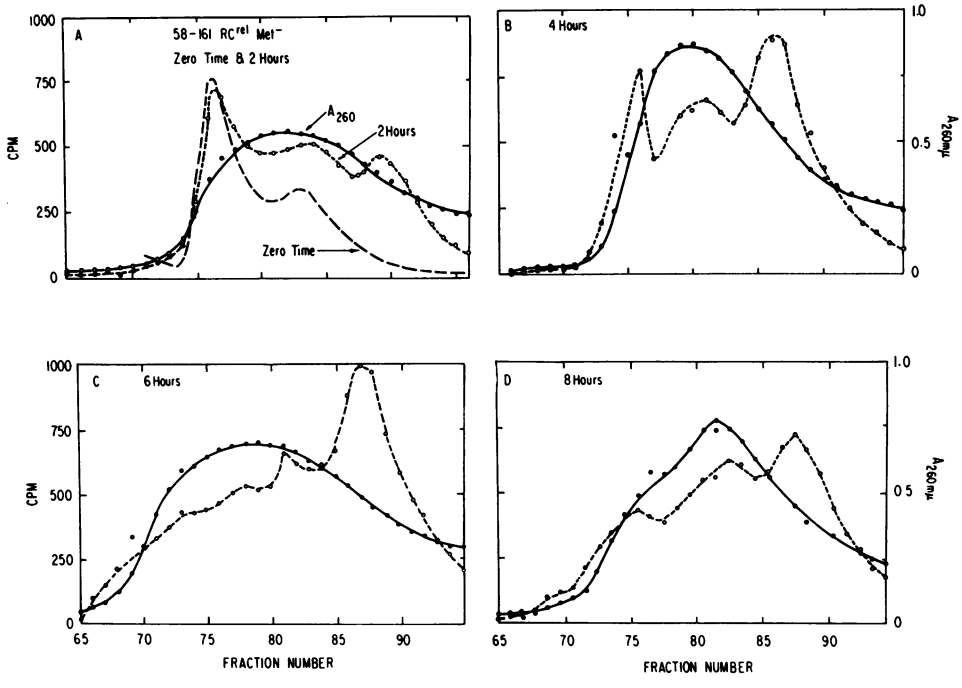

FIG. 1.—Changes in the elution profiles of leucyl-sRNA during methionine deprivation. Soluble RNA was prepared from *E. coli* cells after they had been deprived of methionine for the indicated lengths of time. The RNA was maximally charged with C^{14}-leucine (10 μc/μmole) and chromatographed on MAK columns as described in the text. RNA derived from equivalent weights of cells was applied to the columns to facilitate comparison of the profiles. Total radioactivity applied and percentage recovered after correction for quenching were 18,500 cpm, 88% for the zero time; 33,400 cpm, 82% for the 2-hr sample; 36,000 cpm, 104% for the 4-hr sample; 41,200 cpm, 105% for the 6-hr sample; and 34,700 cpm, 99% for the 8-hr sample. Open and closed symbols represent the radioactivity and the absorbance at 260 mμ, respectively, of each fraction.

sRNA. The total leucine acceptance of the sRNA prepared from methionine-starved cells was approximately twice that of the RNA from cells which were not starved (Table 1), indicating that sRNA produced during methionine starvation in this organism, although deficient in methyl groups, is biologically active.[7–9] Examination of the elution profiles (Fig. 1) from MAK columns of these sRNA samples after enzymatic acylation with C^{14}-leucine reveals some striking changes. In agreement with the studies of Sueoka and Yamane,[15] the leucyl-sRNA from the normally grown cells is resolvable into two components (Fig. 1, zero time). As

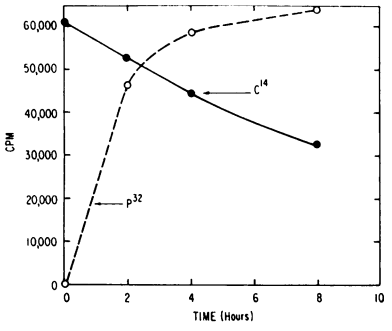

FIG. 2.—Soluble RNA turnover during methionine deprivation. *E. coli* 58–161 (RC^{rel} Met^-) grown in glucose-minimal medium containing 17 μc/liter of C^{14}-methionine was harvested and washed with minimal medium lacking methionine. Washed cells were resuspended in glucose-minimal medium lacking methionine but containing $P^{32}O_4$ (0.25 mc/liter) and incubated at 37° on a rotary shaker. At the indicated times equal aliquots of cells were withdrawn, harvested, and washed. Radiocarbon and radiophosphorus content of sRNA prepared from these cells was measured by liquid scintillation spectrometry.

previously reported,[10] the elution profile of leucyl-sRNA from methionine-starved cells shows the appearance of a third component which elutes later than the two original components. To facilitate the estimation of the changes taking place during methionine deprivation, an amount of sRNA derived from an equal weight of cells was chromatographed in each case. It can be seen in Figure 1 that the relative proportion of the third component increases gradually during the period of methionine deprivation even though the total sRNA and total leucine acceptance do not increase significantly after two hr of starvation (Table 1). This continuing increase in the third component is most easily explained on the basis of the metabolic turnover of cellular RNA during periods of amino acid deprivation previously observed by Goldstein.[6] That a similar degradation and synthesis of sRNA occurs in *E. coli* 58-161 (RC^{rel} Met^-) under conditions identical to those employed in the previous experiment is shown in Figure 2. *E. coli* cells which had been labeled with $C^{14}H_3$-methionine during normal growth were washed and resuspended in minimal medium containing $P^{32}O_4$ but lacking methionine. The progressive loss of the radiocarbon label from the sRNA fraction of these cells during the subsequent incubation and the concomitant incorporation of $P^{32}O_4$ from the medium indicate that both degradative and synthetic reactions occur during the period when no net synthesis of sRNA occurred.

TABLE 1

EFFECT OF METHIONINE DEPRIVATION ON sRNA OF *E. coli* 58-161

Time (hr)	Wet weight of cells (gm)	Total sRNA (A_{260})	Total Amino Acid Acceptor Activity (cpm × 10^5) Leucine	Glutamic acid	Methyl acceptance (cpm/A_{260})
0	2.9	159	2.00	1.00	5
2	3.5	556	4.34	2.08	184
4	3.3	466	4.47	1.91	356
6	3.2	504	4.58	2.17	354
8	3.1	428	4.18	1.93	458

Methionine starvation of cells and preparation of sRNA were carried out as described in *Methods*. The leucine and glutamic acid acceptor activity were determined as previously described.[10, 12] Methyl acceptance of the sRNA was measured with C^{14}-S-adenosylmethionine.[9]

Rechromatography of the fractions from one MAK column indicate that the appearance of the third leucyl-sRNA component is not a chromatographic artifact resulting from the altered composition of the RNA derived from methionine-starved cells. Soluble RNA, isolated from *E. coli* 58-161 (RC^{rel} Met^-) after 4 hr of methionine deprivation, was acylated with C^{14}-leucine and chromatographed on a MAK column. Fractions containing either the first or third components were mixed with equal amounts of the original preparation of unacylated sRNA and each

TABLE 2

EFFECT OF AMINO ACID DEPRIVATION ON sRNA OF DIFFERENT STRAINS OF *E. coli*

	Wet weight of cells (gm)	Total sRNA (A_{260})	Total leucine acceptor activity (cpm × 10^5)
58-161 (RC^{str} Met^- $Biotin^-$)			
Before starvation	2.6	69	1.46
Methionine-starved	2.4	53	0.69
W-1305 (RC^{rel} Met^- Leu^-)			
Before starvation	2.4	62	0.87
Methionine-starved	1.8	94	1.23
Leucine-starved	2.1	123	1.54

Figure 4 presents the results of MAK column analysis of C^{14}-leucyl-sRNA derived from equal amounts of *E. coli* 58-161 (RC^{str}, Met^-, $Biotin^-$) before and after 4 hr of methionine deprivation. It is clear that, despite the degradation, the new component of leucyl-sRNA did not accumulate in organisms with stringently controlled RNA synthesis. Thus, the presence of third peak of leucyl-sRNA appears to be associated with a net synthesis of RNA during amino acid deprivation rather than with a degradation of pre-existing RNA.

Additional evidence that *de novo* synthesis of RNA is necessary for the appearance of the third peak of leucyl-sRNA was provided by using a uracil-requiring mutant derived from *E. coli* W-1305 (RC^{rel} Met^- Leu^-). With this organism, it could be shown that the formation of the new component during methionine deprivation was dependent on the presence of uracil, and thus on sRNA synthesis.

The proposal that the new species of leucyl-sRNA is methyl-deficient suggests also that a relaxed control organism should accumulate this type of RNA when deprived of methionine but not when deprived of other amino acids. This specificity for methionine deprivation is demonstrable with the relaxed control double auxotroph *E. coli* W-1305 (RC^{rel} Leu^- Met^-). Soluble RNA obtained from cells after either leucine or methionine deprivation was charged with C^{14}-leucine and chromatographed as before (Fig. 5, Table 2). The MAK column elution profiles indicate that the sRNA produced during leucine deprivation does not contain the third peak of leucyl-sRNA, while that formed during methionine deprivation contains the new component. Thus, the third component of leucyl-sRNA accumulates only when the biosynthesis of sRNA is allowed to proceed but the methylation of the polyribonucleotide is prevented.

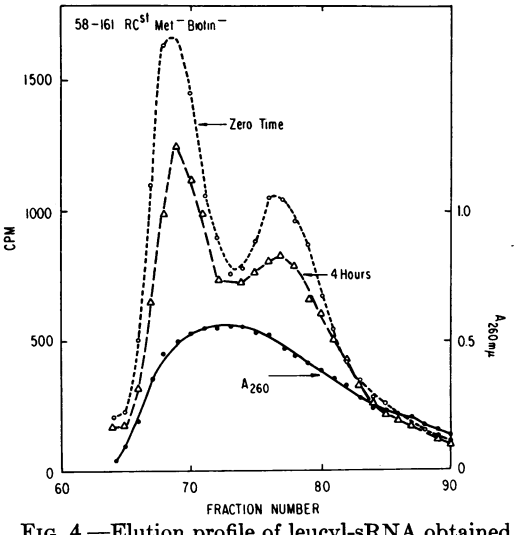

FIG. 4.—Elution profile of leucyl-sRNA obtained from normal and methionine-starved cells of *E. coli* 58-161 archtype (RC^{str} Met^-). Aliquots of sRNA representing equal weights of normal cells (open circles) and methionine-starved cells (open triangles) were maximally charged with C^{14}-leucine (10 μc/μmole) and chromatographed on MAK columns as described in the text.

Discussion.—The observation that the elution profiles from MAK columns of leucyl-sRNA formed during a period of relaxed control are

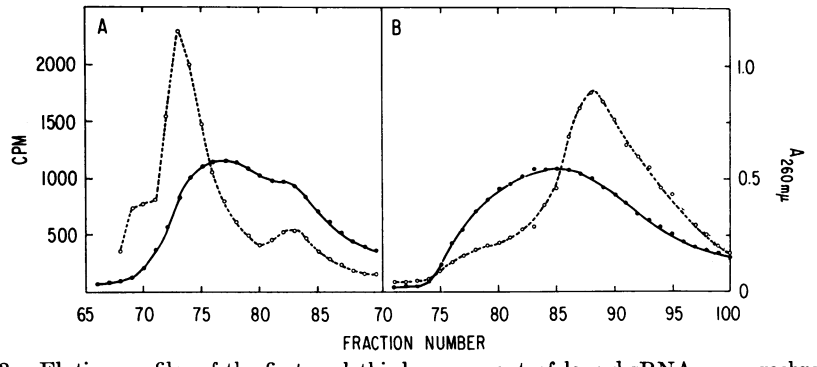

FIG. 3.—Elution profiles of the first and third component of leucyl-sRNA upon rechromatography. Soluble RNA derived from *E. coli* 58-161 (RC^{rel} Met^-) after 4 hr of methionine deprivation was maximally charged with C^{14}-leucine (240 $\mu c/\mu mole$) and chromatographed on a MAK column. Fractions corresponding to the first and third components were separately mixed with 24 $A_{260m\mu}$ of the original uncharged sRNA and rechromatographed. Open and closed symbols represent the radioactivity and absorbance at 260 mμ, respectively, of each fraction.

was rechromatographed. The unaltered elution positions of the first and third C^{14}-leucyl-sRNA components upon rechromatography are shown in Figure 3.

The capacity of a preparation of sRNA to be methylated by S-adenosylmethionine in the homologous RNA methylase reaction is a measure of the extent to which that RNA is deficient in methyl groups.[7, 9, 11] The methyl acceptance of those RNA preparations whose leucyl-sRNA profiles were examined in Figure 1 showed that there was no deficiency of methyl groups in the RNA from cells which were not deprived of methionine and did not exhibit the new species of leucyl-sRNA. It can be seen in Table 1 and Figure 1 that the capacity for methyl acceptance increased in parallel with the proportion of third peak during methionine starvation. This correlation of the increased methyl deficiency in sRNA with the increased concentration of the new component of leucyl-sRNA suggests that the latter is a methyl-deficient species which accumulates during relaxed control and methionine deprivation.[10]

If the new component represents methyl-deficient leucyl-sRNA, a methionine auxotroph that cannot synthesize sRNA *de novo* during methionine starvation (stringent control) would not be expected to form this leucyl-sRNA. This prediction was tested by examining the sRNA obtained from cells of the parental strain of *E. coli* 58-161 that exhibits stringent control of RNA synthesis. The total sRNA and leucine acceptor RNA content of the parental strain before and after a 4-hr deprivation of methionine is shown in Table 2. Comparison of these results with those shown in Table 1 underscore the differences between these mutants. During the deprivation the cellular sRNA increased 300 per cent in the relaxed control auxotroph but decreased 23 per cent in the stringently controlled mutant. This decrease is precisely that which would be predicted if it is assumed that the organisms differ only in their ability to synthesize RNA during amino acid deprivation. From Figure 2 it can be estimated that 25 per cent of the pre-existing sRNA in the relaxed auxotroph was degraded after 4 hr of amino acid deprivation. However, the synthesis of RNA in this organism was sufficient to allow a 200 per cent increase during this period. The stringently controlled mutant unable to synthesize significant amounts of sRNA under these conditions exhibited a net loss in cellular sRNA.

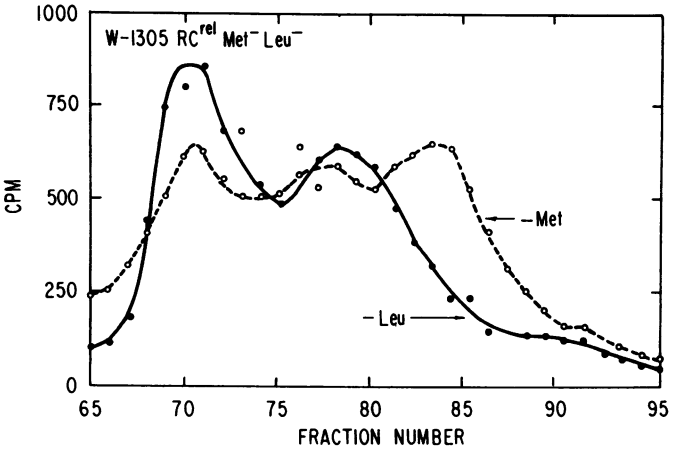

FIG. 5.—Elution profile of leucyl-sRNA obtained from *E. coli* after methionine or leucine deprivation. Cultures of *E. coli* W-1305 (RC^{rel} Leu^- Met^-) were deprived of leucine or methionine for 4 hr and harvested. sRNA extracted from them was maximally charged with C^{14}-leucine, and aliquots containing 31 $A_{260m\mu}$ were chromatographed on MAK columns.

radically different from those of normal leucyl-RNA has provided a device for the examination of the nature of the sRNA formed during relaxed control. Using this chromatographic technique, it has been possible to show that the accumulation of a new species of leucyl-sRNA proceeds essentially linearly over a 6-hr period of methionine deprivation. Studies with both the stringent and relaxed control methionine auxotrophs have demonstrated a limited degradation of the pre-existing sRNA during the period of methionine deprivation. The formation of the new leucyl-sRNA does not appear to be associated with the degradation process, for it appears only during relaxed control when a net synthesis of sRNA takes place.

Mandel and Borek[6] have shown that when a mutant of *E. coli* 58-161 auxotrophic for histidine and methionine is deprived of histidine in the presence of methionine, the accumulated RNA contains methylated bases. The demonstration that a relaxed control organism produces the new component during methionine deprivation, but not during leucine deprivation, indicates that it is a methyl-deficient species of leucyl-sRNA. However, the final decision concerning its methyl content must await a chemical analysis of purified material. Efforts at such a purification and analysis are currently under way.

The sRNA obtained from the relaxed auxotroph after methionine deprivation exhibits an increased acceptance for other amino acids (Table 1) indicating that methyl-deficient forms of these species are also synthesized. However, comparison of the elution profiles of valyl-, glutamyl-, and lysyl-sRNA from normal and methionine-starved cells did not reveal any differences resulting from methionine deprivation. The basis for the unique behavior in this regard of leucyl-sRNA, whether structural or metabolic, is unclear. It is interesting to note, however, that the results of Sueoka and Sueoka[17] also point to the uniqueness of leucyl-sRNA in *E. coli*. They have observed an altered MAK profile of leucyl-sRNA of *E. coli* B after infection with T2 phage. In this case, also, there appears to be no alteration of the elution pattern of the 16 other amino acid-sRNA's examined.

The question arises whether the new component of leucyl-sRNA formed during

methionine deprivation has the potential of being converted to those species of leucyl-sRNA found in normal cells. Attempts to demonstrate directly the conversion of the third component to the normal species during recovery from methionine starvation thus far have yielded equivocal results. Thus, at the present time it is not possible to decide whether the new component is a methyl-deficient precursor of the normal leucyl-sRNA or whether it is a form of leucyl-sRNA that is specifically induced by methionine deprivation.

Summary.—Analysis by chromatography on methylated albumin columns has provided a device for the characterization of a unique species of leucine-sRNA that accumulates during relaxed control. By the use of various related mutants of *E. coli*, evidence was obtained indicating that the formation of this species of leucyl-sRNA is the result of *de novo* synthesis of sRNA in the absence of the methyl donor ordinarily used to complete the synthesis of methylated bases at the polynucleotide level.

The authors are indebted to Dr. A. L. Taylor for isolating the uracil-requiring mutant of *E. coli* W-1305 used in these studies, and to Suzanne J. Gee for her excellent technical assistance.

[1] Pardee, A. B., and L. S. Prestidge, *J. Bacteriol.*, **71**, 677 (1956).
[2] Borek, E., A. Ryan, and J. Rockenbach, *J. Bacteriol.*, **69**, 460 (1955).
[3] Stent, G. S., and S. Brenner, these PROCEEDINGS, **47**, 2005 (1961).
[4] Alföldi, L., G. S. Stent, and R. C. Clowes, *J. Mol. Biol.*, **5**, 348 (1962).
[5] Mandel, L. R., and E. Borek, *Biochem. Biophys. Res. Commun.*, **4**, 14 (1961).
[6] Mandel, L. R., and E. Borek, *Biochemistry*, **2**, 560 (1963).
[7] Starr, J., *Biochem. Biophys. Res. Commun.*, **10**, 181 (1963).
[8] Littauer, U. Z., K. Muench, P. Berg, W. Gilbert, and P. F. Spahr, in *Synthesis and Structure of Macromolecules*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 28 (1963), p. 157.
[9] Peterkofsky, A., C. Jesensky, A. Bank, and A. H. Mehler, *J. Biol. Chem.*, **239**, 2918 (1964).
[10] Peterkofsky, A., these PROCEEDINGS, **52**, 1233 (1964).
[11] Fleissner, E., and E. Borek, these PROCEEDINGS, **48**, 1199 (1962).
[12] Lazzarini, R. A., and A. H. Mehler, *Biochemistry*, **3**, 1445 (1964).
[13] Mandell, J. D., and A. D. Hershey, *Anal. Biochem.*, **1**, 66 (1960).
[14] Bray, G. A., *Anal. Biochem.*, **1**, 279 (1960).
[15] Sueoka, N., and T. Yamane, these PROCEEDINGS, **48**, 1454 (1962).
[16] Goldstein, A., and B. J. Brown, *Biochim. Biophys. Acta*, **44**, 491 (1960).
[17] Sueoka, N., and T. K. Sueoka, these PROCEEDINGS, **52**, 1535 (1964).