*LEUCINE tRNA AND CESSATION OF ESCHERICHIA COLI PROTEIN SYNTHESIS UPON PHAGE T2 INFECTION**

By Tamiko Kano-Sueoka and Noboru Sueoka

DEPARTMENT OF BIOLOGY, AND PROGRAM IN BIOCHEMICAL SCIENCES, PRINCETON UNIVERSITY

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Abstract.—The involvement of tRNA in cellular differentiation has been tested by analyzing aminoacyl-tRNA of *Escherichia coli* after phage T2 infection. One or two minutes after infection, half of one of the five leucine tRNA components (Leu-tRNA$_1$, CUG responding) undergoes a drastic structural change which leads to inactivity of both leucine acceptor activity and codon response. Whether or not the modification causes cessation of host protein synthesis without inhibiting phage-specific protein synthesis has been examined by analyzing polysome-bound leucine tRNA of *E. coli* before and after the phage infection. The results presented in this paper indicate that the amount of Leu-tRNA$_1$ used after infection was greatly reduced as compared to that used in noninfected cells. Studies of the *in vitro* protein-synthesizing system show that T2 mRNA rarely contains the CUG codon. A mechanism by which host mRNA translation is inhibited by the phage infection is proposed from this available information.

Since the discovery of the phage-induced alteration of *Escherichia coli* leucyl-tRNA and the proposition of the adaptor modification hypothesis for cell differentiation in 1964,[1] numerous examples of tRNA alterations in various systems have been reported.[2] However, a crucial test of biological significance of the phenomena has not been possible. Present results on the phage-induced modification of leucine tRNA of *E. coli* give the first experimental support to the idea that the structural modification of tRNA may indeed have a causal effect in cellular differentiation, i.e., in this particular system the cessation of host protein synthesis without affecting phage-specific protein synthesis.

Wettstein (1966)[7] studied *E. coli* tRNA's bound to total ribosomes by MAK column chromatography to examine possible differential usage of synonymous tRNA's and found that in the case of Leu-tRNA, the relative amount of the Leu I peak was much less on ribosomes than that of the supernatant fraction, and that the T4 infection further reduced the relative amount of the Leu I peak. Differential usage of synonymous tRNA's during early embryogenesis of the sea urchin has also been inferred from a comparison on MAK columns of the lysyl-tRNA bound to the particulate fraction and free lysyl-tRNA.[8]

This paper presents the results of further study on the alteration of leucine tRNA after T2 infection by analyzing polysome-bound tRNA. We assume that polysomes are active sites of protein synthesis[9] and that only tRNA's actively participating in protein synthesis are present in this fraction. The study shows that the utilization of Leu-tRNA$_1$, which corresponds to the CUG codon, is greatly reduced after the infection. The results strongly support

the idea that the phage-induced modification of leucyl-tRNA$_1$ of *E. coli* by phage T2 infection may be the primary cause for the cessation of host protein synthesis.

Materials and Methods.—Preparation of bacteriophage T2 and conditions of bacteriophage infection: *E. coli* B, phage T2, and T2-infected *E. coli* B were prepared as previously described.[3]

Preparation of tRNA from various cell components: *E. coli* B cells from 1.2 liters of culture (5×10^8 cells/ml) were divided into two portions and collected by centrifugation. One portion (200 ml) was used to isolate bulk tRNA from whole cells by an ordinary phenol procedure,[3] and the other portion (1 liter) was used to isolate tRNA from polysome, monosome, and supernatant fractions.

(1) *Polysome fractions:* Harvested cells from 1 liter of culture were washed once with Tris-magnesium acetate buffer (0.01 *M* Tris-HCl, pH 7.8, and 0.015 *M* magnesium acetate) and lysed gently by a freeze-thaw-lysozyme method. The lysis was completed by adding sodium deoxycholate to the final concentration of 0.3%.[10] The lysate (2.7 ml) was centrifuged twice at 27,000 *g* for 10 min at 0°C to remove cell debris, and the supernatant was subjected to two successive sucrose gradient centrifugations. A sucrose gradient of 5–20% in Tris-Mg-KCl buffer (0.01 *M* Tris-HCl, pH 7.8, 0.014 *M* magnesium acetate, and 0.06 *M* KCl) was layered on 0.2 ml of a 40% sucrose cushion; 1–1.5 ml of the sample was added to the top and centrifuged in a Spinco model L centrifuge with a SW39 rotor at 37,000 rpm for 2 hr at 0–4°C. A soft pellet formed in the 40% sucrose cushion was suspended in a Tris-Mg-KCl buffer and recentrifuged in another sucrose gradient as before. The two supernatant fractions were combined and saved to prepare the supernatant and monosome fractions described below. When analyzed by sucrose density gradient centrifugation, the ribosomes in the pellet were shown to be composed mainly of polysomes. The pellet was suspended in a large volume of low-magnesium buffer (0.01 *M* Tris HCl, pH 7.3, and 10^{-4} *M* magnesium acetate) to free tRNA from ribosomes.[11] The suspension was kept in ice for 40 min and then centrifuged at 100,000 *g* for 3 hr at 0°C to remove the ribosomes. The supernatant was deproteinized with phenol and precipitated with ethanol. This is designated as a polysomal tRNA fraction, which also contains nucleic acid other than tRNA released from ribosomes.

(2) *Monosomal and supernatant fractions:* The supernatant fraction from the two sucrose gradient centrifugations was spun at 100,000 *g* for 3 hr at 0–4°C to sediment ribosomes. The supernatant from this centrifugation was deproteinized by phenol treatment. This is designated as supernatant tRNA. tRNA was also prepared from the pellets as described for the polysomal tRNA preparation. This is designated as monosomal tRNA.

Aminoacylation and analyses of leucine tRNA's were carried out according to the method previously reported.[3]

In vitro incorporation of leucine from leucyl-tRNA into polypeptides: The reaction mixture contains the following ingredients per 1 ml: 0.1 *M* Tris-HCl buffer, pH 7.8, 0.05 *M* KCl, 3×10^{-5} *M* GTP, 0.008 *M* or 0.01 *M* magnesium acetate, 7.5×10^{-3} *M* phosphoenolpyruvate, 1 μl 2-mercaptoethanol, 4 μg phosphoenolpyruvate kinase (California Biochemical Corp.), 0.04 ml aminoacyl-tRNA synthetase fraction[3] that is free from tRNA (240 μg protein), appropriate amounts of polysomes, and ^{14}C (or ^{3}H) leucyl-tRNA and 19 other ^{12}C aminoacyl-tRNA's from various preparations. The reaction mixture was incubated at 37°C for the desired period, and 2 ml of cold 10% TCA was added to stop the reaction. The tubes were heated in a water bath at 90°C for 20 min, then kept in ice for 30 min. The TCA-precipitable material was collected on glass filters, dried, and counted for radioactivity in a Packard liquid-scintillation counter.

Results.—The tRNA preparations from polysome, monosome, and supernatant fractions as well as bulk tRNA were made from noninfected cells and cells infected for 1.5, 3, 6, and 10 minutes, as described above. Yields of RNA

from the three fractions, estimated from absorbance at 260 mμ and the amount of leucine attached to tRNA, are shown in Table 1. The amount of polysomal tRNA calculated from chargeability indicates that only about 10–14 per cent of the ultraviolet-absorbing material is tRNA. Indeed, when the polysomal tRNA preparation was examined by MAK column chromatography, a large amount (80%) of the ultraviolet-absorbing material was eluted at salt concentrations where the elution of mRNA and ribosomal RNA would be expected.

Leu-tRNA's of those three fractions as well as bulk preparations from the cells infected for different lengths of time were chromatographed on MAK columns, *E. coli* bulk Leu-tRNA with a different radioactivity being used as an internal control. The results of polysome fractions are shown in Figure 1, and T2 0- and 1.5-minute samples of supernatant and monosomal fractions in Figure 2. The percentage of each peak of Leu-tRNA found in the different fractions taken from various time periods is summarized in Table 2. Since the peaks were only partially resolved, these numbers are only estimates.

Leu-tRNA's from bulk preparations gave the expected elution profiles: at 1.5 or 3 minutes after infection the Leu I peak was already low, relative to the Leu II peak, and the presence of some Leu F was noted. By six minutes after infection the amount of Leu I was approximately equal to or a little less than Leu II, and Leu F was no longer present as reported previously.[3]

When Leu-tRNA from the polysomal fraction was examined, a striking difference from bulk tRNA was observed (Fig. 1 and Table 2). First of all, in *E. coli* polysomal tRNA, Leu-tRNA$_1$ represented only 20 per cent of total Leu-tRNA in contrast to 54 per cent in the case of bulk tRNA. This can be

TABLE 1. *RNA fractions and their leucine acceptor activities.*

	T2 (0 min)		T2 (1.5 min)		T2 (3 min)		T2 (6 min)		T2 (10 min)	
Fractions*	(mg)	(cpm × 10^{-3})	(mg)	(cpm × 10^{-3})	(mg)	(cpm × 10^{-3})	(mg)	(cpm × 10^{-3})	(mg)	(cpm × 10^{-3})
Supernatant	1.96	1480	1.79	1308	2.55	1754	2.19	1303	1.92	1091
	(37)†	(66)	(36)	(77)	(47)	(73)	(41)	(62)	(39)	(64)
Monosome	1.58	566	1.40	278	1.68	549	1.66	682	1.60	502
	(30)	(25)	(28)	(16)	(31)	(23)	(31)	(33)	(32)	(30)
Polysome	1.75	194	1.73	124	1.18	91	1.44	101	1.46	100
	(33)	(9)	(35)	(7)	(22)	(4)	(27)	(5)	(29)	(6)
Total	5.29	2240	4.92	1710	5.41	2394	5.29	2086	4.98	1693
	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)

* Supernatant, monosome, and polysome tRNA fractions were prepared according to the method described in *Materials and Methods*. The amount of RNA was estimated from A_{260} with a conversion factor of 0.022 for 1 μg/ml.

† Numbers in parentheses give the percentage of the total.

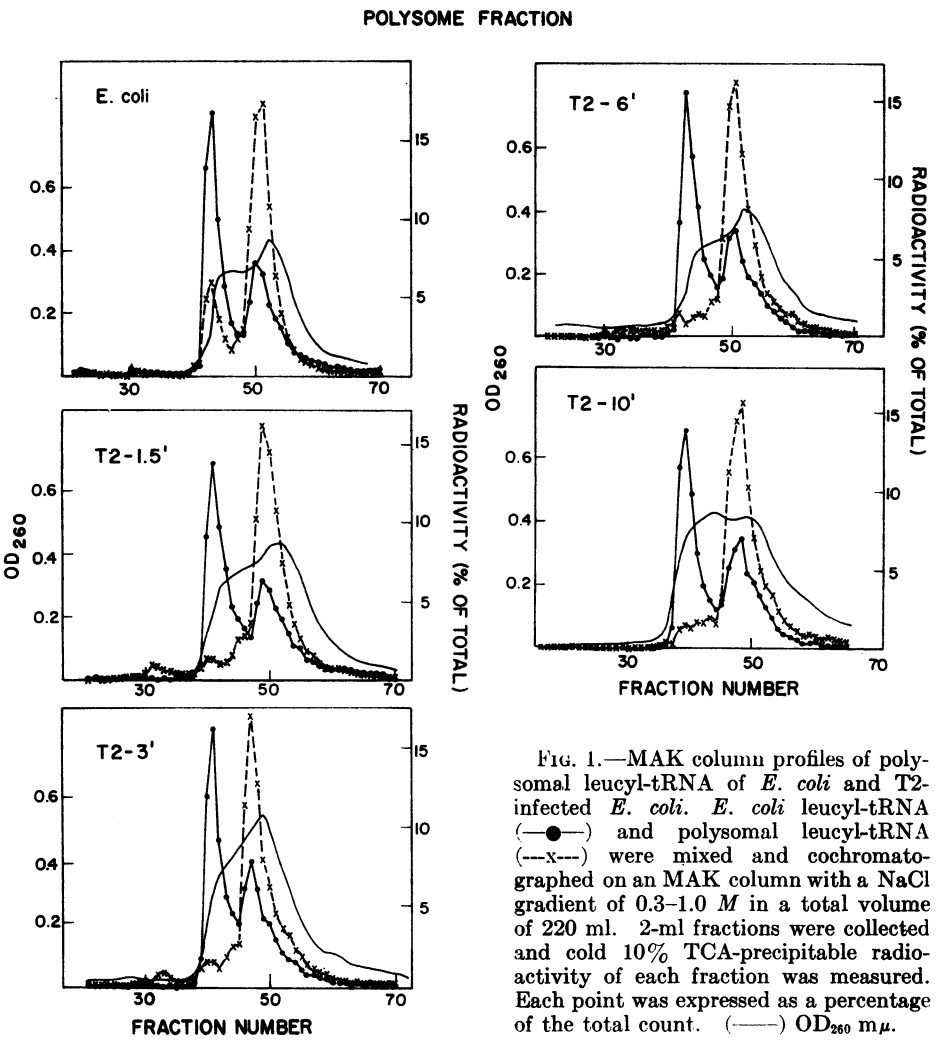

FIG. 1.—MAK column profiles of polysomal leucyl-tRNA of *E. coli* and T2-infected *E. coli*. *E. coli* leucyl-tRNA (—●—) and polysomal leucyl-tRNA (---x---) were mixed and cochromatographed on an MAK column with a NaCl gradient of 0.3–1.0 *M* in a total volume of 220 ml. 2-ml fractions were collected and cold 10% TCA-precipitable radioactivity of each fraction was measured. Each point was expressed as a percentage of the total count. (——) OD_{260} mμ.

interpreted to mean that the frequency of usage of different Leu-tRNA components does not reflect the actual amounts of the components in the cells.[7] Secondly, 1.5 minutes after T2 infection, the amount of the Leu I peak area decreased drastically. The trailing portion of the Leu I region contains another component of Leu-tRNA (Fig. 1). The leading component is Leu-tRNA$_1$, which is the major component and the origin of Leu-tRNA$_F$. The second peak, which appears as a front shoulder of the Leu II peak, can be identified as Leu-tRNA$_3$ by reverse-phase chromatography.[5] At 1.5 minutes after infection, Leu-tRNA$_1$ represented only 6 per cent of the total Leu-tRNA. MAK column elution profiles of polysomal Leu-tRNA from T2 cells infected for 3, 6, and 10 minutes were essentially the same as that of the T2 1.5-minute-infected cells. A very small quantity of Leu-tRNA$_F$ was observed in all polysomal tRNA preparations from T2-infected cells.

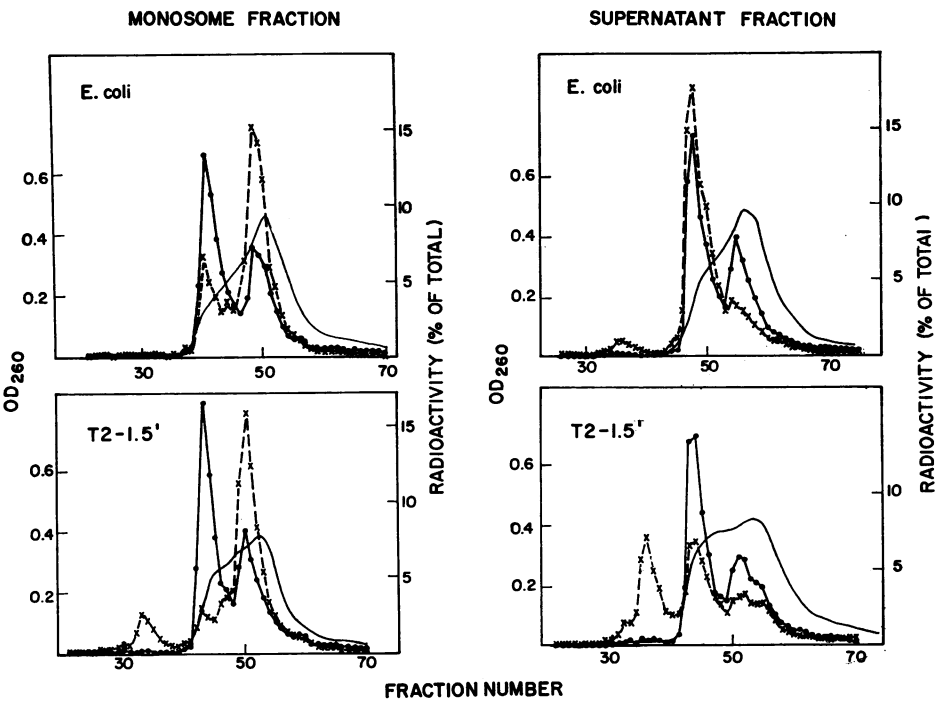

FIG. 2.—MAK column profiles of monosomal and supernatant leucyl-tRNA of *E. coli* and T2-infected *E. coli*. *E. coli* leucyl-tRNA (—●—); monosomal or supernatant Leu-tRNA (---x---); and (——) OD_{260} mμ.

The monosomal tRNA samples showed profiles very similar to those of the polysomal Leu-tRNA samples (Fig. 2 and Table 2). Thus, monosomal Leu-$tRNA_1$ of *E. coli* B constituted 25 per cent of total Leu-tRNA instead of 20 per cent as in the case of polysomal tRNA. Leu-$tRNA_1$ in this fraction was reduced to 14 per cent of total leucyl-tRNA 1.5 minutes after infection and was further reduced to 9 per cent at ten minutes after infection. The monosomal fraction contained 70*S* ribosomes to which degraded mRNA was most likely attached. During the early period of infection, therefore, an abundance of 70*S* ribosomes carrying degraded *E. coli* mRNA could be expected. Furthermore, we could expect that the amount of Leu-$tRNA_1$ in the monosomal fraction continually decreased as the infection proceeded. The polysomal and monosomal tRNA consist of approximately 30 per cent of all chargeable tRNA in the cell (bulk). Analyses of the ribosomal tRNA predict that the supernatant fractions should contain considerably more Leu-$tRNA_1$ than the ribosomal fractions. This was in fact the case, as is shown in Figure 2 and Table 2. A rather large proportion of Leu-$tRNA_F$ was also found in T2 1.5-minute-infected samples. For all samples available, the values of Leu-$tRNA_1$, when calculated by combining the supernatant, monosomal, and polysomal fractions, agreed well with the values obtained from bulk preparation (Table 2).

The above results suggest a greatly reduced usage of Leu-$tRNA_1$ after T2

TABLE 2. *Relative amounts (%) of Leu-tRNA$_F$, Leu-tRNA$_1$, and the remainder of the Leu-tRNA in various fractions.**

	0 min	1.5 min	3 min	6 min	10 min
Bulk					
Leu-tRNA$_F$	0.3	5	11	2	—
Leu-tRNA$_1$					
Observed	54	34	34	32	—
Expected†	52	33	32	27	33
The remainder	46	61	56	66	—
Supernatants					
Leu-tRNA$_F$	4	33	13	8	3
Leu-tRNA$_1$	66	35	40	38	46
The remainder	30	33	47	54	51
Monosomes					
Leu-tRNA$_F$	1	11	9	4	1
Leu-tRNA$_1$	25	14	11	8	9
The remainder	74	75	80	88	90
Polysomes					
Leu-tRNA$_F$	1	4	4	2	1
Leu-tRNA$_1$	20	6	7	8	7
The remainder	79	90	89	90	93

* The relative amounts of leucine tRNA's were estimated from Leu-tRNA fractions shown in Figs. 1 and 2 and from other figures not shown in this paper.

† The values in parentheses were calculated from Leu-tRNA$_1$ of the supernatant, monosomal, and polysomal fractions (this table), and also from relative amounts of Leu-tRNA of the three reactions (Table 1).

infection. This implies that T2 mRNA rarely contains the leucine codon CUG. An *in vitro* system of leucine incorporation into polypeptides was designed to examine the above hypothesis. The system essentially consisted of polysomes, polysomal tRNA of uninfected and T2-infected *E. coli* charged with 20 amino acids (with only leucine being radioactive), and the *E. coli* Leu-tRNA$_1$ fraction, which was charged only with the radioactive leucine. Incorporation of the radioactive leucine from Leu-tRNA into the hot TCA-precipitable fraction was measured (see *Materials and Methods*). As indicated in Table 3, though the results are preliminary, the system incorporated leucine at a relatively high efficiency and, whenever polysomes from T2-infected cells were used, incorporation of leucine from Leu-tRNA$_1$ into peptides was considerably reduced. On the other hand, *E. coli* polysomes incorporated leucine well from Leu-tRNA$_1$ in spite of dilution by bound Leu-tRNA$_1$ on the polysomes. The above results, together with MAK column analyses, strongly support the idea that phage T2 utilizes little, if any, CUG codon for the translation of its own messenger.

Discussion.—The knowledge gained about leucine tRNA's of *E. coli*, the eluting characteristics of five synonymous leucine-tRNA's studied by MAK columns and reverse-phase chromatography,[1, 3, 5] codon responses of those tRNA's before and after infection,[12, 13] and the mode of alteration of Leu-tRNA$_1$ to Leu-tRNA$_F$ after infection[6] has enabled us to carry out critical experiments and interpret data more explicitly. The present study on polysomal tRNA before and after infection indicates that the amount of Leu-tRNA$_1$ is reduced from 20 per cent to 6 per cent of the total Leu-tRNA within 1.5 minutes after infection, and that this value does not change up to 10 minutes after infection. The residual

TABLE 3. *Net radioactive leucine incorporation into polypeptides in vitro from polysomal leucyl-tRNA and Leu-tRNA$_1$ using polysomes from uninfected and T2-infected E. coli.*

		Leu-tRNA	Polysomes: *E. coli* (cpm)	(%)	Polysomes: T2 (6 min) (cpm)	(%)
Expt. 1						
	a	T2 6 min (^{3}H) + Leu-tRNA$_1$ (^{14}C)	157 324	(5)* (13)	172 67	(5) (3)
	b	*E. coli* (^{3}H)	215	(6)	145	(4)
	c	T2 6 min (^{3}H)	163	(5)	244	(7)
Expt. 2						
	a	T2 3 min (^{3}H) + Leu-tRNA$_1$ (^{14}C)	576 690	(25) (21)	566 217	(25) (7)
	b	*E. coli* (^{3}H)	1276	(48)	984	(37)
	c	T2 3 min (^{3}H)	933	(41)	928	(41)

Polysomes were prepared from uninfected and T2 6-min-infected *E. coli* as described in *Materials and Methods*. Polysomal tRNA was prepared from uninfected, T2 3-min-infected, and T2 6-min-infected cells and acylated with ^{3}H-leucine and 19 remaining nonradioactive amino acids. Leu-tRNA$_1$ was prepared by reverse-phase chromatography[4] and acylated with ^{14}C-leucine. Each reaction mixture (0.25 ml) contained the components listed under *Materials and Methods*, and the following where indicated: in expt. 1, 2.22 A_{260} units of *E. coli* polysomes, 2.76 A_{260} units of T2 6-min polysomes, 3390 cpm of *E. coli* polysomal ^{3}H Leu-tRNA, 3410 cpm of T2 6 min-polysomal ^{3}H Leu-tRNA, and 2595 cpm of ^{14}C Leu-tRNA$_1$ of *E. coli;* and in expt. 2, 2.66 A_{260} units of *E. coli* polysomes, 3.31 A_{260} units of T2 6-min polysomes, 2682 cpm of *E. coli* polysomal ^{3}H Leu-tRNA, 2272 cpm of T2 3-min polysomal ^{3}H Leu-tRNA, and 3365 cpm of ^{14}C Leu-tRNA$_1$ of *E. coli*. Magnesium concentration was 0.008 *M* for expt. 1 and 0.01 *M* for expt. 2, and incubation time was 30 min for expt. 1, and 15 min for expt. 2. Also, expt. 1 contained 2×10^{-4} *M* each of 20 nonradioactive amino acids. Expt. 1*a* and expt. 2*a* contained both T2 6-min ^{3}H Leu-tRNA and ^{14}C Leu-tRNA$_1$ in the same test tube.

* Numbers in parentheses indicate the percentage of total incorporated counts out of the input counts.

amount of Leu-tRNA$_1$ (6%) after infection can be interpreted in one of the following ways: (1) some fractions of the host polysomes, whose mRNA contains CUG codons, thus binding Leu-tRNA$_1$, remain stable through the early and middle period of phage infection; (2) the polysomes isolated later than 1.5 minutes after infection contain only phage mRNA, which contains occasional CUG codons; or (3) the residual Leu-tRNA$_1$ is due to a nonspecific binding to polysome fractions. The present data cannot determine which is actually the case. However, it seems clear that phage mRNA carries much less CUG codon for leucine, and that this condition is established immediately after infection (1.5 min) and is still maintained ten minutes after infection when the transition from early to late phase is already taking place under our experimental conditions.

Possible models should account for two phenomena: the fast breakdown of Leu-tRNA$_1$ to 60 per cent of the original level within two or three minutes after infection, and the very infrequent occurrence of the CUG codon corresponding to Leu-tRNA$_1$ in T2 mRNA. A tentative model that seems to best explain the observed phenomenon is given here: *Phage T2 infection induces a specific ribonuclease which nicks Leu-tRNA$_1$ at the site of the translation of E. coli mRNA on ribosomes, which in turn leads to the cessation of host protein synthesis. This process eliminates about half of the Leu-tRNA$_1$ molecules in the cell. Phage mRNA carries little or no CUG codon that corresponds to Leu-tRNA$_1$.*

Although we have no direct evidence, the modification of Leu-tRNA$_1$ on *E. coli* polysomes is favored rather than modification in the unbound state, because the reduction of Leu-tRNA$_1$ is completed within a few minutes after infection. If the ribonuclease were active in the supernatant, a gradual loss of the remaining Leu-tRNA$_1$ might be expected in the bulk or supernatant fraction during infection. This did not occur (Table 2). Other possibilities which do not implicate the biological significance of the modification are not discussed here. The model also seems to be supported by the fact that host protein synthesis stops within three minutes after infection when measured by β-galactosidase activity,[14, 15] a much shorter time than when cessation is due to degradation of host mRNA only, and that degradation of Leu-tRNA$_1$ stops at about the same time. It has also been reported that the translation of M12 RNA does not occur when M12 and T4 doubly infect *E. coli*.[16]

Preliminary results indicate that some modification of Leu-tRNA$_1$ to Leu-tRNA$_F$ can be obtained *in vitro* with extract from T2-infected cells. Further investigation along this line should lead to more decisive evidence of a role of modification as the key step in the cessation of host protein synthesis after virus infection. A recent observation by Weiss *et al.*[17] (1968) suggests synthesis of a phage-specific leucine tRNA at approximately 7.5 minutes after infection. This phenomenon is an additional change in tRNA after infection and does not at the moment relate to the modification of Leu-tRNA$_1$ upon which we have been working.

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