A Factor from *Escherichia coli* Concerned with the Stimulation of Cell-Free Polypeptide Synthesis by Exogenous Ribonucleic Acid. II. Characteristics of the Reaction Promoted by the Stimulation Factor*

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ABSTRACT: A stimulation factor which enhances cell-free polypeptide synthesis in the presence of f_2 ribonucleic acid (RNA) reduces the lag period which follows the addition of the RNA to the complete *Escherichia coli* cell-free system. Preincubation experiments indicate that the factor interacts with the ribosomes prior to the addition of the RNA. This interaction is temperature dependent.

The polyuridylic acid (poly-U) dependent incorporation of phenylalanine is not enhanced by the stimulation factor. The poly-U reaction does not show any lag period. Moreover, poly-U can stimulate the mixed-system *E. coli* ribosomes plus *Euglena gracilis* supernatant. This mixed system has been shown to be unresponsive to f_2 RNA. The results indicate that ribosomes must undergo a reaction promoted by the stimulation factor before interacting with messenger RNA, and that no such reaction is required for their interaction with poly-U. The factor showed some activity with RNA preparations from various cellular sources, but the stimulation with f_2 RNA was considerably greater.

In the preceding paper (Eisenstadt and Brawerman, 1966) it has been shown that the stimulation of cell-free polypeptide synthesis by f_2 ribonucleic acid (RNA) requires the presence of a supernatant factor distinct from the enzymes involved in the formation of peptide bonds. This stimulation factor appears to be species specific, as indicated by the fact that supernatant from *Euglena gracilis* does not support the stimulation of *Escherichia coli* ribosomes by f_2 RNA, and *E. coli* supernatant is similarly inactive with *Euglena* chloroplast ribosomes. Fractionation of the *E. coli* high-speed supernatant leads to a preparation which enhances preferentially the f_2 RNA stimulated incorporation in the *E. coli* cell-free system. It also renders the mixed system, *E. coli* ribosomes plus *Euglena* supernatant, responsive to f_2 RNA. These results were interpreted as indicating the occurrence of an enzymatic step concerned with polypeptide chain initiation in protein synthesis.

The present report deals with the possible nature of the factor effect. The experiments were designed primarily to examine the possibility that the factor promotes the interaction of messenger ribonucleic acid (m-RNA) and ribosomes. Since polyuridylic acid appears to interact with ribosomes far more readily than viral RNA (Barondes and Nirenberg, 1962; Haselkorn and Fried, 1963), the factor requirements of these two types of RNA template were examined. A basic difference in the behavior of f_2 RNA and polyuridylic acid (poly-U) was observed in this respect. The effect of factor on the early kinetics of amino acid incorporation was examined, and preincubation experiments led to the conclusion that the stimulation factor interacts with the ribosomes. The results obtained in this study suggest that ribosomes must undergo a specific reaction promoted by the factor in order to become capable of interacting with m-RNA.

Experimental Section

Cell-Free Preparations. Preincubated S-30, supernatant, ribosomes, and stimulation factor were prepared from *E. coli*, strain B, as described in the preceding report (Eisenstadt and Brawerman, 1966). The ribosomes were not treated with sodium deoxycholate, but were washed three times with 2 mM Tris–HCl, pH 8.0, 0.1 M KCl, 5 mM $MgCl_2$, and 6 mM β-mercaptoethanol. The supernatant fraction from *E. gracilis*, strain Z, was prepared as described previously (Eisenstadt and Brawerman, 1964).

Amino Acid Incorporation Experiments. These were carried out as described previously (Eisenstadt and Brawerman, 1966). Uniformly labeled L-[^{14}C]leucine, 57 μc/μmole, or L-[^{14}C]phenylalanine, 350 μg/μmole, was used in this study.

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TABLE I: Lack of Requirement for Factor in Poly-U-Stimulated Incorporation of Phenylalanine.[a]

Supernatant	No Factor			Factor Added		
	No RNA	f_2 RNA	Poly-U	No RNA	f_2 RNA	Poly-U
None	600	700	2,000	2800	2,800	5,600
Euglena	1800	2200	20,800	1800	8,400	20,100
E. coli	1200	2400	72,400	3200	14,000	71,000

[a] All reaction mixtures (0.2 ml) contained washed *E. coli* ribosomes (0.5 mg/ml) and *E. coli* t-RNA (100 μg/ml). Additions included *Euglena* supernatant, 1.1 mg of protein/ml; *E. coli* supernatant, 0.26 mg of protein/ml; stimulation factor, 0.02 ml/0.2 ml; f_2 RNA, 100 μg/ml; poly-U (Miles Laboratories), 100 μg/ml. Values expressed as counts per minute per milliliter of reaction.

Results

Lack of Factor Requirement in the Poly-U-Dependent Polypeptide Synthesis. The interaction of poly-U with ribosomes has been shown to differ markedly from that of viral RNA (Barondes and Nirenberg, 1962; Haselkorn and Fried, 1963). These investigators report that poly-U appears to have a high affinity for *E. coli* ribosomes, and forms complexes comprising several ribosomes per polynucleotide chain. Moreover, the poly-U-stimulated amino acid incorporation proceeds without noticeable lag. In contrast, viral RNA binds less strongly to the ribosomes, and forms active complexes *in vitro* containing a single ribosome. The incorporation stimulated by RNA also shows a marked lag which can be considerably reduced by preincubation in the absence of amino acids. The behavior of viral RNA suggests that its interaction with ribosomes might require an enzyme-catalyzed step. The poly-U effect, on the other hand, would appear to be independent of any such enzymatic step (Barondes and Nirenberg, 1962).

The above results prompted us to examine the requirement of the poly-U-dependent incorporation for the *E. coli* stimulation factor. As shown in Table I, there is a basic difference between viral RNA and poly-U in this respect. Poly-U effectively stimulates the *E. coli* cell-free system under conditions unfavorable for the stimulation by f_2 RNA. Addition of factor to this system enhances considerably the viral RNA effect, but leaves the poly-U-stimulated incorporation unchanged. Furthermore, the mixed system consisting of *E. coli* ribosomes and *Euglena* supernatant, which cannot be stimulated by f_2 RNA, responds very strongly to poly-U. Addition of factor to the mixed system leads to stimulation by viral RNA, but again the poly-U-dependent incorporation remains unaffected. These results clearly indicate that the factor is not required for the interaction between poly-U and ribosomes. In view of the apparent capacity of poly-U to form complexes with ribosomes without enzymatic mediation, the present results suggest that the stimulation factor might be concerned with such an enzymatic step presumably required by natural m-RNA.

Kinetics of Amino Acid Incorporation in the Presence of

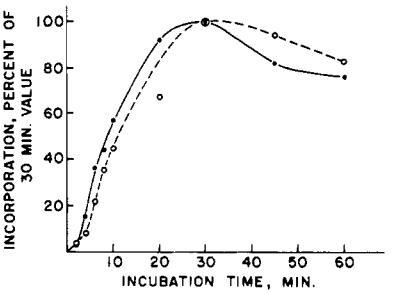

FIGURE 1: Kinetics of phenylalanine incorporation in the presence and absence of stimulation factor. Incubation mixtures (0.6 ml) contained 0.15 ml of S-30 and 100 μg of f_2 RNA; amount of factor added, 0.01 ml. Total incorporations after 30 min, in counts per minute per milliliter, were 1330 in the absence of factor (open circles, dashed lines), and 22,390 in the presence of factor (solid line, closed circles).

Stimulation Factor. Although the factor increases considerably the extent of polypeptide synthesis by the *E. coli* cell-free system in the presence of f_2 RNA, it has little effect on the over-all time course of the reaction. In the experiment described in Figure 1, the incorporation in the presence of stimulation factor is seventeen times greater than in its absence, but in both cases, the maximum incorporation is attained after about 30 min. An effect on the early kinetics is evident however. In the presence of factor, the lag period before the onset of rapid polypeptide synthesis is considerably reduced. In order to determine the nature of the lag period, and the effect of the factor on this lag, the following experiments were designed. The complete incorporation mixture was preincubated for 4 min in the absence of RNA under a variety of conditions, and the duration of the lag after addition of f_2 RNA was examined. When the preincubation is carried out in the absence of factor, and the latter is added just before the RNA, the accelerated polypeptide synthesis begins at a linear rate after a short lag period (Figure 2). If factor is present during the

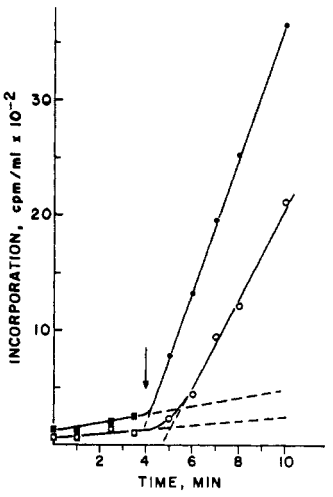

FIGURE 2: Kinetics of phenylalanine incorporation in the presence of f_2 RNA after preincubation of S-30 with stimulation factor. S-30 preincubated for 4 min at 37° in complete incorporation mixture (0.6 ml total volume), in the presence of factor (I, closed squares) or equivalent amount of buffer (10 mM Tris, pH 7.6, 6 mM β-mercaptoethanol) (II, open squares). Samples (0.05 ml) taken at specified time intervals. At 4 min (arrow), buffer added to reaction I and factor to reaction II, followed immediately by f_2 RNA. Incorporations in the presence of RNA represented by circles. Dashed lines represent extrapolated curves. Reaction mixtures contain S-30, 0.015 ml/0.6 ml; factor, 0.03 ml/0.6 ml; f_2 RNA, 100 μg/ml.

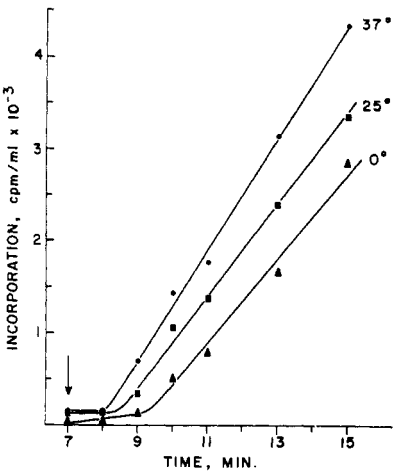

FIGURE 3: Kinetics of phenylalanine incorporation after preincubation with stimulation factor at different temperatures. S-30 preincubated for 4 min at 0 (triangles), 25 (squares), and 37° (circles) in complete incorporation mixture, including factor, but without RNA. Reactions kept in ice for 3 min, then brought to 37° and f_2 RNA added at the same time (arrow). For other details see Figure 2.

preincubation, the lag is reduced considerably. This effect was observed consistently in many experiments. Thus, it appears that the factor interacts with a component of the cell-free system in such a manner that the system becomes more susceptible to stimulation by the RNA. In order to determine whether this interaction is temperature dependent, the preincubations with factor were carried out at 0, 25, and 37°. The reactions were kept in ice for 3 min, then supplemented with f_2 RNA and brought to 37°. As shown in Figure 3, the duration of the lag after addition of RNA varied inversely with the temperature during preincubation. The longer lag periods in this experiment as compared to those shown in Figure 2 are due to the fact that the initial temperature was 0 instead of 37°.

The nature of the component which interacts with the factor was investigated by omitting from the preincubation mixture either the ribosomes or the high-speed supernatant. The reduction in the lag period could then be expected to occur only if the proper component were present during preincubation with the factor. The shortest lag period was obtained when the preincubation was carried out in the presence of the ribosomes (Figure 4). This effect was observed consistently in three separate experiments. Thus the factor appears to exert its effect by interacting with the ribosomes.

The preincubation experiments were also carried out with poly-U as template instead of viral RNA. It was shown in the preceding section that the factor is not required for the stimulation of amino acid incorporation by poly-U. As indicated in Figure 5, the early kinetics of this reaction are also unaffected by the factor. Little, if any, lag is evident after addition of poly-U to the reaction mixture without factor. With f_2 RNA, on the other hand, there is a considerable lag when the reaction is carried out in the absence of factor (Figure 5). This experiment indicates that the effect of preincubation with factor cannot be attributed to some activation of enzymes concerned with peptide bond formation, since the system without factor shows full activity immediately after addition of poly-U.

Effect of Stimulation Factor with RNA Preparations from Various Sources. Different RNA preparations were examined with respect to enhancement of template activity by the factor. As shown in Table II, the response to factor varies widely with the nature of the RNA. By far the best stimulation is obtained with f_2 RNA. Rat liver cytoplasmic RNA, which has a low template activity, is only slightly affected by the factor. *E. coli* RNA shows a better response, and a highly active RNA fraction from *E. gracilis* is affected to about the same extent. The response with the nonviral RNA preparations tended to vary from experiment to experiment, but it was always greatest with f_2 RNA. The selective effect of the factor is most apparent when the RNA's from *Euglena* and

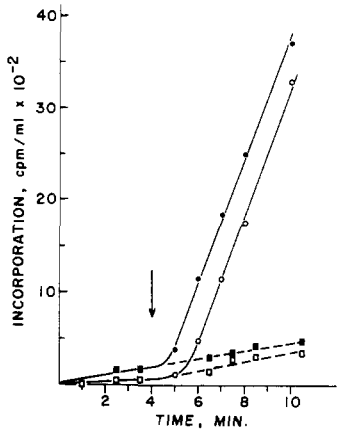

FIGURE 4: Kinetics of phenylalanine incorporation after preincubation of ribosomes and of supernatant with stimulation factor. Washed *E. coli* ribosomes (I, closed squares) and supernatant (II, open squares) preincubated for 4 min at 37° in complete incorporation mixture (1.1-ml total volume) in the presence of factor but without RNA. At 4 min (arrow), supernatant added to I and ribosomes to II, followed immediately by addition of f_2 RNA (0.05 ml) to 0.45-ml portion of each reaction. Incorporation with RNA represented by circles, and without RNA by squares; dashed lines, incorporation in portions of reactions to which RNA was not added. Reaction mixtures contained ribosomes, 0.5 mg/ml; supernatant, 0.26 mg of protein/ml; factor, 0.1 ml/1.1 ml; f_2 RNA, 100 μg/ml.

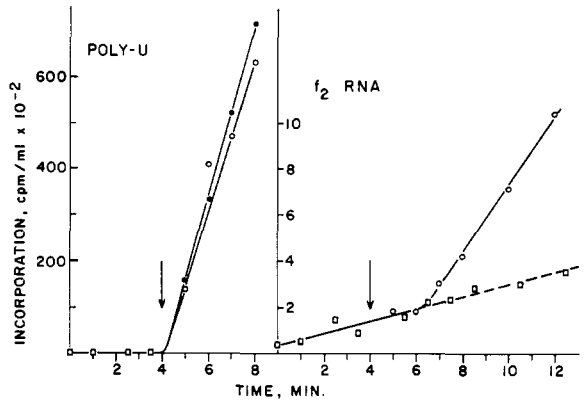

FIGURE 5: Kinetics of poly-U-stimulated phenylalanine incorporation after preincubation of ribosomes in the presence and absence of stimulation factor. Washed ribosomes preincubated for 4 min at 37° in complete incorporation mixture, then supernatant and RNA template added. For experimental details see Figure 4. Open circles, reactions preincubated without factor; closed circles, preincubated with factor; squares and dashed lines, incorporation without RNA. Poly-U used at concentration of 100 μg/ml.

f_2 are compared. Without factor, the *Euglena* RNA fraction shows more template activity than f_2 RNA, but in the presence of factor the situation is reversed. This differential effect is difficult to understand. It could be that the reaction promoted by the factor is specific for certain classes of m-RNA. It is also possible that the factor effect tends to be masked with preparations containing m-RNA as only a minor component.

Inhibition of the Factor Effect by Excess Supernatant. In order to determine the optimal conditions for the expression of the factor effect, experiments were carried out with varying proportions of ribosomes and supernatant. As shown in Figure 6A, amino acid incorporation in the presence of factor shows a maximum within a narrow range of supernatant concentrations. Amounts of supernatant higher than 0.01 ml/0.2 ml of reaction lead to a strong inhibition of the factor effect. In the absence of factor, the extent of polypeptide synthesis continues to rise with supernatant concentrations up to 0.04 ml/0.2 ml (Figure 6B). Increasing the ribosome concentration also reduces the factor effect, but to a much smaller extent (Figure 6C). The proportion of ribosomes relative to supernatant producing the most pronounced factor effect is twice as high as in the usual S-30 preparations.

The inhibitory effect of excess supernatant appears to be related specifically to the action of the stimulation factor, since polypeptide synthesis in the absence of added factor is not affected in the same manner. This indicates that a component of the supernatant interacts either with the factor itself, or with a reaction product of the factor. The effect of excess ribosomes could be due to the presence of a small amount of the same component in the ribosome preparation.

TABLE II: Effect of Factor on Stimulation of Leucine Incorporation by Different Ribonucleic Acids.[a]

Type of RNA (μg/ml)	Incorporation		
	No Factor	Factor Present	Stimulation[b]
None	1190	1640	1.4
Rat liver (175)	1340	2040	1.5
(525)	1420	2460	1.7
Euglena, pH 8.5 (39)	1710	3450	2.0
(96)	2570	4790	1.9
E. coli (200)	1670	3690	2.2
(500)	2990	5310	1.8
f_2 (50)	1370	6210	4.5
(100)	2010	9940	4.9

[a] Reaction mixtures (0.20 ml) contained 0.02 ml of S-30 and 0.02 ml of stimulation factor. Rat liver RNA prepared from cytoplasm as described previously (Brawerman *et al.*, 1965); *Euglena* RNA obtained by differential phenol extraction at pH 8.5 (Brawerman, 1963). *E. coli* prepared from disrupted cells by the procedure used for rat liver RNA. [b] Stimulation indicates the enhancement of incorporation by factor.

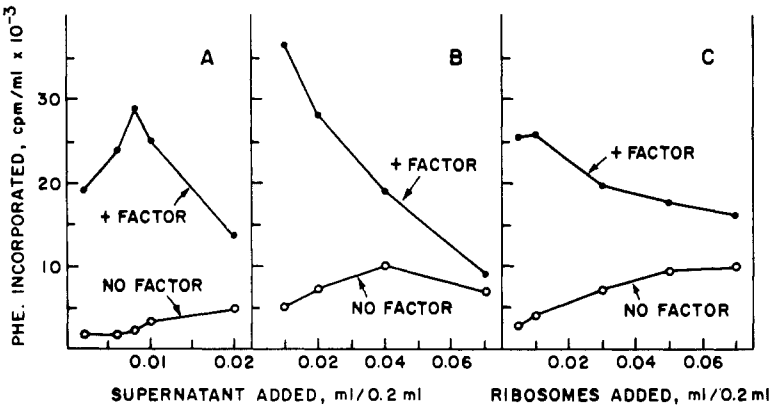

FIGURE 6: Effect of excess supernatant and ribosomes on stimulation of polypeptide synthesis by factor. Incubation mixtures (0.2 ml) contained 0.01 ml of washed ribosomes in experiments A and B, and 0.02 ml of supernatant in C. Ribosome suspension contained 5.5 mg of protein/ml and supernatant had 5.2 mg of protein/ml. Amount of factor used in A, 0.013 ml; in B and C, 0.02 ml. f_2 RNA present in a concentration of 100 μg/ml.

Discussion

In the present report, the preparation of stimulation factor is shown to have two effects on the polypeptide synthesis promoted by f_2 RNA. The extent of the reaction is greatly increased, and the response of the cell-free system to RNA is more rapid. Neither of these two effects is observed when poly-U is used as template RNA. The poly-U-dependent reaction appears to proceed maximally without factor, and the cell-free system responds to this polymer without any noticeable lag even in the absence of stimulation factor. Thus the action of the factor can probably be interpreted in terms of some basic difference in the behavior of poly-U and viral RNA in the cell-free system for polypeptide synthesis. One difference which has been noted by Barondes and Nirenberg (1962) and Haselkorn and Fried (1963) is that poly-U readily forms polysomal complexes with ribosomes, while viral RNA appears to bind only a single ribosome *in vitro*. This behavior suggests that ribosomes can bind to poly-U throughout the polypeptide chains, while viral RNA has only a single binding site, presumably the initiation site for protein synthesis. The interaction of ribosomes with viral RNA appears to require an enzymic step while the poly-U–ribosome interaction takes place at 0° in the absence of supernatant (Barondes and Nirenberg, 1962). The stimulation factor described in this report could be concerned with the ribosome–viral RNA interaction. The present results would then indicate that ribosomes must undergo a reaction promoted by the factor in order to become capable of interacting with m-RNA.

Although the above interpretation is compatible with the available data, a different possible mode of action of the stimulation factor must also be considered. It has been shown that *N*-formylmethionine serves as the initial amino acid residue in the synthesis of f_2 coat protein *in vitro* (Adams and Capecchi, 1966; Webster *et al.*, 1966). Thus the enzymes required for the formylation of methionyl t-RNA (Marcker and Sanger, 1964; Marcker, 1965) must be present in the cell-free system. If the system were deficient in one of the components of this reaction, the initiation of coat protein synthesis could probably not take place. Thus the stimulation factor preparation could exert its effect by providing an essential component for the formylation reaction. This interpretation of the factor effect is compatible with most of the available data. Preincubation of the cell-free system with factor would lead to formylation of methionine and result in a more rapid response to viral RNA. Moreover, polyphenylalanine synthesis would not require any prior formylation, and poly-U should be independent of any factor requirement. Some of the present data, however, cannot be explained in terms of *N*-formylmethionine formation. The factor appears to exert its effect directly on the ribosomes, while, if formylation were involved, an interaction with the supernatant should be expected. Also the absolute requirement for the factor when *Euglena* supernatant is used does not appear to be compatible with a formylation step. The *Euglena* cell-free system is capable of f_2 coat protein synthesis (Schwartz *et al.*, 1965). If initiation by *N*-formylmethionine is a prerequisite for this process, *Euglena* supernatant should be capable of formylating methionine. The results with the mixed *E. coli* and *Euglena* cell-free systems (Eisenstadt and Brawerman, 1966) indicate that the stimulation factor must be of the same species as the ribosomes. This tends to support the idea that the factor interacts directly with the ribosomes. A possible role of the stimulation factor in the formylation reaction cannot be ruled out, however, without more direct evidence.

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The Effect of Sodium Chloride on Esterification of Leucine to Transfer Ribonucleic Acid by Heterologous Aminoacyl Transfer Ribonucleic Acid Synthetases*

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ABSTRACT: The esterification of leucine to *Escherichia coli* or yeast transfer ribonucleic acid (t-RNA) by their respective homologous enzymes is unaffected by NaCl. However, the heterologous esterification reaction is markedly influenced by NaCl. In the presence of 0.12 M NaCl, both the rate of esterification and the yield of leucyl-t-RNA are appreciably inhibited. The level of inhibition is related to the concentration of NaCl. The evidence suggests that the leucyl-t-RNA synthetase undergoes a reversible modification in the presence of NaCl to a form that can no longer attach leucine to heterologous t-RNA. The pyrophosphate exchange reaction of the yeast leucyl-t-RNA synthetase is unaffected by NaCl. The K_m for *E. coli* leucine acceptor RNA is approximately 2.5×10^{-8} M for the *E. coli* enzyme and 1.6×10^{-7} M for the yeast enzyme. The K_m for yeast leucine acceptor RNA for the yeast enzyme is 4×10^{-7} M.

The initial enzymatic step unique to the process of protein synthesis involves the esterification of the free amino acid to t-RNA[1] (Hoagland *et al.*, 1958). While there is considerable evidence that there is a single aminoacyl-t-RNA synthetase for each of the naturally occurring amino acids (Berg, 1961),[2] there appear to be multiple species of t-RNA for the individual amino acids. It is assumed that the various species of t-RNA which can carry a single amino acid have the capacity to find their way to different positions in a protein sequence as a result of a unique interaction with template RNA. The process by which an aminoacyl-t-RNA synthetase selects the proper species of t-RNA for acylation presents an interesting problem in protein–nucleic acid interaction. Although a number of investigators have purified aminoacyl-t-RNA synthetases[3] and purified species of t-RNA are becoming increasingly available (Brown, 1963; Goldstein *et al.*, 1964; Muench and Berg, 1966), a clear picture of the mechanisms of t-RNA "recognition" or "discrimination" has not been formulated. A supplementary approach to the study of recognition of t-RNA by aminoacyl-t-RNA synthetases involves the use of heterologous enzymes (Yamane and Sueoka, 1963; Peterkofsky, 1964). The present work describes the effect of NaCl in specifically inhibiting heterologous aminoacylation of t-RNA and describes some experiments designed to characterize this phenomenon.

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[1] Abbreviations used in this work: ATP, adenosine triphosphate; GSH, glutathione; PP_i, inorganic pyrophosphate; t-RNA, amino acid acceptor RNA; leucyl-t-RNA, the esterified form of the t-RNA; TCA, trichloroacetic acid.

[2] Yu and Rappaport (1966) have recently presented evidence for two distinct leucyl-t-RNA synthetases in *E. coli*.

[3] The recent paper by Baldwin and Berg (1966) includes a summary of references to these enzymes.