THE INCORPORATION OF RADIOCARBON FROM ATP AND AMINO ACID INTO NUCLEIC ACIDS OF *ESCHERICHIA COLI*

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SUMMARY

1. The incorporation of the adenylate moiety of labeled ATP into soluble cytoplasmic RNA of *E. coli* and the incorporation of [^{14}C]isoleucine into RNA and proteins has been observed.

2. Fractionation of *E. coli* preparations yielded one fraction which catalyzed incorporation of adenine nucleotide into the intranucleotide positions of RNA and another which catalyzed incorporation into the terminal position of RNA.

3. Some characteristics of the enzymes responsible for nucleotide-amino acid end-group labeling have been described. The adenylate incorporating activities have been resolved from the enzymes which activate amino acids and which probably catalyze the incorporation of amino acids into RNA.

4. The evidence presented suggests that incorporation of amino acids into *E. coli* proteins may involve analogous reactions to those described for mammalian systems.

INTRODUCTION

Studies on incorporation of radioactive amino acids into proteins of mammalian tissues have suggested that enzymes present in the non-particulate fraction of cytoplasm catalyze a carboxyl activation of amino acids by ATP[1-4] yielding enzyme-bound amino acyl adenylates, and transfer of amino acyl groups to low molecular weight ribonucleic acid molecules[5-9]. Subsequently, there is a transfer of the amino acyl moiety of such ribonucleic acid–amino acid complexes to ribonucleoprotein particles[7,10]. Although amino acid activating enzymes of the type found in mammalian tissues have also been found in bacteria[11,12], studies with *S. aureus*[13] and *A. faecalis*[14] demonstrated that radioactive amino acids may be incorporated into proteins of these species by a system containing a particulate fraction but apparently lacking amino acid-activating activity of the type found in mammalian tissues[14]. These observations suggest that incorporation of amino acids into bacterial proteins may occur by a mechanism different than that described for mammalian tissues, but do not exclude other pathways of amino acid incorporation. The present studies, carried out with

Abbreviations: ATP, adenosine triphosphate; RNA, ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; CTP, cytidine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate. A.S., ammonium sulphate. RNase, ribonuclease; DN-ase, deoxyribonuclease.

preparations obtained from *E. coli* show that amino acid incorporation into ribonucleic acid and into protein can occur by a pathway which seems to be analogous to that found in animal tissues. The similarity in the nature and precursors of the nucleotide-amino acid end sequence in soluble cytoplasmic RNA, recently elucidated in mammalian preparations[15–17], are consistent with the sequence of reactions which appear to be associated with incorporation of amino acids into mammalian proteins.

Previous studies in this laboratory[8,9] with purified tryptophan-activating enzyme indicated that the amino acyl moiety of enzyme-bound amino acyl adenylate was enzymically transferred to soluble cytoplasmic RNA. Under conditions where considerable transfer of amino acid from enzyme-amino acyl adenylate complex to RNA was observed, the adenylate moiety was not incorporated into RNA. These results suggested that the adenylate portion of enzyme-bound amino acyl adenylate is not the direct precursor of terminal adenine nucleotide in RNA[15,17,18]. Similar studies with an amino acid-activating enzyme preparation from *Escherichia coli* also indicated that although enzyme-bound amino acids are transferred to *E. coli* RNA, transfer of enzyme-bound adenylic acid does not occur. Considerable incorporation of ^{14}C from labeled ATP into RNA present in the activating enzyme fraction was observed, however, and control incubations of activating enzymes in presence of [^{14}C]ATP and added RNA also resulted in incorporation of radioactivity into RNA. These results suggested the presence, in the amino-acid-activating enzyme fraction, of an activity responsible for incorporation of adenine nucleotide from ATP into RNA which did not involve intermediary participation of amino acyl adenylate. The incorporation of precursors of the nucleotide-amino acid end sequence into RNA of *E. coli* is described in this communication.

EXPERIMENTAL

Preparation of E. coli *enzyme fractions*

E. coli were grown for 16–18 h at 37° in a medium containing 0.1 % K_2HPO_4, 0.1 % KH_2PO_4, 0.1 % NaCl, 0.07 % $MgSO_4$, 0.4 % $(NH_4)_2SO_4$, 0.05 % Na citrate, 1 % N–Z CASE and 0.5 % glucose at pH 7.4. All succeeding operations were carried out at 4°. Cells were collected by centrifugation, washed once with cold distilled water, resuspended in 0.03 *M* Tris buffer, pH 7.0 (1 g wet wt. of cells/10 ml buffer), and 35-ml aliquots sonically disrupted for 7 min. The sonicate was centrifuged at 100,000 × *g* (average) for 2 h and the residue discarded. The pH of the supernatant was adjusted to pH 4.9 with 1 *M* acetic acid and the residue obtained by centrifugation at 30,000 × *g* for 30 min (pH 4.9-residue) was suspended in 0.1 *M* Tris buffer, pH 7.0. The resuspended residue was reprecipitated at pH 4.9 and resuspended in Tris buffer pH 7.0 two times. The final suspension contained 200 mg wet wt. of residue/ml buffer. The yield of pH 4.9-residue was 20 % (wet wt. basis) of initial weight of cells. The pH 4.9-residue suspension was diluted with an equal volume of 0.1 *M* Tris buffer, pH 7.0, 2 g of solid $(NH_4)_2SO_4$ were added/10 ml of suspension, and the solution stirred for 20 min. The precipitate was collected by centrifugation at 13,000 × *g* for 10 min, resuspended in a minimal amount of distilled water and dialyzed against running water for 5 h (20 % A.S. fraction). To the supernatant of the 20 % A.S. fraction, 2 additional g of solid $(NH_4)_2SO_4$ were added and the resulting residue was treated similarly to the 20 % A.S. fraction to yield the 40 % A.S. fraction.

The precipitation with $(NH_4)_2SO_4$ was continued and the 60% A.S. fraction was obtained.

Preparation of pH 4.9-residue nucleic acid

The pH 4.9-residue, recycled three times at pH values of 4.9 and 7.0 as described above, was diluted with 5 volumes of 0.02 *M* potassium phosphate buffer, pH 7.0 and extracted with water-saturated phenol at 4° (see ref. 19). After precipitation of potassium nucleate with 66% ethanol and dialysis against running water for 3 h, the preparation was lyophilized and resuspended in H_2O (20 mg/ml).

Methods

Incubations were carried out at 37.5° in 1 ml volumes in presence of the following components: KCl, 20 μmoles; $MgCl_2$, 10 μmoles; NaF, 1 μmole; Tris buffer, pH 8.0, 100 μmoles; phosphoenolpyruvic acid 10 μmoles; pyruvate kinase, 40 μg; nucleic acid, isolated from the pH 4.9-residue, 4 mg; CTP, 0.5 μmoles. Enzyme preparations were added as described. Radioactive precursors used were [^{14}C]ATP (1 μmole, 80,000 counts/min), [^{14}C]ADP (1 μmole, 90,000 counts/min), [^{14}C]AMP (1 μmole, 90,000 counts/min) or [^{14}C]isoleucine (0.07 μmole, 170,000 counts/min). Following the incubation period, 4 ml of cold 0.4 *M* $HClO_4$ were added and samples allowed to stand at 4° for 30 min. In experiments with labeled nucleotides, nucleic acids were isolated by two extractions of the washed acid-insoluble residue with 10% sodium chloride–0.01 *M* sodium Versenate at pH 7.0 for 30 min, followed by precipitation with ethanol and dialysis. Extractions in presence of Versene resulted in lower blanks in unincubated controls or in incubations with boiled enzyme preparations. When incubations were carried out with less than 2 mg of added nucleic acid, a corresponding amount was added as carrier immediately prior to addition of perchloric acid. In experiments with labeled amino acid, nucleic acid-amino acid complex was isolated by two extractions with 10% NaCl at 100° for 30 min[7]; extractions were carried out under acidic conditions, not neutralized as in the isolation of nucleotide-labeled nucleic acids. The residual proteins were isolated for radioactivity determinations as described[8].

Chromatography of nucleic acids hydrolyzed in 0.2 *M* NaOH at 80° for 3 h was carried out in ethanol–1 *M* NH_4 acetate, pH 7.5 (7:3); similar results were obtained with 2 other solvent systems. Chromatography of the acid-soluble nucleotides, following precipitation of perchloric acid as insoluble potassium salt, was also carried out with the solvent system described above.

RESULTS

The incorporation of [^{14}C]isoleucine into RNA and proteins of pH 4.9-residue obtained from *E. coli* sonicates, previously centrifuged at 100,000 × *g* for 45 min instead of 2 h as described above, is shown in Fig. 1. Incorporation of amino acid into RNA occurred first and loss of radioactivity was accompanied by incorporation of ^{14}C into proteins. Similar experiments with pH 4.9-residue as described above (2 h centrifugation at 100,000 × *g*) or with any of the subsequent fractions derived from it did not result in incorporation into protein; protein labeling described here is probably due to incomplete removal of particulates which are also sedimented at pH 4.9 and are capable of accepting activated amino acid from RNA-amino acid complex.

Incubation of the 100,000 × *g* supernatant (sonicate supernatant) obtained from sonically disrupted *E. coli* cells with [^{14}C]ATP (Table I) led to incorporation of radiocarbon into nucleic acids isolated: similarly, incubations with pH 4.9-residue or any of the $(NH_4)_2SO_4$ fractions derived from it also resulted in labeling of nucleic acid. The 20% A.S. fraction catalyzed primarily incorporation of ^{14}C from ATP

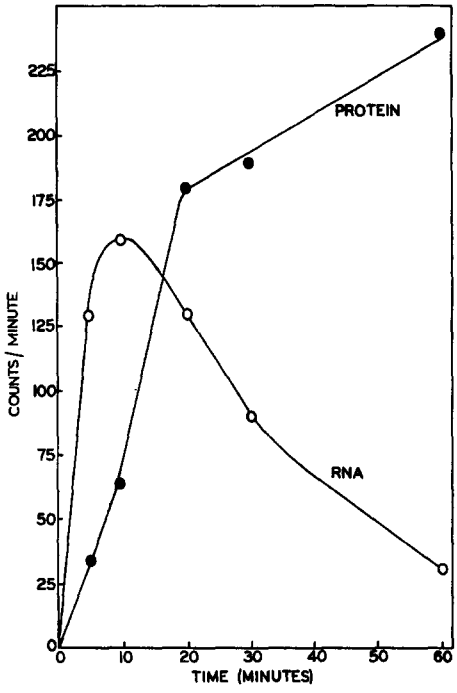

Fig. 1. O—O, Incorporation of [^{14}C]isoleucine into RNA and ●—●, protein. Incubations were carried out with pH 4.9-residue prepared from *E. coli* sonicate previously centrifuged for 45 min at 100,000 × *g*, and [^{14}C]isoleucine, in the absence of added nucleic acid. Other incubation conditions described in text.

TABLE I

INCORPORATION OF RADIOCARBON FROM LABELED ATP INTO NUCLEIC ACIDS OF *E. coli* IN THE PRESENCE OF VARIOUS ENZYME PREPARATIONS

Incubations as described in text, contained [^{14}C]ATP and sonicate supernatant, 18 mg protein, pH 4.9-residue, 11 mg protein, 40% A.S. fraction, 4 mg protein, or Ca–PO_4 eluate, 0.3 mg protein. Incubations were carried out for 20 min.

Preparation	*Specific activity**	*% of initial activity*
Sonicate supernatant	0.16	100
pH 4.9-residue	0.18	85
40% A.S. fraction	1.67	54
Ca–PO_4 eluate	4.8	7

* Specific activity: mμmoles of [^{14}C]adenine nucleotide incorporated into the total nucleic acid fraction isolated, per mg of enzyme protein. Values are corrected for non-enzymic adsorption of ^{14}C observed in unincubated controls or in incubations with boiled enzyme preparations (0.05 mμmole/mg protein).

into intranucleotide (internal) sequences in nucleic acid; chromatographic analysis of alkaline hydrolysates of labeled nucleic acid revealed approx. 70 % as 3'-adenylic acid and 30 % as adenosine (Table II). The recovery of about 60 % of the radioactivity in adenosine from similar analysis of nucleic acids labeled with 40 % A.S. fractions indicated that this preparation catalyzed more extensively incorporation into the

TABLE II

DISTRIBUTION OF ISOTOPE IN NUCLEIC ACID LABELED WITH [^{14}C]ATP

Incubation conditions as described in text; incubation time, 10 min.

Enzyme fraction	*Specific activity**	*% initial activity***	*% termival adenylate****
20 % A.S.	1.73	17	30
40 % A.S.	1.80	54	61
60 % A.S.	0.63	11	60

* Specific activity (see under Table I).
** Per cent of activity observed in the 100,000 × *g* supernatant of sonicated *E. coli* cells.
*** Per cent of total radiocarbon in labeled nucleic acids recovered chromatographically as [^{14}C]adenosine following alkaline hydrolysis.

terminal adenine nucleotide sequence. Analogous analyses of nucleic acids labeled with 60 % A.S. fractions revealed similar results to those obtained with 40 % A.S. fractions; however, the quantity of enzyme and specific enzymic activity were considerably lower in the former. Further attempts to purify the enzyme present in the 40 % A.S. fraction with Ca–PO_4 gel, DEAE, or XE-64 resin, yielded a 30-fold purified preparation (as compared to sonicate supernatant) but resulted in 90 % loss of activity chromatographed (Table I). It was found that ability to catalyze incorporation into the terminal sequence of nucleic acid was lost gradually when 40 % A.S. fraction was stored at —15°; however, activity responsible for incorporation into the internal part of the nucleic acid chain appeared to be stable.

In contrast to results described by HURWITZ[20], the labeled product formed in incubations described here was rendered acid-soluble on incubation with alkali or RN-ase but not DN-ase, suggesting that labeled adenine nucletoide is incorporated into RNA of *E. coli* under the conditions described above.

The incorporation of [^{14}C]isoleucine into RNA was also catalyzed by sonicate supernatant, pH 4.9-residue, and the various ammonium sulfate fractions. Fig. 2 presents the time (A) and RNA-dependent (B) incorporation of ^{14}C from ATP and isoleucine in presence of 40 % A.S. fraction and supernatant obtained after treatment of the 40 % A.S. fraction with 2 % protamine sulfate (Protamine Super). Considerable incorporation of [^{14}C]adenine nucleotide occurred with 40 % A.S. fraction in absence of added RNA, due to the presence of nucleic acid in this enzyme fraction; addition of RNA to incubations resulted in a corresponding increase in incorporation of radiocarbon into RNA (2B). Treatment of 40 % A.S. fraction with protamine resulted in removal of approx. 98 % of the nucleic acid and 60 % of the protein from this preparation. A more marked RNA-dependent incorporation of [^{14}C]ATP into RNA was observed in incubations with Protamine Super. The effect of added RNA on incorporation of [^{14}C]isoleucine is also presented in Fig 2B. Although incorporation

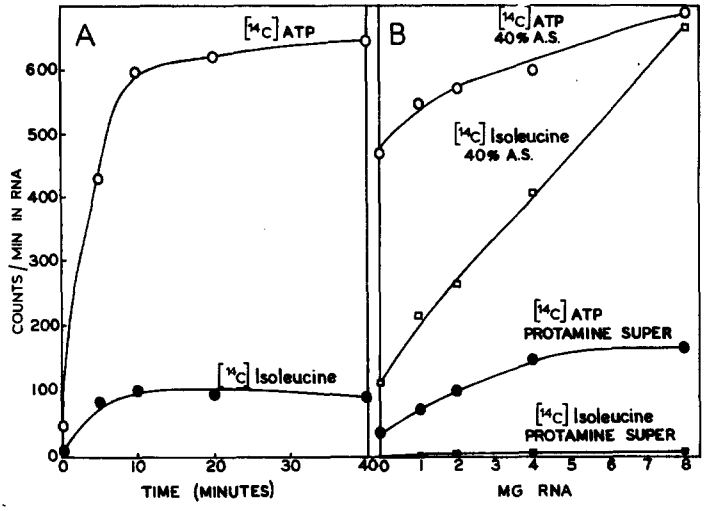

Fig. 2. A. O—O, time-dependent incorporation of [^{14}C]ATP; ●—●, [^{14}C]isoleucine into RNA. Incubation conditions as described in text; enzyme preparation, 40% A.S. fraction, 4 mg protein. B. O—O, the effect of added RNA on the incorporation of [^{14}C]ATP and, □—□, [^{14}C]isoleucine into RNA; 40% A.S. fraction; ●—●, [^{14}C]ATP and ■—■, [^{14}C]isoleucine; Protamine Super.

of [^{14}C]ATP into RNA was catalyzed by Protamine Super, incorporation of [^{14}C]-isoleucine was not detected. This preparation appears to be devoid of amino acid-activating enzymes, as evidenced by failure to observe an amino acid dependent ATP-pyrophosphate exchange reaction with several groups of amino acids. On the other hand, the 40% A.S. fraction catalyzed amino acid dependent exchange of pyrophosphate and ATP with most amino acids tested.

The effect of various incubation components on incorporation of [^{14}C]ATP and [^{14}C]isoleucine into RNA is presented in Table III. Omission of an ATP-generating system or of RNA resulted in a slight decrease (20%) in incorporation of [^{14}C]ATP;

TABLE III

EFFECT OF VARIOUS INCUBATION COMPONENTS ON THE INCORPORATION OF [^{14}C]ATP AND [^{14}C]ISOLEUCINE INTO RNA

Incubation conditions as described in text. Enzyme preparation, 40% A.S. fraction; incubation time, 10 min.

Incubation mixture	*[^{14}C]ATP**	*[^{14}C]isoleucine***
Complete system	1.64	134
Complete-ATP-generating system	1.31	22
Complete-RNA	1.31	58
Complete-CTP	1.18	125
Complete + P–P (2 μmoles)	1.83	—
Complete + P–P (25 μmoles)	0.04	20
Complete + PO_4 (25 μmoles)	1.60	120

* Incubations with radioactive ATP. Values are expressed as specific activity (see under Table I).

** Incubations with radioactive L-isoleucine. Values are expressed as total counts/min incorporated into RNA.

similar experiments with pH 4.9-residue revealed that incorporation was 50 % lower in absence of an ATP-generating system; probably due to presence of ATP-ase in this preparation. Incorporation of [^{14}C]isoleucine was markedly lower in absence of an energy generating system. In the absence of CTP, incorporation of [^{14}C]ATP was about 30 % lower than in its presence while [^{14}C]isoleucine incorporation was not significantly affected. Whereas at low levels of pyrophosphate incorporation of [^{14}C]ATP was slightly stimulated, higher levels were completely inhibitory; monophosphate at equimolar levels did not have any effect on incorporation. Similar results were obtained with [^{14}C]isoleucine incubated in presence of 25 μmoles of pyro- and monophosphate.

Some of the characteristics of the adenine nucleotide incorporating system are presented in Fig. 3. The pH optimum of the reaction, pH 8 (Fig. 3A), is similar to that reported by HECHT *et al.*[17] with ascites cell preparations. The enzyme concentration curve (Fig. 3B) was somewhat linear over the concentration range of 4 mg of protein leveling off at higher concentrations, probably due to limiting amounts of RNA present in these incubations. Magnesium ions were found to be essential (Fig. 3C); manganese at equimolar concentrations could replace Mg^{++} and addition of 10 μmoles of Mn^{++} to incubation mixtures containing Mg^{++} resulted in 35 % stimulation of incorporation. Radioactivity from labeled ADP as well as ATP was

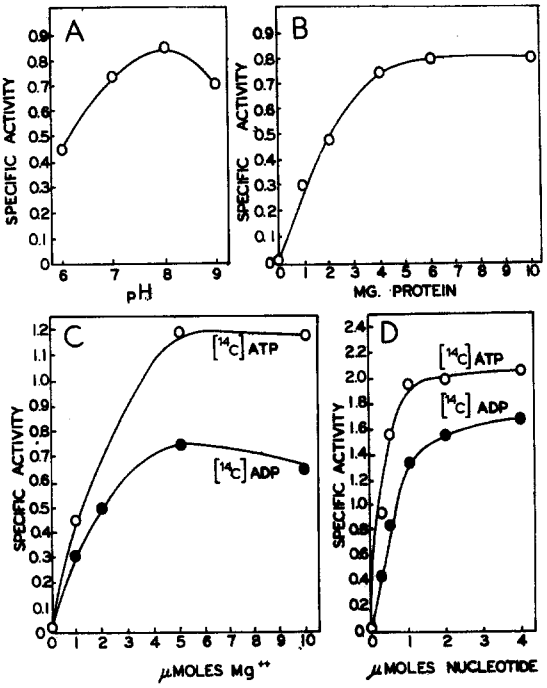

Fig. 3. Characteristics of the adenine nucleotide incorporation system. Incubation conditions as described in text; enzyme preparation, 40 % A.S. fraction; incubation time, 10 min. A. Effect of pH on the incorporation of [^{14}C]ATP into RNA. B. Effect of enzyme concentration on the incorporation of [^{14}C]ATP into RNA. C. O—O, effect of Mg^{++} on the incorporation of [^{14}C]ATP; ●—●, [^{14}C]ADP into RNA (PEP and Py-kinase omitted). D. O—O, effect of labeled substrate concentration on the incorporation of [^{14}C]ATP and ●—●, [^{14}C]ADP into RNA (PEP and Py-kinase omitted).

incorporated into RNA under these conditions; ATP, however, appeared to be more effective than ADP in this process (Fig. 3D). In subsequent experiments with more purified enzyme preparations and [^{14}C]ADP, a more pronounced requirement for an ATP-generating system in incorporation af adenine nucleotide was evident. Chromatographic analysis of alkaline hydrolysates of [^{14}C]RNA labeled in presence of 40 % A.S. fraction and [^{14}C]ADP revelead a pattern of labeling similar to that observed with [^{14}C]ATP. Analyses of the acid-soluble nucleotides in incubations with [^{14}C]ADP, in absence of an ATP-generating system, revealed that approximately 65 % had been converted to ATP within 2 min. These and other results obtained suggest that incorporation of [^{14}C] from ADP probably occurs via the intermediary formation of ATP. In contrast to observations made with labeled ATP and ADP, [^{14}C]AMP was not incorporated in absence of an ATP-generating system.

Preincubation of the enzyme preparations and RNA to remove enzymically the terminal cytidylic and adenylic moieties[16,17] was unsuccessful, probably due to the presence of nuclease activity which rendered RNA incapable of accepting labeled adenine nucleotide even in presence of CTP. Evidence was also obtained which indicated that preincubation of enzyme for 10 min, in absence of RNA, resulted in marked loss of activity. Incorporation of [^{14}C]isoleucine into "preincubated" RNA was considerably lower than that described above in Table III, however, addition of CTP resulted in 30 % stimulation of incorporation under these conditions.

DISCUSSION

Low molecular weight cytoplasmic RNA appears to be involved in incorporation of amino acids into proteins. The formation of RNA-amino acid complex from free amino acids and ATP[5,6,8,9,15,21,22], or from synthetic amino acyl adenylates[8,9], and the transfer of amino acid from RNA to microsomal proteins[7,10] are consistent with this suggestion. The observation presented here, that uptake of [^{14}C]-isoleucine into protein is preceded by incorporation into and release of radioactivity from RNA, is also consistent with the role of RNA as a participant in amino acid incorporation. This RNA appears to contain a specific nucleotide end-sequence consisting of cytosine nucleotides and a terminal adenine nucleotide[16,17,23,24]. Evidence has been presented which suggests that amino acid occurs bound to the 3′ (or 2′)-hydroxyl group of the terminal adenylate moiety of RNA[15,17,18]. The incorporation of cytidine and adenine nucleotides into the terminal sequence of RNA has been reported[17,23–26] and it has been shown that these nucleotide end-units are required for binding of amino acids to RNA[17].

HERBERT[26] has reported the presence of two activities in pigeon liver responsible for incorporation of adenine nucleotides into RNA. One of these, present in the nuclear fraction, catalyzes incorporation into the interior of the RNA molecule; the other, present in cytoplasm, catalyzes incorporation into the end-group position. The presence of separate enzymes which catalyze intranucleotide incorporation of adenine nucleotide[25,27] and of cytidine nucleotide[28,29] from their respective triphosphates into RNA has been reported. The results presented here indicate that the supernatant obtained by high speed centrifugation of sonically-disrupted *E. coli* cells contains enzymes which catalyze amino acid activation, incorporation of amino acids into RNA and protein, and incorporation of the adenylate moiety of ATP

into interior and end-group positions of RNA. A partial resolution of these activities has been obtained. The evidence obtained suggests that, as in the case with mammalian preparations, terminal incorporation of cytidine and adenine nucleotides may be necessary for binding of activated amino acids to RNA in *E. coli*. Unfortunately, due to the presence of nuclease in the purified enzyme preparations, it has not yet been possible to prepare active RNA in which the terminal nucleotide sequence has been removed. Attempts to prepare stable *E. coli* preparations, free of interfering enzymes such as nuclease and myokinase, are in progress.

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