The Enzymic Synthesis of Amino Acyl Derivatives of Ribonucleic Acid

I. THE MECHANISM OF LEUCYL-, VALYL-, ISOLEUCYL-, AND METHIONYL RIBONUCLEIC ACID FORMATION*

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The enzymic formation of enzyme-bound amino acyl adenylates from adenosine triphosphate and amino acid (Equation 1) has been recognized for several years (1–8) and enzymes specific for certain of the amino acids have been isolated in a number of laboratories (2, 9, 10). These same enzymes are now known (11–15) to catalyze a second reaction involving the transfer of the amino acyl moiety from the adenosine phosphate moiety to a specific type of ribonucleic acid (Equation 2). The over-all reaction catalyzed by such amino acyl ribonucleic acid synthetases is summarized in Equation 3.

**Experimental Procedure**

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‡ Enzymes which catalyze these amino acid-selective ATP-PP exchange and ATP-dependent amino acid hydroxamate formation have been referred to as amino acid-activating enzymes (1). Inasmuch as these activities are partial manifestations of the over-all reaction leading to amino acyl RNA formation (12, 16, 17) we propose to designate this class of enzymes as amino acyl RNA synthetases and the enzyme specific for a single amino acid, e.g., leucine, as leucyl RNA synthetase. This nomenclature, we feel, is consistent with the practice of including some indication of the nature of the product formed in the reaction. Moreover, it minimizes any ambiguity arising from situations in which amino acid activation occurs by reactions not involving amino acyl RNA formation, e.g., S-adenosyl methionine (18), glutamine (19), glycineamide ribonucleotide (20, 21) formation, and very likely the formation of peptides (22).
C\textsuperscript{14}-labeled Amino Acids — The uniformly C\textsuperscript{14}-labeled amino acid mixture was obtained from the protein of Chromatium grown in the presence of NaHCO\textsubscript{3} as carbon source (31). The protein was hydrolyzed in 6 \times HCl at 110\degree \text{C} for 18 hours. The specific activity of the amino acids was 2.5 to 3.0 \times 10\textsuperscript{5} c.p.m. per \mu g atom of carbon. DL-Leucine-1-C\textsuperscript{14}, DL-valine-1-C\textsuperscript{14}, and L-methionine-CH\textsubscript{3}-C\textsuperscript{14} were purchased from Isotope Specialties, Inc., and uniformly labeled L-isoleucine-C\textsuperscript{14} was obtained from Volk Radiochemical Company. The specific activities of the amino acids ranged from 3 to 17 \times 10\textsuperscript{4} c.p.m. per \mu mole counted in a windowless gas flow counter.

Miscellaneous—PP\textsubscript{32} was made as previously described (29). Nucleoside mono-, di-, and triphosphates were obtained from the Sigma Chemical Company, and unlabeled amino acids were purchased from the California Foundation for Biochemical Research or from Nutritional Biochemicals. As pointed out elsewhere (26), it was necessary in certain cases to use synthetic preparations of the amino acids to avoid trace contaminations by other amino acids.

Methods

Measurement of Amino Acyl RNA Formation — Depending upon the experiment, one of two assays for amino acyl RNA formation was carried out. The first determined the yield of amino acyl RNA formed when the enzyme, ATP, and amino acids were present in excess and the amount of acceptor RNA was limiting. The standard conditions for this measurement were as follows. The incubation mixture contained in a total volume of 0.5 ml, 50 \mu moles of sodium cacodylate buffer, pH 7.0; 0.5 \mu mole of ATP; 1.0 \mu mole of MgCl\textsubscript{2} (for leucyl- and valyl RNA formation) or 5.0 \mu moles of MgCl\textsubscript{2} (for isoleucyl- and methionyl RNA formation); either 0.3 \mu mole of DL-leucine-1-C\textsuperscript{14}, 0.4 \mu mole of DL-valine-1-C\textsuperscript{14}, 0.03 \mu mole of uniformly labeled L-isoleucine-C\textsuperscript{14} or 0.3 \mu mole of L-methionine-CH\textsubscript{3}-C\textsuperscript{14}; 0.2 to 1.0 \mu mole of acceptor RNA nucleotide; 100 \mu g of crystalline beef serum albumin; 2 \mu moles of reduced glutathiones; 5 \mu moles of potassium chloride (for methionyl RNA formation); and either 0.9, 0.5, 7, or 3 \mu g of protein of the leucyl-, valyl-, isoleucyl-, or methionyl RNA synthetase preparations, respectively. The mixture was incubated at 30\degree for 20 minutes (a time which was sufficient for the reaction to come to completion) and the reaction was stopped by the addition of 0.5 to 1.5 mg of carrier yeast RNA and 3 ml of a cold solution containing 0.5 \mu NaCl and 67\% ethanol. After 5 minutes at 0\degree, the precipitate was centrifuged and washed three times by resuspension in the ethanol-salt mixture. The precipitate was dissolved in 1 ml of 1.5 \times NH\textsubscript{4}OH, and a suitable aliquot was dried in small dishes and counted in a windowless gas flow counter. The results are expressed as millimicromoles of amino acid bound per \mu mole of acceptor RNA nucleotide. Data to be presented below (Fig. 5) show that, under these conditions, the amount of each of the amino acids bound is proportional to the amount of acceptor RNA added.

In contrast to the first assay, which determined the yield of product, the second assay measured the rate of amino acyl RNA formation and was carried out under the conditions described above, except with less enzyme and more acceptor RNA (1.0 to 2.0 \mu moles of RNA nucleotide). The reaction rate was proportional to enzyme concentration over the range shown in Fig. 1.

Measurement of Amino Acyl Adenylicate Formation — The capacity of each of the enzymes to form amino acyl adenylates was measured by the amino acid-dependent exchange of ATP and PP\textsubscript{32} (20). Inasmuch as the rate of amino acyl adenylate formation was the rate determining step in the overall exchange reaction (2, 29), the amino acid-dependent incorporation of PP\textsubscript{32} into ATP actually measured the rate of amino acyl adenylate formation.

For comparisons of amino acyl adenylate and amino acyl RNA formation under the same conditions, the following assay was used. In a volume of 1.0 ml were 100 \mu moles of sodium cacodylate buffer, pH 7.0, 5 \mu moles of MgCl\textsubscript{2}, 2 \mu moles of ATP, 2 \mu moles of PP\textsubscript{32} (specific activity, 0.5 to 1.0 \times 10\textsuperscript{5} c.p.m. per \mu mole), 2 \mu moles of the L-form of leucine, valine, isoleucine, or methionine, 200 \mu g of serum albumin, 4 \mu moles of reduced glutathione (where indicated above), 10 \mu moles of KCl (where indicated above), and enough enzyme to give an incorporation of 0.01 to 0.3 \mu mole of PP\textsubscript{32} into ATP. The mixture was incubated at 30\degree for 15 minutes and the ATP was isolated and counted as previously described (2). All values were corrected for any ATP\textsubscript{32} formed in the absence of amino acid. This blank was always less than 5\% of that observed with amino acid.

Results

Required Components for Enzymatic Synthesis of Amino Acyl RNA Compounds — Formation of the amino acyl RNA derivatives was observed in the presence of ATP, Mg\textsuperscript{2+}, a specific RNA
**TABLE I**

**Requirements for amino acyl RNA formation by amino acyl RNA synthetases from E. coli**

The incubation mixtures and conditions used for measuring the rate of formation of each amino acyl RNA derivative are described under "Methods." The column headings refer to the isolated enzymes which are relatively specific for the amino acids listed (26).

<table>
<thead>
<tr>
<th>Components</th>
<th>Leucine</th>
<th>Valine</th>
<th>Isoleucine</th>
<th>Methionine</th>
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<tr>
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<td>21.0</td>
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<td>3.3</td>
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<td>Minus ATP</td>
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<td>&lt;0.02</td>
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<td>Minus RNA</td>
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<td>&lt;0.2</td>
<td>&lt;0.02</td>
<td>&lt;0.1</td>
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<tr>
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<td>&lt;0.2</td>
<td>&lt;0.02</td>
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</table>

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**TABLE II**

**Formation of ATP from valyl RNA, AMP, and PP**

The complete system contained, per ml, 100 μmoles of sodium cacodylate buffer, pH 7.0, 2 μmoles of MgCl₂, 50 μmoles of potassium fluoride, 200 μg of serum albumin, 2.38 μmoles of valine-C¹⁴ as valyl RNA, 106 μg of valyl RNA synthetase protein, 0.10 μmole of AMP, and 0.06 μmole of PP₃ (3.4 × 10⁶ c.p.m. per μmole). Valyl RNA was hydrolyzed to free acceptor RNA and valine by heating at 55° for 15 minutes at pH 9. The incubation was at 30° for 15 minutes and the reaction was stopped by boiling for 2 minutes. An aliquot of the reaction mixture was removed and the amount of valyl RNA remaining was determined by the amount of radioactivity still precipitable after the addition of the NaCl-ethanol mixture described under "Methods." After the addition of unlabeled ADP, ATP, and PP, to the remainder of the reaction mixture, the nucleotides were adsorbed on charcoal. After the charcoal was washed several times with 0.01 M NaOH and chromatographed on a Dowex 1-C₁³ column (32). In control experiments in which PP, was omitted, there was no disappearance (<5%) of valyl RNA or ATP when added in the amounts obtained in the experiment. Over 90% of the radioactivity was eluted with the carrier ATP, whereas in the experiment with hydrolyzed valyl RNA, less than 5% of the P³₂ appeared with the ATP. In the former case, the specific activity of the ATP was essentially constant over the entire peak. The isolated material was further identified as ATP by the following two experiments. After reaction of the ATP³₂ with glucose and hexokinase, 45% of the P³₂ was isolated in the glucose 6-phosphate and 55% in the ADP. With an excess of valyl RNA synthetase, L-valine, and unlabeled PP₃, under conditions of the ATP-PP₃ exchange reaction (36), 59% of the T³₂ in the ATP was found in the PP₃ fraction.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Valyl RNA</th>
<th>ATP</th>
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</thead>
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<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Complete</td>
<td>1.19</td>
<td>0.06</td>
</tr>
<tr>
<td>Complete system with hydrolyzed valyl RNA</td>
<td>0.04</td>
<td>0.05</td>
</tr>
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</table>

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**Fig. 2. Reversibility of amino acyl RNA synthesis.** The C¹⁴ labeled mixed amino acyl RNA was prepared by incubating 2000 μmoles of cacodylate buffer, pH 7.0, 90 μmoles of MgCl₂, 8 μmoles of ATP, 1.17 × 10⁶ c.p.m. of the C¹⁴-amino acid mixture, 93 μmoles of acceptor RNA, and 8.0 mg of protein of a sonic extract of E. coli in a volume of 20 ml for 60 minutes at 30°. The product was isolated by the addition of NaCl to a concentration of 1.5 m followed by 2 volumes of cold ethanol. After chilling the mixture, the precipitated product was removed by centrifugation and extracted with 0.01 M cacodylate buffer, pH 7.0. Denatured protein was removed by centrifugation, and the process of precipitation, buffer extraction, and removal of denatured protein was repeated twice more. The final product was dissolved in 0.02 M succinate buffer, pH 6.

The amino acyl RNA was incubated with 20 μmoles of cacodylate buffer, pH 7.0, 15 μmoles of MgCl₂ and, where indicated, approximately 2 μmoles of C¹⁴-amino acid mixture prepared from Chromatium, 2 μmoles of AMP, 2 μmoles of PP₃, 4 μmoles of P₃, and 0.2 mg of protein of a sonic extract of E. coli in a volume of 0.5 ml, for the indicated time at 30°. The amount of amino acid remaining bound to the RNA was determined by measuring the amount of C¹⁴-amino acid still precipitable by 0.1 M perchloric acid. The abbreviation used is: Enz, enzyme.
**Table III**

**Determination of equilibrium constant of L-valyl RNA formation**

For each experiment, 350 µmoles of sodium cacodylate buffer, pH 7.0, 7 µmoles of MgCl₂, 0.7 mg of serum albumin, 175 µmoles of KF, and the reactants shown in the table were incubated in a volume of 3.5 ml. The valyl RNA was labeled with L-valine-1-C¹⁴ (6 X 10⁶ c.p.m. per µmole). Samples were removed at 2, 3, 4, 5, 10, 15, and 20 minutes and the valyl RNA formed or remaining was determined as described in the standard assay procedure. In each case, the reaction was followed until no further change in the amount of valyl RNA could be detected. Completion of the reaction occurred by 5 minutes, and no change was measurable up to 20 minutes. The concentration of RNA is expressed as millimicromoles of valine-specific acceptor sites. The concentration of the RNA was calculated by the difference between the amount of valine-specific acceptor RNA added and the amount of valyl RNA formed or from the amount of valyl RNA which disappeared. Inasmuch as the concentrations of each of the other components was large compared to the amount of reaction which had occurred, the initial concentrations of each were used in the calculation. In separate experiments, it was shown that under these conditions there was no detectable destruction of the valine acceptor RNA chains nor was there any disappearance of ATP, AMP, or PP₃ (<4%) when added separately.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Initial concentrations (µmols/ml)</th>
<th>Final concentrations</th>
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<td>PP₃</td>
<td>ATP</td>
</tr>
<tr>
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<td>0.40</td>
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<td>0.40</td>
</tr>
<tr>
<td>2</td>
<td>0.40</td>
<td>0.51</td>
<td>0.80</td>
</tr>
<tr>
<td>3</td>
<td>0.40</td>
<td>0.51</td>
<td>0.80</td>
</tr>
<tr>
<td>4</td>
<td>0.40</td>
<td>0.51</td>
<td>0.80</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* K = \( \frac{(\text{AMP})(\text{PP₃})(\text{Valyl RNA})}{(\text{ATP})(\text{Valine})(\text{RNA})} \)

![Fig. 3](image)

Fig. 3. The equilibrium position for the valine incorporation reaction. The incubation conditions are described in Table III. O—O, experiment 1; O—O, experiment 2; O—O, experiment 3; O—O, experiment 4.

That the previous observations do represent reversal of amino acyl RNA synthesis was established by the finding that incubation of C¹⁴-valyl RNA with AMP, PP₃, and the specific valyl RNA synthetase resulted in essentially complete removal of the valine from the RNA and stoichiometric formation of ATP (Table II). No ATP₃ formation is observed when an equivalent amount of RNA and valine is substituted for the valyl RNA.

Determination of the equilibrium constant for valyl RNA formation was made by measuring the steady state concentration of valyl RNA in the presence of the other components of the system (Table III and Fig. 3). The average \( K'_e \) value of 0.32 showed that there was little change in free energy resulting from the formation of valyl RNA at the expense of the cleavage of ATP.

**Existence of Specific Acceptor RNA for Each Amino Acid**

An examination of the kinetics of amino acyl RNA formation showed that the reaction proceeded linearly with time and then reached a limit (Fig. 4). This limit was not appreciably increased (less than 5%) by the addition of up to 5 times more enzyme, ATP, or amino acid, whether added initially or when the reaction had stopped. The addition of 2.6 µg of crystalline inorganic pyrophosphatase did not affect the extent of amino acyl RNA formation. However, the addition of acceptor RNA, either at the beginning of the reaction or at the time the reaction ceased, lead to an increased yield of amino acyl RNA. If in each case the reaction was allowed to proceed to completion in the presence of varying amounts of acceptor RNA, the amount of amino acyl RNA formed was a linear function of the amount of acceptor RNA added (Fig. 5). It should be noted, however, that the yield of each amino acyl RNA was different. Thus, although...
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**FIG. 4.** Kinetics of amino acyl RNA formation. The conditions used were those described for the usual assay of amino acyl RNA formation except that 1.2, 0.1, 0.4, or 0.5 pg of leucyl-(L), valyl-(V), isoleucyl-(I), or methionyl-(M) RNA synthetase protein, respectively, and 1.0 pmole of acceptor RNA nucleotide were used.

**FIG. 5.** Formation of amino acyl RNA as a function of the amount of acceptor RNA. The conditions used were those described under “Methods.” Abbreviations are as in Fig. 4.

The RNA acted stoichiometrically with each amino acid, for a given amount of acceptor RNA the amount of amino acyl RNA formed varied with the amino acid.

Two possible interpretations of this result are that (a) there was a single binding site which reacted with each amino acid to a different extent, or (b) there existed different and specific sites for the individual amino acids. These alternatives were distinguished by the following experiments (Table IV). When leucine, valine, and methionine were present together, the total amount of amino acid linked to the acceptor RNA was equal to the sum of the amounts obtained when each amino acid was present by itself (Experiment 1). Moreover, saturation of the acceptor RNA with one amino acid (e.g. L-valine) did not affect the amount of any other amino acid (e.g. L-leucine or L-methionine) which could subsequently be linked to the acceptor RNA (Experiment 2). These data ruled out the common binding site hypothesis but were consistent with the existence of a limited and fixed number of binding sites, each specific for a particular amino acid.

Further support for this view has come from studies on the destruction of amino acid acceptor sites by periodate (28). This work showed that periodate oxidation of acceptor RNA destroys the ability to accept all amino acids. However, similar treatment of leucyl-, valyl-, or methionyl RNA followed by removal of the amino acid yielded preparations of RNA which could accept only that amino acid which was linked to the RNA during the exposure to periodate. Since, according to currently accepted ideas of RNA structure, polynucleotide chains are un-
branch and therefore the only cis-hydroxyl configuration resides on the terminal nucleotide with a free 3'-hydroxyl group, it may be inferred that each amino acid is linked exclusively to either the 2'- or 3'-hydroxyl group of the terminal nucleotidyl ribose unit of individual RNA molecules. For the leucine- and valine-specific polynucleotide chains this terminal nucleotide is adenylate (28), although it is now clear that, in the acceptor RNA of E. coli, adenylate is the sole terminal nucleotide containing a free 3'-hydroxyl group on the ribose moiety (27).

Heterogeneity of Acceptor RNA Chains Reacting with Single Amino Acid—The conclusion stated above predicts that acceptor RNA represents a heterogeneous population of polynucleotide chains, each chain being specific for a particular amino acid. It is essential before considering any analysis of the chemical basis of the amino acid specificity of acceptor RNA to know whether there exists a second order of heterogeneity, namely, whether all the chains reacting with a particular amino acid are identical. The following experiments suggested that they are not.

Acceptor RNA from E. coli bound methionine to a different extent depending upon whether the methionyl RNA synthetase from E. coli or yeast was used (Fig. 6). Although the amount of methionine fixed was a direct function of the acceptor RNA added, the slopes of the two curves differed by a factor of about 2.5; that is, 2.5 times more methionine was bound per unit of RNA when the synthetase from E. coli was used as compared with the one from yeast. Although the addition of more yeast methionyl RNA synthetase, ATP, or methionine did not increase the yield of methionyl RNA, it was clear that there still were sites available to accept methionine. This is shown by the experiment (Table V) in which the E. coli synthetase was added when the reaction with the yeast enzyme had come to completion. The reciprocal experiment, in which the acceptor RNA was retested with methionine with the E. coli enzyme and then exposed to the yeast enzyme, showed no additional formation of methionyl RNA. It may be inferred from this result that of the polynucleotide chains specific for methionine, 40% can function with either enzyme, whereas 60% of the chains are available only to the E. coli synthetase.

Support for this interpretation was obtained by the periodate oxidation technique for selectively inactivating those polynucleotide chains not linked to amino acids (28). Samples of methionyl RNA prepared with the E. coli or yeast synthetases were treated with periodate, reisolated, and then the amino acids were removed with alkali. The regenerated acceptor RNA preparations were retested for their capacity to accept methionine with each of the enzymes (Table VI). When the methionyl RNA was prepared with E. coli enzyme, all sites specific for methionine survived the periodate oxidation when tested with either enzyme. On the other hand, when methionyl RNA was prepared with the yeast enzyme, 60% of the chains which accept methionine were inactivated as judged by the test with the enzyme from E. coli but all were conserved when assayed with the yeast enzyme. These data show that within the population of acceptor RNA molecules there were at least two distinguishable classes of polynucleotide chains which could accept methionine.

Nature of Enzymes Catalyzing Amino Acyl RNA Formation—The enzyme preparations used in the present studies were purified on the basis of their activity for amino acyl adenylate formation (26). Although these same preparations catalyzed the formation of the amino acyl RNA derivatives, it was not clear which of the following hypotheses was operative.

1. The formation of the specific enzyme-amino acyl adenylate complex may be followed by a nonenzymic transfer of the amino acid to the RNA.
2. There may be a single amino acid-specific enzyme which catalyzes the formation of an amino acyl adenylate and the transfer of that amino acid from the adenylate moiety to the acceptor RNA.
3. There may be required in addition to the enzyme forming the amino acyl RNA derivative (a) separate specific amino acyl transferases for linking each amino acid residue to the appropriate acceptor RNA, or (b) a single amino acyl transferase which
transfers any amino acyl group but only to the appropriate acceptor RNA chain. Although nonenzymic acylation of RNA by amino acyl adenylates has been observed, the amino acids appeared to be bound to any RNA and in a variety of linkages (24). Such a mechanism would therefore not account for the fact that only a particular fraction of RNA functions as an amino acid acceptor (11, 27). It also seems unlikely that a nonenzymic mechanism would manifest the high degree of specificity inherent in linking each amino acid exclusively to the terminal nucleotide of a particular RNA chain. Furthermore, the different yields of methionyl RNA produced in the presence of two different methionyl RNA synthetases are inconsistent with a nonenzymic transfer reaction. Since hypotheses 2 and 3 predict that the synthesis of a given amino acyl RNA derivative is preceded by the formation of the corresponding amino acyl adenylate, it is implicit in either alternative that the formation of each amino acyl RNA compound must be at least as specific with respect to the amino acid as is the synthesis of the amino acyl adenylate. Table VII shows that with four enzymes from E. coli and one from yeast only that amino acid which is converted to the adenylate is linked to the acceptor RNA. The only deviation from an exact correlation of the two specificities is the case of the isoleucyl RNA synthetase. Although this enzyme can form both isoleucyl- and valyl adenylates (26), it synthesizes only isoleucyl RNA. The reasons why the valyl moiety is not transferred to the acceptor RNA (which can accept valine from the valyl RNA synthetase fraction) remain to be determined.

Hypothesis 2, in contrast to 3, predicts that the ratio of the activity for amino acyl adenylate formation and amino acyl RNA synthesis must be constant throughout the purification of the enzymes. Any alteration in this ratio during purification would suggest the existence of separable activities. Table VIII shows that the ratio of activities for methionyl adenylate and methionyl RNA formation was constant during the course of an approximately 100-fold purification of the methionyl RNA synthetase from yeast. Similar findings have been made with the leucyl- and methionyl RNA synthetases of E. coli. The ratio of leucyl adenylate to that of leucyl RNA formation was measured as described elsewhere (26), and the ratio of amino acyl RNA synthesis was determined as described under "Methods."

### Table VI

<table>
<thead>
<tr>
<th>Source of enzyme used to measure methionyl RNA formation</th>
<th>Methionyl RNA formation</th>
<th>Periodate-treated methionyl RNA prepared with E. coli enzyme</th>
<th>Yeast enzyme</th>
<th>umoles/umole RNA nucleotide</th>
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</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>Original RNA</td>
<td>0.25</td>
<td>0.23</td>
<td>0.10</td>
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<tr>
<td>Yeast</td>
<td>Original RNA</td>
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### Table VII

<table>
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<th>Enzyme</th>
<th>Amino acid tested</th>
<th>Amino acyl adenylate formation</th>
<th>Amino acyl RNA formation</th>
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<td>Valine</td>
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<td>Methionine (E. coli)</td>
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<td>Valine</td>
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<td>&lt;0.001</td>
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<td></td>
<td>Valine</td>
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<td>&lt;0.001</td>
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<tr>
<td></td>
<td>Phenylalanine</td>
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### Table VIII

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Methionyl adenylate formation (A)</th>
<th>Methionyl RNA formation (B)</th>
<th>A/B × 10^3</th>
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<tr>
<td>Crude extract</td>
<td>0.61</td>
<td>0.23</td>
<td>2.6</td>
</tr>
<tr>
<td>Alcohol Fraction 2</td>
<td>14.8</td>
<td>5.2</td>
<td>2.8</td>
</tr>
<tr>
<td>Ammonium sulfate Fraction 1</td>
<td>23.2</td>
<td>8.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Ammonium sulfate Fraction 2</td>
<td>44.0</td>
<td>16.2</td>
<td>2.7</td>
</tr>
<tr>
<td>Alumina C7 gel eluate</td>
<td>55.7</td>
<td>19.7</td>
<td>2.8</td>
</tr>
</tbody>
</table>

* The various enzyme fractions were prepared as previously described (35) and the specific enzyme activities for the formation of methionyl adenylate and methionyl RNA were measured as described under "Methods."
TABLE IX
Comparison of rate of amino acyl adenylate and amino acyl RNA formation under similar conditions

The rate of amino acyl RNA formation was measured as already described, and the synthesis of amino acyl adenylates was determined by the amino acid-dependent ATP-PPi exchange (26). To compare the two rates, however, the ATP-PPi exchange reaction was carried out under the same conditions used for amino acyl RNA formation, except that unlabeled amino acid and 0.002 M ATP and PPi were added.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Amino acyl adenylate formation</th>
<th>Amino acyl RNA formation</th>
<th>A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucyl RNA synthetase</td>
<td>120</td>
<td>2.8</td>
<td>43</td>
</tr>
<tr>
<td>Valyl RNA synthetase</td>
<td>574</td>
<td>26.4</td>
<td>22</td>
</tr>
<tr>
<td>Isoleucyl RNA synthetase</td>
<td>450</td>
<td>3.3</td>
<td>145</td>
</tr>
<tr>
<td>Methionyl RNA synthetase (E. coli)</td>
<td>244</td>
<td>3.0</td>
<td>81</td>
</tr>
<tr>
<td>Methionyl RNA synthetase (yeast)</td>
<td>22.1</td>
<td>0.007</td>
<td>3200</td>
</tr>
</tbody>
</table>

and (b) if the hypothetical enzyme catalyzing the transfer of the amino acyl group to RNA were present in excess in both the crude and subsequent enzyme fractions. The first point can only be answered by more extensive purification studies. The second objection, however, is eliminated by the observation that with each enzyme preparation it is the formation of the amino acyl adenylate which is by far the faster reaction, i.e. the transfer of the amino acyl group to the RNA is the rate-limiting step (Table IX). Note that this difference in the rate of amino acyl adenylate- and amino acyl RNA formation is of the order of 20- to 140-fold and in one case is about 3000 times. These data imply that the transfer of the amino acyl moiety from the enzyme-amino acyl adenylate complex to the acceptor RNA is the rate-limiting reaction. It should be pointed out that the slow transfer of methionine by the yeast methionyl RNA synthetase may be due to the use of the acceptor RNA from E. coli.

At present, our data are consistent with the hypothesis that a single enzyme catalyzes both the formation of a specific enzyme-amino acyl adenylate complex and the transfer of the amino acyl group to the acceptor RNA.

DISCUSSION

The findings reported here and those presented recently by other workers (16, 17) indicate strongly that the so-called “amino acid-activating enzymes” are in essence amino acyl RNA synthetases. Whereas the initial reaction between ATP, amino acid, and a specific enzyme results in the formation of an enzyme-bound amino acyl adenylate, in the presence of the appropriate acceptor RNA chain, the amino acyl moiety is transferred to the RNA and more specifically to the 2'- or 3'-hydroxyl group of the terminal nucleotidyl ribose. A mechanism of this type not only minimizes spontaneous destruction of the highly unstable free amino acyl adenylate under physiological conditions (34, 36), but it also eliminates the requirement of additional specific enzymes to form each amino acyl RNA derivative. Indeed, it has recently been shown (37) that synthetic tryptophanyl adenylate, in the presence of purified tryptophanyl RNA synthetase, serves as tryptophan donor to amino acid acceptor RNA. From a mechanistic view, the amino acyl RNA synthetases are analogous to the enzymes which catalyze the formation of acyl-CoA derivatives (38-40), pantothentic acid (41), and carnitine (42) in that there is a primary formation of an enzyme-bound acyl adenylate and a subsequent transfer of the acyl moiety to an acceptor molecule.

Recently Zilleg et al. (43) reported that the yield of amino acyl RNA is a function of the amount of amino acyl RNA synthetase added. We have not observed this phenomenon in our studies. Rather, only the initial rate of amino acyl RNA formation is influenced by the amount of enzyme present. The final yield of amino acyl RNA is with sufficient time, independent of enzyme concentration and depends entirely on the amount of acceptor RNA present. In our early studies of valyl RNA synthesis, we observed that the yield of valyl RNA did vary with the amount of enzyme added. This, however, was found to be due to inactivation of the enzyme during the course of the reaction, and it could be circumvented by the addition of serum albumin to the incubation mixture. Under these latter conditions, the enzyme continues to act until the acceptor RNA is saturated with respect to valine.

The finding that amino acyl RNA synthesis is reversible is surprising in light of the ester linkage between amino acid and the acceptor RNA. The K_m of 0.32 for valyl RNA synthesis and the values of 0.7 and 0.37 reported for threonyl RNA synthesis (16, 17) indicate that the amino acyl moiety is maintained at a high energy level. Whether this thermodynamic activation of the amino acid is a consequence of an adjacent hydroxyl group on the ribose or to some other structural feature of the combination remains to be determined.

An interesting aspect of the mechanism of amino acyl RNA synthesis concerns the basis of the specificity in linking each amino acid to the appropriate polynucleotide chain. This question may be considered on the basis of the two reactions catalyzed by the enzyme: in the first phase, the enzyme forms a specific enzyme-amino acyl adenylate complex and in the second this complex reacts with a specific acceptor RNA chain to form the appropriate amino acyl RNA derivative. With respect to the first phase of the reaction, it is clear from studies with the purified amino acyl RNA synthetases that they exhibit a relatively high degree of selectivity for a single naturally occurring amino acid. The significance of the slight activity sometimes noted with other amino acids is difficult to assess in the absence of more precise data concerning the purity of the enzyme preparations and the amino acid substrates (26). There are two exceptions, however, which should be noted. The purified isoleucyl RNA synthetase forms valyl adenylate as well as isoleucyl adenylate, and the valyl RNA synthetase forms threonyl adenylate (26). In both cases, the K_m for the “unnatural” substrate is about 100-fold higher than that for the “natural” one, so that with equal concentrations of the “natural” and “unnatural” amino acids, the enzyme reacts almost exclusively with the “natural” substrate.

In an analysis of the factors which control the transfer of the amino acyl moiety to its specific acceptor RNA chain, several aspects must be considered. First, we might ask, “What portions of the synthetase-amino acyl adenylate complex function in selecting the appropriate acceptor RNA chain?” Our data suggest that both the amino acid and protein moieties function in this selection. The fact that the enzyme-isoleucyl adenylate complex transfers isoleucine to the isoleucine-specific RNA chain but that the same enzyme in combination with valyl adenylate does not transfer the valine to any acceptor RNA chain empha-
sizes the role of the amino acid side chain. Similarly, the ob-
servation that different amounts of methionyl RNA are formed
when methionyl adenylate is linked to two different proteins
points to a specific function for the protein in the selection of
the correct RNA chain. There is no information at present con-
cerning the chemical structures of the RNA chains which allow
for the “recognition” between a specific enzyme-amino acid
adenylate complex and its appropriate RNA chain. Clearly, the
terminal nucleotide, to which the amino acid is bound, cannot
account for this specificity since for each amino acid this unit is
adenylic acid (16, 24, 25). Whether the differentiation between
acceptor RNA chains relies on differences in nucleotide sequence,
configuration, or to some unknown factors remains to be deter-
mined. The indications that there may be heterogeneity
amongst RNA chains specific for a single amino acid may serve
to complicate the analysis of this problem.

SUMMARY

Purified enzymes from Escherichia coli which form L-leucyl-
L-valyl-, L-isoleucyl-, or L-methionyl adenylates also catalyze
the formation of the corresponding amino acyl ribonucleic acid
derivatives. Each amino acid is bound through its carboxyl
group to the terminal deoxynucleotide (2'- or 3'-hydroxyl end) of
specific polynucleotide chains. The synthesis of amino acyl ribo-
nucleic acid derivatives is reversible, and in the case of L-valyl
ribonucleic acid formation the equilibrium constant is 0.32.
Indications were obtained that the polynucleotide chains specific
for accepting L-methionine are heterogeneous.

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