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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[1] is summarized in Equation 3.

$$\text{AMP-PP} + \text{RCHNH}_2\text{COOH} + \text{enzyme} \xleftrightarrow{Mg^{++}} \text{enzyme—AMP}\overset{\overset{O}{\|}}{C}\text{CHNH}_2\text{R} + \text{PP}_i \quad (1)$$

$$\text{Enzyme—AMP}\overset{\overset{O}{\|}}{C}\text{CHNH}_2\text{R} + \text{RNA—OH} \rightarrow \text{RNA—O}\overset{\overset{O}{\|}}{C}\text{CHNH}_2\text{R} + \text{AMP} \quad (2)$$

$$\text{AMP-PP} + \text{RCHNH}_2\text{COOH} + \text{RNA—OH} \xleftrightarrow{Mg^{++}} \text{RNA—O}\overset{\overset{O}{\|}}{C}\text{CHNH}_2\text{R} + \text{AMP} + \text{PP}_i \quad (3)$$

By the above reaction, the amino acids are bound to the acceptor ribonucleic acid through an ester linkage to the 2′- or 3′-hydroxyl group of the terminal nucleotidyl ribose moiety (23–25), and where this has been examined, each amino acid is linked to a terminal adenylic acid (16, 23–25).

The results of our investigations on the mechanism of amino acyl ribonucleic acid formation are reported in the present communication. The purification and characterization of the specific amino acyl ribonucleic acid synthetases and the amino acid-acceptor ribonucleic acid from *Escherichia coli* are presented in the following papers (26, 27). The fourth communication (28) describes the enzymic removal and resynthesis of the 3′-hydroxy-ended trinucleotide portion of the acceptor ribonucleic acid.

EXPERIMENTAL PROCEDURE

Materials

Enzymes—In some of our earlier studies and in several experiments reported here, extracts of *E. coli* and a mixture of uniformly C^{14}-labeled amino acids were used as a means of generating highly labeled amino acyl adenylates. Extracts were prepared from cells grown as described in Paper II (26) by treatment of a washed cell suspension (4 ml of 0.05 M glycylglycine buffer, pH 7.0, per g wet weight of cells) in a cooled Raytheon 10 kc sonic oscillator for 15 minutes or by disruption in a Waring Blendor with glass beads (26). Both types of extract were dialyzed for about 24 hours against 30 to 40 volumes of 0.01 M Tris buffer, pH 8.0.

The leucyl-, valyl-, isoleucyl-, and methionyl RNA synthetases from *E. coli* were prepared as described in Paper II of this series (20); the isolation of the methionyl RNA synthetase from yeast has been reported previously (2, 29).

Crystalline inorganic pyrophosphatase (30) was kindly supplied by Drs. G. Perlmann and M. Kunitz.

Amino Acid-Acceptor RNA Preparations—The acceptor RNA was isolated as described by Ofengand *et al.* (27), and in almost all cases, the material eluted from Ecteola (Brown Company) was used. The concentration of the acceptor RNA is expressed in terms of its nucleotide content and determined by its optical density at 260 mμ in 0.01 N KOH with a value of 10.0 as equal to 1 μmole of RNA nucleotide.

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[1] Enzymes which catalyze an amino acid-dependent 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^{14}-labeled Amino Acids—The uniformly C^{14}-labeled amino acid mixture was obtained from the protein of *Chromatium* grown in the presence of $NaHC^{14}O_3$ as carbon source (31). The protein was hydrolyzed in 6 N HCl at 110° for 18 hours. The specific activity of the amino acids was 2.5 to 3.0 × 10^5 c.p.m. per μg atom of carbon. DL-Leucine-1-C^{14}, DL-valine-1-C^{14}, and L-methionine-CH_3-C^{14} were purchased from Isotope Specialties, Inc., and uniformly labeled L-isoleucine-C^{14} was obtained from Volk Radiochemical Company. The specific activities of the amino acids ranged from 3 to 17 × 10^6 c.p.m. per μmole counted in a windowless gas flow counter.

Miscellaneous—PP_i[-2] 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 μmoles of sodium cacodylate buffer, pH 7.0; 0.5 μmole of ATP; 1.0 μmole of $MgCl_2$ (for leucyl- and valyl RNA formation) or 5.0 μmoles of $MgCl_2$ (for isoleucyl- and methionyl RNA formation); either 0.3 μmole of DL-leucine-1-C^{14}, 0.4 μmole of DL-valine-1-C^{14}, 0.03 μmole of uniformly labeled L-isoleucine-C^{14} or 0.3 μmole of L-methionine-CH_3-C^{14}; 0.2 to 1.0 μmole of acceptor RNA nucleotide; 100 μg of crystalline beef serum albumin; 2 μmoles of reduced glutathione; 5 μmoles of potassium chloride (for methionyl RNA formation); and either 0.9, 0.5, 7, or 3 μg of protein of the leucyl-, valyl-, isoleucyl-, or methionyl RNA synthetase preparations, respectively. The mixture was incubated at 30° 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 M NaCl and 67% ethanol. After 5 minutes at 0°, 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 N NH_4OH, 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 μmole 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 μmoles of RNA nucleotide). The reaction rate was proportional to enzyme concentration over the range shown in Fig. 1.

Measurement of Amino Acyl Adenylate 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_i[32] (26). Inasmuch as the rate of amino acyl adenylate formation was the rate-determining step in the over-all exchange reaction (2, 29), the amino acid-dependent incorporation of PP_i[32] into ATP actually measured the rate of amino acyl adenylate formation.

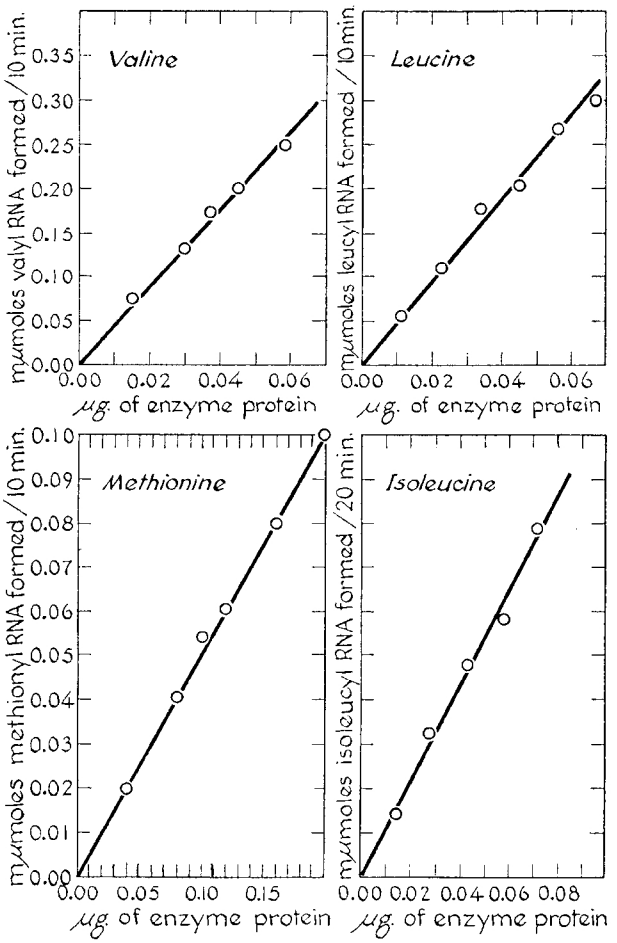

FIG. 1. Linear relationship of the rate of amino acyl RNA formation with enzyme concentration. See text for conditions.

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 μmoles of sodium cacodylate buffer, pH 7.0, 5 μmoles of $MgCl_2$, 2 μmoles of ATP, 2 μmoles of PP_i[32] (specific activity, 0.5 to 1.0 × 10^5 c.p.m. per μmole), 2 μmoles of the L-form of leucine, valine, isoleucine, or methionine, 200 μg of serum albumin, 4 μmoles of reduced glutathione (where indicated above), 10 μmoles of KCl (where indicated above), and enough enzyme to give an incorporation of 0.01 to 0.3 μmole of PP_i[32] into ATP. The mixture was incubated at 30° for 15 minutes and the ATP was isolated and counted as previously described (2). All values were corrected for any ATP^{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^{++}, 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).

Components	Leucine	Valine	Isoleucine	Methionine
	μmoles/mg/hour			
Complete	3.1	21.0	3.3	3.3
Minus ATP	<0.03	<0.2	<0.02	0.1
Minus RNA	<0.03	<0.2	<0.02	<0.1
Minus Mg^{++}	0.33	0.6	0.15	<0.1
Minus enzyme	<0.03	<0.2	<0.02	<0.1

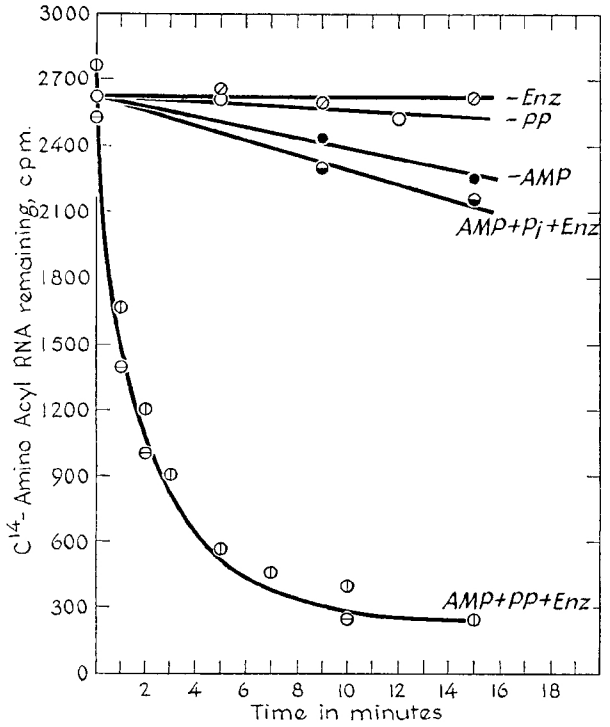

FIG. 2. Reversibility of amino acyl RNA synthesis. The C^{14} labeled mixed amino acyl RNA was prepared by incubating 2000 μmoles of cacodylate buffer, pH 7.0, 90 μmoles of $MgCl_2$, 8 μmoles of ATP, 1.17×10^7 c.p.m. of the C^{14}-amino acid mixture, 93 μmoles of acceptor RNA, and 8.0 mg of protein of 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 $MgCl_2$ and, where indicated, approximately 2 μmoles of C^{12}-amino acid mixture prepared from *Chromatium*, 2 μmoles of AMP, 2 μmoles of PP_i, 4 μmoles of P_i, 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 C^{14}-amino acid still precipitable by 0.6 M perchloric acid. The abbreviation used is: Enz, enzyme.

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 $MgCl_2$, 50 μmoles of potassium fluoride, 200 μg of serum albumin, 2.38 mμmoles of valine-C^{14} as valyl RNA, 106 μg of valyl RNA synthetase protein, 0.10 μmole of AMP, and 0.06 μmole of PP_i^{32} (3.4×10^8 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_i to the remainder of the reaction mixture, the nucleotides were adsorbed on charcoal. After the charcoal was washed several times with 0.01 M PP_i, the nucleotides were eluted with 50% ethanol containing 0.3 M NH_4OH and chromatographed on a Dowex 1-Cl^- column (32). In control experiments in which PP_i 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 P^{32} 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 ATP^{32} with glucose and hexokinase, 45% of the P^{32} was isolated in the glucose 6-phosphate and 55% in the ADP. With an excess of valyl RNA synthetase, L-valine, and unlabeled PP_i, under conditions of the ATP-PP_i^{32} exchange reaction (26), 95% of the P^{32} in the ATP was found in the PP_i fraction.

Conditions	Valyl RNA			ATP	
	Initial	Final	Δ	Final	Δ
	mμmoles			*mμmoles*	
Complete system	1.19	0.06	−1.13	1.11	+1.06
Complete system with hydrolyzed valyl RNA	0.04			0.05	

fraction isolated from *E. coli*, a C^{14}-labeled amino acid, and the purified enzyme fraction capable of converting that amino acid to the corresponding amino acyl adenylate (Table I). In each case, omission of any one of the cited components resulted in a marked decrease in the rate of amino acyl RNA synthesis. Substitution of the ATP by UTP, GTP, CTP, dATP, or ADP lead to a decrease in the rate to less than 1%. Ribosomal RNA (27) from *E. coli*, or the equivalent fractions from animal and other bacterial sources, and synthetic polynucleotides prepared with polynucleotide phosphorylase (31), failed to function as amino acid acceptors under these conditions (32).

Reversibility of Amino Acyl RNA Formation—Equation 3 predicts that amino acyl RNA formation is reversible. Substantiation of this prediction was given by the following experiments. When amino acid-acceptor RNA, to which a mixture of C^{14}-labeled amino acids had been linked, was incubated with AMP, PP_i and a dialyzed extract of *E. coli*, there was a rapid removal of the labeled amino acids from the RNA (Fig. 2). This occurred whether or not a pool of unlabeled amino acids was added. If either the AMP, PP_i, or the extract was omitted, or if UMP or CMP replaced AMP, or if P_i was substituted for PP_i, the rate

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 $MgCl_2$, 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-C^{14} (6×10^6 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 PP_i (<4%) when added separately.

Experiment No.	Initial concentrations							Final concentrations Valyl RNA	K^*
	AMP	PP_i	ATP	Valine	Valyl RNA	RNA	Valyl RNA synthetase		
	μmoles/ml						*units/ml*	*μmoles/ml* × 10^4	
1	0.40	1.02	0.40	0.52	1.36×10^{-3}	0	2.3	2.1	0.36
2	0.40	0.51	0.80	0.52	1.36×10^{-3}	0	1.2	5.4	0.32
3	0.40	0.51	0.80	0.52	0	1.36×10^{-3}	0.3	4.8	0.28
4	0.40	0.51	0.80	0.52	1.36×10^{-3}	0	0.3	5.1	0.30
Average									0.32

$$^*K = \frac{(AMP)(PP_i)(Valyl\ RNA)}{(ATP)(Valine)(RNA)}$$

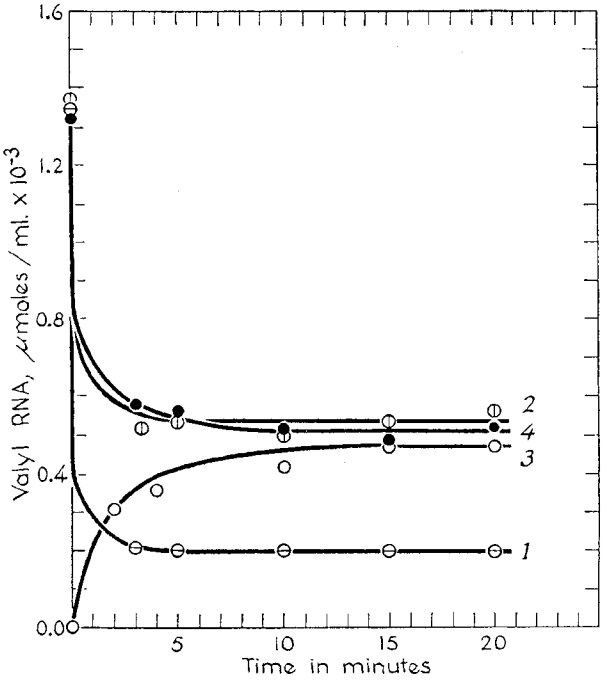

FIG. 3. The equilibrium position for the valine incorporation reaction. The incubation conditions are described in Table III. ⊖——⊖, experiment 1; ⊕——⊕, experiment 2; ○——○, experiment 3; ●——●, experiment 4.

of amino acid removal from the RNA occurred at less than 1% the rate. The failure to remove amino acids from the RNA in the presence of only AMP and the enzyme was also consistent with the formation of enzyme-bound amino acyl adenylates by the reverse as well as the forward reaction (7, 8).

That the previous observations do represent reversal of amino acyl RNA synthesis was established by the finding that incubation of C^{14}-valyl RNA with AMP, PP_i^{32}, and the specific valyl RNA synthetase resulted in essentially complete removal of the valine from the RNA and stoichiometric formation of ATP^{32} (Table II). No ATP^{32} 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_{eq} value of 0.32[2] 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

[2] It should be pointed out that the calculation of the K_{eq} does not take into account the concentrations of possible complexes of the phosphorylated derivatives (*e.g.* ATP, RNA, etc.) with Mg^{++}.

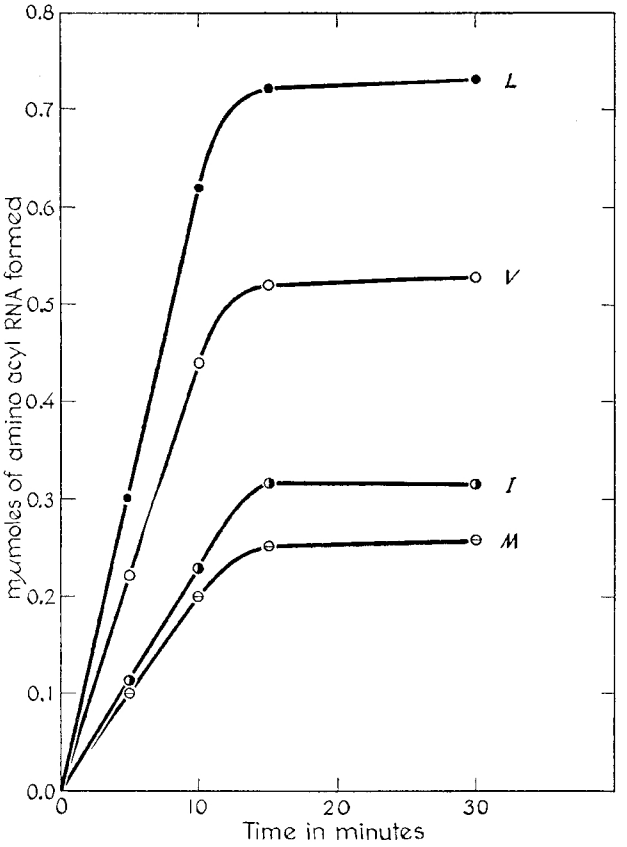

FIG. 4. Kinetics of amino acyl RNA formation. The conditions used were those described for the usual assay of amino acyl RNA formation except that 1.2, 0.1, 0.4, or 0.5 μg of leucyl-(*L*), valyl-(*V*), isoleucyl-(*I*), or methionyl-(*M*) RNA synthetase protein, respectively, and 1.0 μmole of acceptor RNA nucleotide were used.

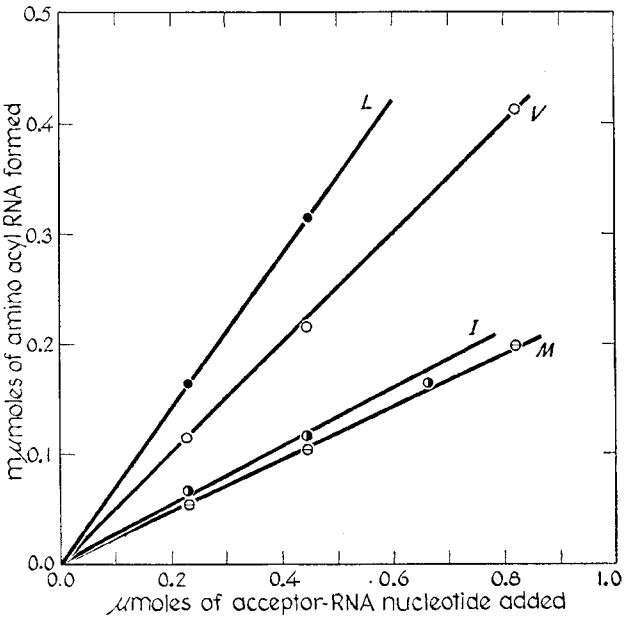

FIG. 5. Formation of amino acyl RNA as a function of the amount of acceptor RNA. The conditions used were those described under "Methods." Abbreviations are as in Fig. 4.

TABLE IV

Separate sites for linking amino acids to acceptor RNA

Experiment 1. The reaction mixture (0.5 ml) contained 20 μmoles of sodium cacodylate buffer, pH 7.0, 1 μmole of $MgCl_2$, 0.2 μmole of ATP, either 0.25 μmole of DL-leucine-1-C^{14} (5.1×10^6 c.p.m. per μmole), 0.25 μmole of DL-valine-1-C^{14} (6.1×10^6 c.p.m. per μmole) or 0.16 μmole of L-methionine-CH_3-C^{14} (3.0×10^6 c.p.m. per μmole), 0.9 μmole of acceptor RNA, and 100 μg of a sonic extract of *E. coli*. The incubation was for 20 minutes at 30°. In the experiment with a mixture of the three amino acids, all were present initially.

Experiment 2. The incubation mixtures and conditions were as described under "Methods." When the incorporation of two amino acids was examined, the second amino acid and the appropriate enzyme were added after 20 minutes, and the incubation was continued for an additional 20 minutes.

Experiment No.	Amino acid added	Incorporation
		total c.p.m.
1	Leucine	3759
	Valine	1646
	Methionine	468
	Mixture of above	5832
	Calculated sum	5873
2	Valine	947
	Leucine	1038
	Valine, then leucine	2006
	Calculated sum	1985
	Valine	947
	Methionine	448
	Valine, then methionine	1434
	Calculated sum	1395

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-

branched 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 adenylic acid (28), although it is now clear that, in the acceptor RNA of *E. coli*, adenylic acid 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 reacted to a limit 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 each amino acyl adenylate either (*a*) separate specific amino acyl transferases for linking each amino acyl residue to the appropriate acceptor RNA, or (*b*) a single amino acyl transferase which

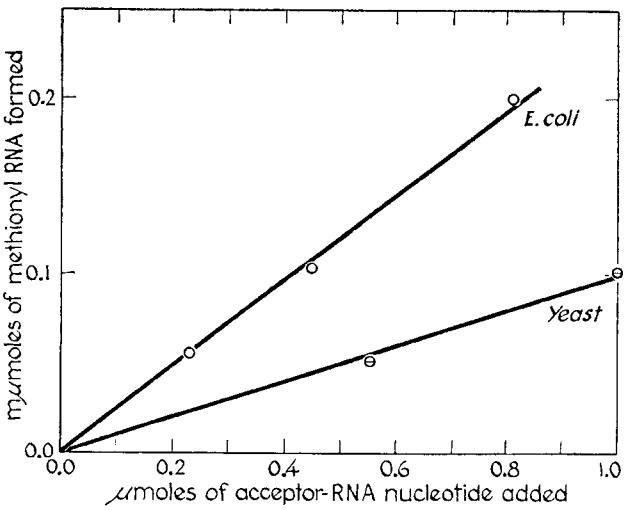

FIG. 6. Formation of methionyl RNA by methionyl RNA synthetases from yeast and *E. coli*. The conditions used are those described under "Methods."

TABLE V

Formation of methionyl RNA by methionyl RNA synthetases from E. coli and yeast

The reaction mixtures contained, in a volume of 0.5 ml, 50 μmoles of sodium cacodylate buffer, pH 7.0, 1 μmole of $MgCl_2$, 0.5 μmole of ATP, 0.06 μmole of L-methionine-CH_3-C^{14} (7.5×10^6 c.p.m. per μmole), 5 μmoles of potassium chloride, 100 μg of serum albumin, 0.81 μmole of acceptor RNA, and either 30 μg of protein of methionyl RNA synthetase from *E. coli* or 125 μg of protein of the similar enzyme from yeast added as indicated in the table. The incubation was at 30°.

Enzyme additions	Total time of incubation	Methionyl RNA formation
	min	*mμmole/μmole nucleotide*
E. coli enzyme at time zero	20	0.28
	40	0.27
E. coli enzyme at time zero and again at 20 minutes	40	0.28
E. coli enzyme at time zero; yeast enzyme at 20 minutes	40	0.28
Yeast enzyme at time zero	20	0.12
	40	0.14
Yeast enzyme at time zero and again at 20 minutes	40	0.13
Yeast enzyme at time zero; *E. coli* enzyme at 20 minutes	40	0.28
Yeast and *E. coli* enzyme at time zero	20	0.27

TABLE VI

Evidence for heterogeneity among acceptor RNA chains for L-methionine

Methionyl RNA was prepared under the usual conditions with either the methionyl RNA synthetase from *E. coli* or from yeast and was isolated as already described. Both samples were treated with sodium metaperiodate in 0.1 M sodium succinate buffer at pH 5.6 in the ratio of 1 μmole of periodate per 14 μmoles of acceptor RNA nucleotide. The RNA samples were reisolated and the bound methionine removed by 0.1 M glycine buffer, pH 10.2, at 30° for 60 minutes. The two RNA preparations were again recovered and then tested as acceptors of methionine with each of the methionyl RNA synthetase preparations as described in Table V.

Source of enzyme used to measure methionyl RNA formation	Methionyl RNA formation		
	Original RNA	Periodate-treated methionyl RNA prepared with	
		E. coli enzyme	Yeast enzyme
	mμmoles/μmole RNA nucleotide		
E. coli	0.25	0.23	0.10
Yeast	0.10	0.10	0.10

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 (34). 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 preparation) 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 formation to that of leucyl RNA formation was 40 in crude extracts and 43 in the purified preparation (50-fold purified). The analogous ratios with methionine in the crude and purified preparations (75-fold purified) were 79 and 81, respectively.

The failure to observe separation of amino acyl adenylate and amino acyl RNA formation might also result (*a*) if the enzymes involved fractionated identically in the procedures we have used,

TABLE VII

Specificity of enzyme preparations for amino acyl adenylate and amino acyl RNA formation

The rate of amino acyl adenylate formation was measured as described elsewhere (26), and the rate of amino acyl RNA synthesis was determined as described under "Methods."

Enzyme	Amino acid tested	Amino acyl adenylate formation	Amino acyl RNA formation
		μmoles/mg/hour	
Leucine	Leucine	358	3.2
	Valine	13.2	0.18
	Methionine	8.0	<0.01
	Isoleucine	<3.0	<0.01
Valine	Valine	560	25
	Leucine	<0.5	<0.01
	Isoleucine	<4.0	<0.01
	Methionine	2.0	<0.01
Isoleucine	Isoleucine	768	3.3
	Leucine	31	<0.07
	Valine	416	<0.03
	Methionine	41	0.07
Methionine (*E. coli*)	Methionine	356	3.5
	Leucine	4.0	<0.01
	Valine	<3.0	<0.01
	Isoleucine	<3.0	<0.01
Methionine (Yeast)	Methionine	44	0.016
	Leucine	<0.4	<0.001
	Valine	<0.4	<0.001
	Phenylalanine	<0.4	<0.001

TABLE VIII

Methionyl adenylate and methionyl RNA formation in various fractions obtained during purification of methionyl RNA synthetase from yeast

Enzyme fraction*	Methionyl adenylate formation (A)	Methionyl RNA formation (B)	A/B × 10^{-3}
	μmoles/mg/hour	*mμmoles/mg/hour*	
Crude extract	0.61	0.23	2.6
Alcohol Fraction 2	14.8	5.2	2.8
Ammonium sulfate Fraction 1	23.2	8.0	2.9
Ammonium sulfate Fraction 2	44.0	16.2	2.7
Alumina C_γ gel eluate	55.7	19.7	2.8

* 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-PP_i^{32} exchange (26). To compare the two rates, however, the ATP-PP_i^{32} 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 PP_i^{32} were added.

Enzyme	Amino acyl adenylate formation (A)	Amino acyl RNA formation (B)	A/B
	μmoles/mg/hour		
Leucyl RNA synthetase	120	2.8	43
Valyl RNA synthetase	574	26.4	22
Isoleucyl RNA synthetase	460	3.3	140
Methionyl RNA synthetase (*E. coli*)	244	3.0	81
Methionyl RNA synthetase (yeast)	22.1	0.007	3200

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 adneylate- 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), pantothenic acid (41), and carnosine (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 Zillig *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_{eq} 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 observation 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 concerning the chemical structures of the RNA chains which allow for the "recognition" between a specific enzyme-amino acyl 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 determined. 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 nucleotide (2′- or 3′-hydroxyl end) of specific polynucleotide chains. The synthesis of amino acyl ribonucleic 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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