uridine triphosphate; CTP or CRPPP, cytidine triphosphate; and GTP, guanosine triphosphate.

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*AN ENZYMATIC MECHANISM FOR LINKING AMINO ACIDS TO RNA**

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INTRODUCTION

For the past several years we have been interested in the general problem of acyl group activation. Our initial studies were concerned primarily with the enzymatic mechanism of acetyl CoA formation.[1, 2] The results of these investigations demonstrated the existence of a new type of reaction in which the carboxyl group of acetate reacts with ATP and becomes linked to the phosphate of adenosine-5′-phosphate. This "activated" acetyl group is subsequently transferred, by the same enzyme, to coenzyme A (eqs. [1]–[3]).

$$\text{ATP} + \text{acetate} \rightleftharpoons \text{adenyl acetate} + \text{PP} \quad (1)$$

$$\text{Adenyl acetate} + \text{CoA} \rightleftharpoons \text{acetyl Coa} + \text{AMP} \quad (2)$$

$$\text{ATP} + \text{acetate} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + \text{AMP} + \text{PP} \quad (3)$$

The widespread occurrence of this type of reaction in the activation of higher fatty acids,[3–6] inorganic acids,[7–9] and other acyl compounds[10, 11] has been demonstrated by a number of investigators. Of particular interest was the finding by Hoagland et *al.*[12] and others[13–16] of enzymes which carry out a reaction analogous to that shown in equation (1) with amino acids. Purification and characterization of some of these enzymes showed, first, that the enzymes were specific for a single amino acid and, second, that the product of the reaction was the adenyl amino acid

presumably existing tightly bound to the enzyme.[13-16] Direct evidence supporting the hypothesis of adenyl amino acid formation was provided by the chemical synthesis of several adenyl amino acids and the demonstration of their conversion to ATP in the presence of PP and the appropriate enzyme.[17-19]

One of the major questions concerning the metabolism of adenyl amino acids is their biologic function. One hypothesis suggests that these amino acid derivatives are the immediate precursors of protein synthesis, and support for this idea is derived from studies of amino acid incorporation into protein by subcellular particles.[12] Our approach to the question of adenyl amino acid utilization was directed toward identifying the immediate acceptor of the "activated" amino acid. The information obtained from our earlier studies on acetate activation suggested that the enzymes which carry out the formation of the adenyl amino acids might also catalyze the transfer of the amino acid to a suitable acceptor. We felt that the isolation and characterization of such an acceptor would provide information pertinent not only to the details of adenyl amino acid formation but perhaps also to the mechanism of protein synthesis.

In the present paper we shall describe an enzymatic reaction which links amino acids to ribonucleic acid (RNA). This reaction requires, in addition to the amino acid and a specific RNA fraction, ATP, Mg^{++}, and the corresponding amino acid–activating enzyme. For a given amino acid, the activation reaction and subsequent transfer to RNA appear to be catalyzed by a single enzyme, i.e., the amino acid–activating enzyme. Moreover, our studies indicate that there is a fixed number of sites available for each amino acid per unit of RNA. Our hypothesis to depict this reaction is summarized by the following reactions:

$$\text{ATP} + \text{amino acid} \rightleftharpoons \text{adenyl amino acid} + \text{PP} \quad \text{(activation step)} \quad (4)$$

$$\underline{\text{Adenyl amino acid} + \text{RNA} \rightleftharpoons \text{RNA-amino acid} + \text{AMP} \quad \text{(transfer step)}} \quad (5)$$

$$\text{ATP} + \text{amino acid} + \text{RNA} \rightleftharpoons \text{RNA-amino acid} + \text{AMP} + \text{PP}$$

$$\text{(over-all reaction)} \quad (6)$$

EXPERIMENTAL RESULTS

If one incubates a mixture of C^{14}-labeled amino acids with a dialyzed sonic extract of *Escherichia coli* in the presence of ATP, the amino acids are converted to an acid-insoluble form which is stable to repeated washings with cold 0.3 *M* perchloric acid. Purification of the bacterial extract revealed that two fractions are required for this conversion. One of these fractions can activate a number of amino acids, and the other, which is composed largely of ribonucleic acid, appears to function as the amino acid acceptor.

Using a single labeled amino acid as substrate, e.g., 1-C^{14}-valine, the system can be reconstructed with a purified valine-activating enzyme[20] and a specific RNA fraction isolated from *E. coli*. The complete requirements for the reaction are shown in Table 1. It is clear that there is an absolute requirement for ATP, RNA, and the enzyme. The effect of Mg^{++} is best shown in the second experiment, where less enzyme was used and the rate rather than the final yield of the reaction was determined. Under these conditions Mg^{++} increases the rate of the reaction about ten times.

Properties of the RNA–Amino Acid Complex.—The RNA–amino acid complex can

be precipitated with cold 0.3–0.5 *M* perchloric acid and is readily distinguished from protein-bound amino acids by the following criteria. Treatment of the acid-precipitated product with 5 per cent perchloric acid at 100° for 15 minutes or exposure to 0.01 *N* alkali at 25° for 5 minutes completely converts the amino acids to an acid-soluble form. Even much milder treatments, such as incubation in Tris buffer, pH 8.6, at 37° or exposure to hydroxylamine (pH 7.0), result in the disappearance of the amino acids from the acid-insoluble fraction (Table 2). In the presence of 0.8 *M*

TABLE 1

REQUIREMENTS FOR INCORPORATION OF C^{14}-VALINE*

Exp. No.	Components	C^{14}-Valine Incorporated (mμmole)
1	Complete	0.33
	Minus RNA	0.00
	Minus ATP	0.00
	Minus enzyme	0.00
2	Complete	0.11
	Minus Mg^{++}	0.01

* The reaction mixture contained in a total volume of 0.25 ml., 20 μmoles of sodium cacodylate buffer pH 7.0; 1 μmole of $MgCl_2$; 0.2 μmole of ATP 0.42 μmole of C^{14}-DL-valine (specific activity 6.05 × 10^6 c.p.m. per μmole) 0.15 ml. of RNA containing 12 μmoles of pentose nucleotide per ml., and 0.14 μg. of the valine-activating enzyme, having an activity of 66 units per μg. of protein in the ATP-PP exchange reaction (see Berg, *J. Biol. Chem.*, 222, 1025, 1956). Time 10 minutes; temperature 30°. In experiment 2, 0.10 ml. of RNA and 0.03 μg. of the valine-activating enzyme were used.

TABLE 2

PROPERTIES OF RNA–AMINO ACID COMPOUND*

Treatment	Time (Min.)	C.p.m. Linked to RNA
Tris buffer, pH 8.6, 37°	0	2,681
	5	1,344
	10	684
	20	237
No hydroxylamine, pH 7, 30°	5	2,616
0.4 *M*	5	1,489
0.8 *M*	5	703
1.6 *M*	5	248
Product at zero time		2,755
Cacodylate buffer, pH 7, 30°	10	2,090
Plus 0.10 μg/ml RNAase	10	15
Plus 3.0 μg/ml DNAase	10	2,348

* *Procedure:* C^{14}-labeled product was isolated by precipitation with acid, and aliquots were treated as described above. The radioactivity of the resultant acid-precipitable material was determined.

hydroxylamine at pH 7 and 30°, the half-life for removal of the linked amino acids is 3 minutes, while under the same conditions in the absence of hydroxylamine the half-life is 62 minutes. Exposure of the RNA–amino acid compound to pancreatic ribonuclease completely converts the amino acids to an acid-soluble form. Similar treatment with pancreatic deoxyribonuclease has no effect, even though added DNA is almost completely degraded. Studies are now in progress to determine whether treatment with ribonuclease results in cleavage of the RNA–amino acid linkage or whether the complex is degraded to smaller acid-soluble fragments with the amino acids still attached.

The amount of amino acid incorporated into the acid-insoluble fraction is a

linear function of the amount of RNA added (Fig. 1). This is true whether C^{14}-labeled valine and the purified valine-activating enzyme are used (curve *A*) or whether a mixture of C^{14}-labeled amino acids[21] and an extract of *E. coli* are used (curve *B*). The reaction proceeds at a rate which is proportional to the amount of amino acid–activating enzyme used and then stops. Addition of more enzyme, amino acid, or ATP produces no further increase in the amount of amino acid incorporated. Addition of more RNA at this point, however, results in a further incorporation of amino acid which is proportional to the amount of RNA added. The incorporation of a given amino acid is independent of the incorporation of other amino acids. For example, with three labeled amino acids together (leucine, valine, and methionine), the total amount of radioactivity linked to the RNA is equal to the sum of the amount incorporated with each amino acid alone.

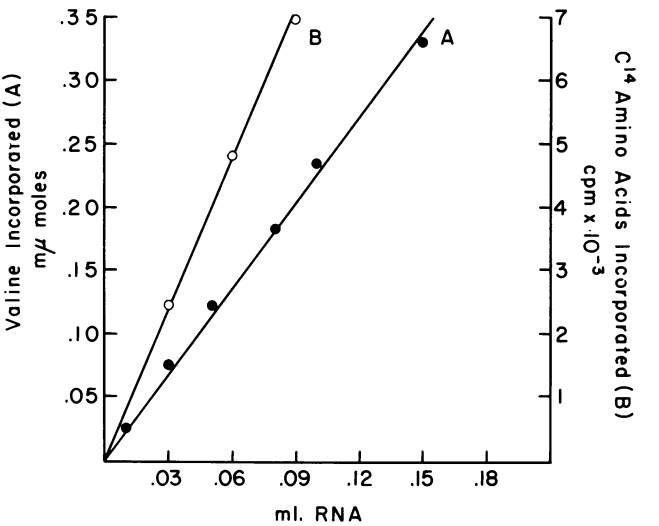

FIG. 1.—Dependence of amino acid incorporation on RNA. *Curve A:* reaction mixture same as in Table 1. *Curve B:* The reaction mixture contained in a total volume of 0.50 ml., 50 μmoles of sodium cacodylate buffer, pH 7.0; 1.5 μmoles of $MgCl_2$; 0.2 μmole of ATP; 9.0×10^5 c.p.m. of the labeled amino acid mixture,* and 0.04 ml. of dialyzed sonic extract of *E. coli* (2 mg. protein/ml). The RNA preparation used was the same as that described in Table 1. Time 20 minutes; temperature 30°. *Hydrolysate of protein from *Chromatium* grown in $C^{14}O_2$ (see n. 21). Specific activity 7.3×10^5 c.p.m. per μmole of carbon.

Properties of the RNA.—The preparation of RNA used in these studies was obtained from dried cells of *E. coli* by extraction with hot detergent,[22] followed by salt and alcohol fractionation, and adsorption and elution from charcoal. The ability to act as an acceptor of amino acids is not destroyed by heating at 60° for periods up to 30 minutes, nor is it significantly inpaired after precipitation at pH 2. At pH 7.0, the most purified material showed an ultraviolet absorption maximum at 260 mμ and a minimum at 230 mμ. The 260 mμ: 230 mμ ratio is 2.33, and the 260 mμ: 280 mμ ratio is 1.91. The preparation contains, relative to the pentose nucletide content, 3 per cent of deoxypentose nucleotide[23] and between 3 and 5 per cent of protein.[24]

Specificity of RNA Acceptor.—Using the assay system described in Figure 1, *B*, a number of RNA preparations isolated from a variety of sources have been tested for their ability to function as amino acid acceptors. These included RNA from yeast (prepared in the same way as the active RNA from *E. coli*), *Azotobacter vinelandii*, turnip yellow mosaic virus, tobacco mosaic virus, and rat liver.[25] The activity with these preparations was 5 per cent or less of that found with the RNA fraction from *E. coli*. We have also tested several enzymatically synthesized polyribonucleotides[26] such as polyadenylic acid, polyuridylic acid, polyadenylic-uridylic acid, and the mixed polymer containing all four naturally occurring nucleotides. These, too, were inert as amino acid acceptors. Moreover, not all the RNA which can be extracted from *E. coli* is active; over 50 per cent of the cellular RNA can be precipitated from the original detergent extract with sodium chloride (1.5 *M*), but this fraction has little or no activity as an acceptor of amino acids. It is not yet clear, however, whether the lack of activity with the various RNA preparations tested is due to a fundamental inability to accept amino acids or whether it is due to some difference in the physical state of the RNA. Further work is in progress to determine this point.

Specificity of Amino Acid Incorporation.—We have examined the question of whether an amino acid–activating enzyme is specific with regard to the amino acid which it links to RNA. Table 3, experiment 1, shows that the purified valine-ac-

TABLE 3

INCORPORATION OF AMINO ACIDS BY VALINE-ACTIVATING ENZYME AND BY EXTRACTS OF *E. coli**

Exp. No.	Amino Acid	Amino Acid Incorporation (MμMoles per Ml. RNA)
1	Valine	1.78
	Leucine	0.00
	Phenylalanine	0.00
	Methionine	0.00
2	Valine	5.44
	Leucine	14.7
	Phenylalanine	4.90
	Methionine	3.39
	Tryptophane	2.00

* The reaction mixture contained in a total volume of 0.25 ml., 20 μmoles of sodium cacodylate buffer, pH 7.0 1.5 μmoles of $MgCl_2$ 0.2 μmole of ATP; 0.2 μmole of C^{14}-DL-valine (specific activity 6.05×10^6 c.p.m per μmole); 0.2 μmole of C^{14}-DL-phenylalanine (specific activity 1.1×10^6 c.p.m. per μmole); 0.25 μmole of C^{14}-DL-leucine (specific activity 5.1×10^6 c.p.m. per μmole); 0.18 μmole of C^{14}-L-methionine (specific activity 2.95×10^6 c.p.m. per μmole); or 0.52 μmole of C^{14}-DL-tryptophane (specific activity 4.67×10^6 c.p.m. per μmole). Experiment 1 contained 0.03 μg. of the valine-activating enzyme and 0.10 ml. of the RNA preparation described in Table 1. Experiment 2 contained 0.05 ml. of the dialyzed sonic extract of *E. coli* (2 mg protein/ml) and 0.05 ml. of a more purified RNA preparation containing 12.5 μmoles of pentose nucleotide/ml. Time: 20 minutes; temperature, 30°.

tivating enzyme catalyzed only the incorporation of valine. Likewise, a purified methionine-activating enzyme,[14] which incorporated 0.87 mμmole of methionine/ml of the RNA preparation, incorporated less than 0.04 mμmole of leucine, valine, or phenylalanine. The same RNA preparation, however, can accept a number of different amino acids, provided that a source of the appropriate activating enzymes is present. With an extract of *E. coli* which can activate a large number of amino acids,[13] leucine, phenylalanine, tryptophane, methionine, and valine become linked to the RNA (Table 3, exp. 2). The extent of incorporation of each of these amino

acids is, as mentioned earlier, dependent on the amount of RNA present. Thus it appears that the incorporation of a given amino acid onto RNA requires the corresponding amino acid–activating enzyme.

Of particular significance in understanding this reaction is the question of whether the amino acid–activating and –incorporating activity are due to the same enzyme. To study this question, we examined the methionine-activating enzyme[14] at various stages of purity for activity with respect to methionine activation and incorporation onto RNA (Table 4). The activation step may be determined separately by meas-

TABLE 4

COMPARISON OF METHIONINE ACTIVATION AND INCORPORATION ACTIVITY AT VARIOUS STAGES OF PURIFICATION*

Fraction	Specific activity (Units per Mg. of Protein)	Activity A† (Units per Ml.)	Activity B‡ (Units per Ml.)	A/B
Crude extract	0.20	4.5	6.1	0.74
A-2	5.4	23.1	33.2	0.70
AS-1	9.2	15.7	23.2	0.68
AS-2b	15	17.5	24.2	0.72
Cγ eluate	19	2.7	3.9	0.69

* A detailed description of the purification procedure will be published at a later date. The specific activity is expressed as units of methionine activation activity (Activity B) per mg. of protein (see n.14).

† Activity A refers to the incorporation of methionine onto RNA expressed as units per ml. One unit of activity is equal to the incorporation of 0.34 mμmole of methionine in 10 minutes at 30°.

‡ Activity B refers to the methionine-activation activity expressed as units per ml. One unit of activity is equal to the incorporation of 1 μmole of PP^{32} into ATP in 15 minutes in the standard exchange assay described previously (see n. 14).

uring the methionine-dependent exchange of PP^{32} with ATP[14], and the incorporation reaction was measured as described earlier. It is clear that, over an approximately hundred fold purification of the enzyme, the two activities were purified to the same extent. We have concluded from these data that the amino acid-activating enzyme also catalyzes the transfer of the activated amino acid to the RNA.

DISCUSSION

We have provided evidence in this communication for an enzymatic reaction linking amino acids to RNA. The resemblance of this mechanism to that demonstrated for acyl CoA formation is of some interest. In each case the formation of the adenyl acyl derivative and the subsequent transfer of the acyl moiety to an appropriate acceptor appear to be carried out by a single enzyme. Such a mechanism would tend to stabilize the relatively labile adenyl amino acid intermediate by combination with the enzyme until, in the presence of RNA, it is converted to the more stable RNA-amino acid derivative. We have also carried out some experiments to determine whether, like the acetate activation reaction, amino acid incorporation onto RNA is reversible. Although these experiments are not yet complete, it is clear that the bound amino acids are readily removed from the RNA in the presence of AMP and PP, resulting in the formation of ATP (see eqs. [4]–[6]). This observation may explain the finding by Holley[27] of an amino acid–dependent, RNAase-sensitive, exchange of AMP-C^{14} with the adenyl moiety of ATP. Experiments are now in progress with the synthetic adenyl amino acids to obtain more detailed information on the mechanism and specificity of the amino acid transfer to RNA.

The significance of the present observations on the linking of amino acids to RNA with regard to the mechanism of protein synthesis is not clear. Many of the current ideas concerning protein synthesis visualize this process as involving, first, the

activation of the free amino acids to some higher-energy level, followed by the orientation of these activated amino acids in a predetermined sequence on a nucleic acid "template." Such sequentially arranged amino acids then undergo polymerization and eventual conversion to a polypeptide chain having a specific configuration.[28-32] The most attractive hypothesis at present, although by no means the only one, is that the linking of amino acids to RNA represents the so-called template stage mentioned above. Consistent with this view is the finding by Hoagland *et al.*[33] that the amino acids of an RNA–amino acid compound from rat liver are incorporated into peptide linkage by the liver microsome fraction. Although the evidence for this view is still incomplete, it is of some interest to consider our present information with respect to the template hypothesis.

Some of the questions which are posed by the template hypothesis are: (1) What is the nature of the linkage between the amino acid and the nucleic acid? (2) What is the minimal size of the structural unit required to bind a single amino acid? (3) What are the determinants which specify the positioning of a given amino acid on the nucleic acid?

With regard to the first question, we do not yet have sufficient data to describe the precise chemical linkage between the amino acids and the RNA. The marked lability of the isolated RNA–amino acid complex to alkali and hydroxylamine and the relative stability at low pH closely resemble that found with the synthetic adenyl amino acids.[17, 18] The major difference appears to be the slightly greater stability of the RNA–amino acid compound at neutral pH. This resemblance suggests that the amino acid carboxyl groups are linked to the phosphates of the RNA If such is the case, the amino group of an adjacent linked amino acid might readily attack the acyl phosphate linkage to form a peptide bond. Wieland *et al.*[19] have reported that mixing of an adenyl amino acid with free amino acids leads to the formation of peptides in which the carboxyl group of the activated amino acid is transferred to the amino group of the second amino acid.

Our experiments suggest that there are a fixed number of sites specific for a given amino acid associated with the RNA. Under the conditions we have used, these sites appear to function independently, that is, there is no enhancement or inhibition of incorporation when more than one amino acid is used as substrate. Our studies thus far do not enable us to make any estimate of the minimal size of the amino acid–binding site. From the extent of incorporation observed with 5 amino acids (Table 3, exp. 2), it appears that one amino acid is bound per approximately 400 nucleotide units. This figure of approximately 400 nucleotides is, of course, maximal, and further studies with more amino acids and more purified RNA preparations are required to arrive at the minimal figure.

The question of what structural features of the RNA determine the positioning of the amino acids has been stated in more general terms by Gamow *et al.*[32] This problem, which has come to be known as the "coding" problem, has been analyzed in terms of how the nucleotide sequence in nucleic acid determines the order of amino acids in polypeptide chains. Thus far it has not been possible to arrive at a unique solution of this problem.[32, 34] One aspect of this problem which has previously been alluded to only briefly is the role of the amino acid–transferring enzyme in determining the position of the amino acid on the RNA.[31] Particularly, we may ask whether the specificity of the activating enzyme is such that it will transfer the

activated amino acid only to a specific nucleotide sequence or configuration (or both) of the RNA. That such a specificity on the part of the enzyme may exist can be inferred from studies on the specificity of the acyl group acceptor in the case of fatty acid activation With these particular enzymes,[1, 3-6] CoA—but not other sulfhydryl compounds—is the fatty acid acceptor. Moreover, in acetate activation, pantetheine, a closely related derivative of CoA, is inactive as acyl acceptor.[35] If this specificity on the part of the amino acid–activating enzyme does exist, then the sequential arrangement of amino acids in protein may be determined both by the nucleotide sequence of the RNA and the specificity of the individual amino acid–activating enzymes. This distinction becomes important if we consider whether the homologous amino acid–activating enzymes from different sources have the same or different specificities with regard to the acceptor site on the RNA. The foregoing arguments pose the question of whether, in all species, a given amino acid is always associated with the same structural or sequential arrangement of nucleotides. We are presently investigating this question by studying the incorporation of a single amino acid onto RNA, using the appropriate amino acid–activating enzyme from different sources and with RNA isolated from various species.

SUMMARY

An enzymatic system for linking amino acids to RNA has been described. The incorporation of a single amino acid requires the corresponding amino acid–activating enzyme, a specific RNA fraction, ATP, and Mg^{++}. Our experiments indicate that the amino acid–activating enzyme catalyzes both the formation of the adenyl amino acid and the transfer of the amino acid moiety to RNA. Experiments with several amino acids indicate that there are specific binding sites for these amino acids associated with the RNA.

* This work was supported by a grant from the United States Public Health Service. The following abbreviations have been used: ATP, adenosine triphosphate; AMP, adenosine-5′-monophosphate; CoA, coenzyme A; DNA, deoxyribonucleic acid; PP, inorganic pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; RNA, ribonucleic acid.

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SOME PROBLEMS CONCERNING THE ACTIVATION OF AMINO ACIDS

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Any enzyme system that is proposed to account for the activation of amino acids prior to their conversion to proteins must have certain characteristics: (1) the reaction must involve all the naturally occurring amino acids; (2) there may reasonably be a correspondence between the degree of activation of a given amino acid and the frequency of occurrence of that amino acid in proteins; and (3) the proposed reaction should be present in all living cells.

Hoagland[1] discovered an amino acid-dependent exchange between inorganic pyrophosphate (PP) and ATP in extracts of rat liver that appears to reflect a system for the carboxyl activation of amino acids. DeMoss and Novelli[2] found this exchange reaction widely distributed in micro-organisms. Since the exchange reaction seemed to involve many of the naturally occurring amino acids and also because the reaction was found in partially resolved systems that catalyze the incorporation of amino acids into proteins, the amino acid-dependent PP-ATP exchange system has been implicated as the enzyme system responsible for the activation of amino acids for the biosynthesis of proteins.[3–5]

My purpose in this discussion is to report some observations on the activation of amino acids, as reflected by the PP-ATP exchange reaction, that are inconsistent with the idea that this system is responsible for the activation of amino acids for protein biosynthesis. These observations may be real or artifacts, but in either