*BINDING OF AMINO ACIDS TO THE END GROUP OF A SOLUBLE RIBONUCLEIC ACID**

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Introduction.—One of the intermediate steps in the process of protein synthesis appears to be the formation of a RNA-amino acid compound.[1-10] The acceptors of the amino acids are low molecular weight RNA molecules, which contain a specific nucleotide end grouping consisting of one or two cytosine nucleotides and a terminal adenine nucleotide.[11] Terminal addition of nucleotides to RNA has been observed in several laboratories.[12-16]

In our previous report[17] it has been shown that these nucleotide end units of the RNA are required for the binding to RNA of all the 14 amino acids that were tested. The evidence here presented indicates that the activated amino acids are transferred to the 2′ or 3′ hydroxyl group of the terminal adenine nucleotide of these nucleotide end groupings of specific RNA molecules. These data extend our preliminary report[17] and support recent findings of Zachau, Acs, and Lipmann.[18]‡

Materials and Methods.—Substrates: L-leucine-1-C^{14}, L-valine-1-C^{14}, L-isoleucine-1-C^{14}, L-norvaline-1-C^{14}, and L-alloisoleucine-1-C^{14} were prepared by Dr. R. B. Loftfield according to the methods previously described.[19] Other C^{14}-labeled amino acids were purchased from the California Corporation for Biochemical Research, Los Angeles, California. The amino acids were dissolved in distilled water, neutralized and stored at −20°. ATP-8-C^{14} was purchased from Schwarz Laboratories, Inc. and CTP-C^{14} was prepared as described.[11] The unlabeled nucleotides were purchased from the Sigma Chemical Company. Deoxy-CTP was kindly donated by Dr. J. F. Koerner. One μmole of $MgCl_2$ was added per μmole of nucleotide and the neutralized solutions were stored at −20°. Pyruvate kinase and the silver barium salt of phosphopyruvic acid were purchased from C. F. Boehringer and Son, Mannheim, Germany, and the latter was converted to the potassium salt. RNA was prepared from rat liver, ascites cell, and calf liver cell fractions and from whole Baker's yeast, as described earlier.[2, 20, 21] RNA isolated from the 105,000 × *g* supernatant cell fraction is referred to as soluble RNA or S-RNA.

Preparation of the pH 5 fraction: The 105,000 × *g* supernatant (S_3) fraction and the pH 5 precipitable fraction derived from it are prepared from mouse Ehrlich ascites cells as described.[11, 22] The pH 5 fraction is dissolved in medium A (0.25 *M* sucrose, 0.005 *M* $MgCl_2$, 0.025 *M* KCl, and 0.05 *M* Tris-HCl§ buffer pH 7.6), and is stored at −20°. The "preincubated pH 5 fraction" is prepared as follows: the pH 5 fraction is thawed, and the small amount of insoluble material is removed by centrifugation. The supernatant fraction is incubated at 37° for 30 min. in the absence of added nucleoside triphosphates and amino acids. It is then chilled and diluted with two volumes of ice cold distilled water. The pH is adjusted to 5.2 with 1 *M* acetic acid, and the precipitate (the pH 5 fraction) is isolated and redissolved in medium A. The pH is adjusted to 7.6 with 0.1 *N* KOH. The undissolved material is discarded. The pH 5 fraction is incubated again at 37° for

20 min., and is then chilled and reprecipitated two times, as described above. A third incubation-reprecipitation cycle has been carried out in some cases. Unless otherwise stated all enzyme solutions have been prepared in medium A.

Isolation of RNA for radioactivity determination: The RNA is isolated according to the general procedure described previously.[2] An acid-insoluble precipitate is obtained by the addition of perchloric acid to a final concentration of 0.4 *N*. The precipitate is washed four times with 0.2 *N* perchloric acid (using a glass homogenizer each time to disperse the pellet), once with 0.2 *N* perchloric acid–95 per cent ethanol (1:5), and three times with 95 per cent ethanol. Lipids are further extracted with ethanol-ether (2:1) during 10 min. at 50°, and finally the precipitate is washed once with 95 per cent ethanol. The nucleic acids are then extracted from the washed precipitate with 10 per cent NaCl *without neutralization* at 100° for 30 min. The sodium salts of the nucleic acids are precipitated from the NaCl extract by addition of 2.5 volumes of cold 95 per cent ethanol, and are reprecipitated as outlined previously.[11] The estimations of the RNA and radioactivity are likewise carried out as described.[11]

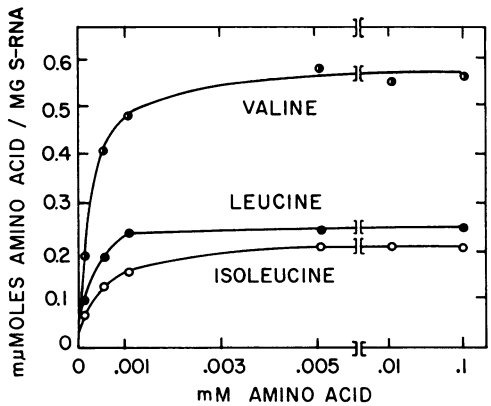

FIG. 1.—*Amino acid concentration curve.* Preincubated pH 5 fraction (0.75 ml) in medium A was incubated at 37° for 10 minutes in the presence of 10 mM ATP and 0.05 mM CTP with increasing concentrations of valine-C^{14} (4.29×10^6 cpm per μmole) or isoleucine-C^{14} (11.7×10^6 cpm per μmole), or leucine-C^{14} (1.82×10^6 cpm per μmole) in a total volume of 1 ml ◑ = valine, ● = leucine, and ○ = isoleucine.

Formation and isolation of valine amides:[23] Aliquots of the chilled incubation mixture, containing pH 5 fraction, CTP, ATP, and valine-C^{14} are rapidly added to alcoholic ammonia at −78°. The samples are stoppered, shaken, and kept at −20° for 30 min. Thereafter the ammonia is allowed to escape during a 12- to 16-hr period at −20°, and then most of the remaining ammonia is removed by blowing an air stream over the samples kept at 0°. The samples are acidified with 100 per cent trichloroacetic acid and placed on 20 × 1 cm columns of Dowex-1 hydroxide resin. Valine amide is washed out of the column with distilled water while free valine is retained. The valine amide is concentrated, plated on copper planchets, and counted.

Results.—Conditions for amino acid incorporation into the RNA of the pH 5 fraction of ascites cells: In a previous publication[2] Hoagland *et al.* described conditions for the incorporation of amino acids into RNA and protein in rat liver cell fractions. Subsequently it was found advantageous to use cell fractions of ascites cells, because the activity of degradative enzymes is lower.[11] The new conditions used are therefore described. Figure 1 shows the substrate requirement for the incorporation of valine, leucine, and isoleucine into the S-RNA of the preincubated pH 5 fraction. In each case a concentration of 10^{-3} mM very nearly saturates the incorporation system. In order to obtain maximal incorporation of amino acids into RNA a concentration of 1 mM ATP is required, in the presence of phospho-

pyruvate and pyruvate kinase (Fig. 2). In the absence of the ATP generating system, 10 mM ATP is needed. A pH curve for the binding of valine to RNA is shown in Figure 3. The conditions used provide an excess of RNA and a limiting amount of enzyme. Optimal activity is observed between pH 7.8 and pH 8.6 and at a higher pH the activity drops rapidly. This is probably due to the instability of RNA-amino acid at the higher pH range.[2]

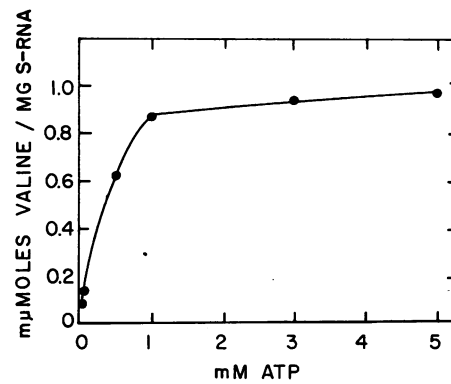

FIG. 2.—*ATP requirement for amino acid incorporation into RNA.* Preincubated pH 5 fraction (0.75 ml) in medium A was incubated at 37° for 10 minutes in the presence of 5 mM phosphopyruvate, 10 μg pyruvate kinase, 0.1 mM CTP and 0.02 mM valine-C^{14} (4.29×10^6 cpm per μmole), with increasing amounts of ATP in a total volume of 1 ml.

As has been demonstrated previously,[2] this system incorporates the amino acid only into the RNA of the nonparticulate fraction of the cell (S-RNA). No species specificity for the source of the S-RNA is indicated, as illustrated in more detail in Figure 4. S-RNA of the mammalian cells and RNA from whole Baker's yeast both are good acceptors of the amino acids, and the incorporation is proportional to the amount of RNA added in every case. Equivalent quantities of microsomal and nuclear RNA of the mammalian cells, however, do not accept the amino acids.

The enzymes activating leucine and valine and transferring these amino acids to RNA remain active for at least 2 hrs. of incubation at 37°. This is shown in Figure 5, where the transfer of leucine to *added* RNA, present in excess, is measured during a 10-min interval, after the enzymes have been preincubated as long as 110 min. It has not, however, been determined to what extent the enzymes in-

TABLE 1

EFFECT OF PREINCUBATION OF THE pH 5 FRACTION ON THE INCORPORATION OF LEUCINE AND VALINE INTO RNA, AND ON THE ACTIVATION OF THESE AMINO ACIDS

	pH 5 Fraction	
	Control, cpm per mg RNA	Preincubated, cpm per mg RNA
Incorporation of leucine into RNA	1,510	25
Incorporation of valine into RNA	2,000	174
	Per Cent Exchange[a]	
$P^{32}P^{32}$ Exchange into ATP		
No amino acid added	0.89	0.65
Plus leucine	5.18	3.51
Plus valine	6.10	6.03

[a] Per cent exchange, cpm in ATP one half cpm added as PP.

The control pH 5 fraction was precipitated twice at pH 5.2 and dissolved in medium A. This fraction was incubated at 37° for 30 min. and chilled to yield the preincubated pH 5 fraction. The amino acid incorporation into RNA was carried out as follows: 0.75 ml of the pH 5 fraction was incubated at 37° for 10 min. in the presence of 10 mM ATP and 0.1 mM leucine-C^{14} (2.07×10^6 cpm per μmole) or 0.1 mM valine-C^{14} (1.83×10^6 cpm per μmole) in a total volume of 1 ml.

$P^{32}P^{32}$ was prepared by pyrolysis of P^{32}-labeled orthophosphate. The exchange of $P^{32}P^{32}$ into ATP was determined as follows: 0.05 ml. of the pH 5 fraction in medium A was incubated for 7 min. at 37° with 5 mM ATP, 5 mM $P^{32}P^{32}$ (containing 356,000 cpm) and 10 mM amino acids where indicated. The final volume was 0.2 ml. The reaction was stopped with trichloroacetic acid and ATP and PP were separated by charcoal adsorption according to the method of Crane and Lipmann.[24]

volved in the incorporation of other amino acids into RNA are stable under these conditions.

Effect of CTP on the incorporation of amino acids into preincubated S-RNA: Following 30-min. preincubation of the pH 5 fraction at 37° in the absence of added nucleoside triphosphates and amino acids, a marked decrease in the binding of valine and leucine to the *endogenous* S-RNA is observed (Table I). The pyrophosphate exchange with ATP shows that preincubation under these conditions has a relatively small effect on the activating enzymes. Since the data in Figure 5 show that the enzymes transferring the amino acids to S-RNA are stable, an alteration of the RNA acceptor during the preincubation period is suggested. In previous experiments[11] it has been shown that under such preincubation conditions the S-RNA loses its capacity to accept the terminal adenine nucleotides, which are linked to cytosine nucleotides. In the presence of CTP, the required cytosine nucleotides are incorporated into the S-RNA, thereby providing the necessary sites onto which the adenine nucleotides can add (Table 2). Table 2 shows that in the presence of CTP plus ATP, valine (or leucine) can be incorporated into the S-RNA of the preincubated pH 5 fraction, whereas there is little or no incorporation of valine (or leucine) into S-RNA in the absence of CTP. Neither GTP plus ATP, nor UTP plus ATP can produce this effect. Thus CTP is essential for both the addition of the adenine nucleotide to S-RNA, and for the binding of the amino acids to this same S-RNA fraction.

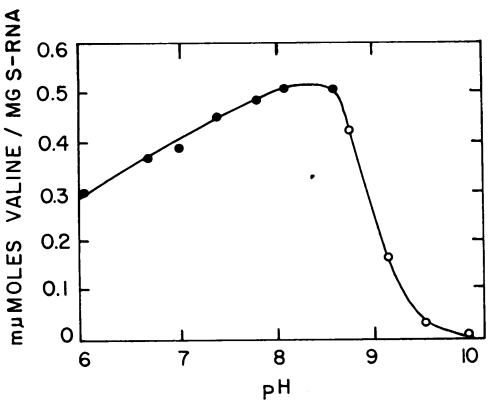

FIG. 3.—*pH curve for incorporation of valine into RNA.* The preincubated pH 5 fraction was dissolved in medium A (minus its buffer), by adjustment of the pH to 7.6 with dilute KOH. Samples containing 0.75 ml of the preincubated pH 5 fraction were incubated with 10 μmoles of ATP and 0.05 μmole of CTP in a total volume of 0.89 ml for 10 minutes at 37°, to form RNA-pCpCpA.[11] The samples were chilled and 0.1 ml of 0.5 M Tris-maleate buffer ●, or 0.5 M sodium bicarbonate-sodium carbonate buffer O were added. 0.04 μmole of valine-C^{14} (4.29×10^6 cpm per μmole) were then added and the incubation was continued for 10 minutes at 37°. The final volume was 1 ml. The samples were chilled, and perchloric acid was added to stop the reaction. The mμmoles of valine incorporated per mg of RNA are given at the various pH values. (A parallel set of flasks was treated in the same manner except that C^{12}-valine was added. At the end of the incubation the pH of the incubation mixture was measured. In no case was the change of pH during the incubation greater than 0.15 pH unit).

Table 3 shows that all of the 14 natural amino acids and the three amino acid analogues tested are incorporated into the RNA of the preincubated pH 5 fraction to a much greater extent when CTP is added to the incubation system. The variation in the relative incorporation of these amino acids into the S-RNA may be due to differences in the yield and stability of specific amino acid activating enzymes and differences in the amounts of specific S-RNA molecules which may serve as acceptors of individual amino acids. The presence of RNA molecules specific for the individual amino acids is suggested by the absence of competition between natural amino acids for sites on the S-RNA, and by the additive incorporation of

TABLE 2

CTP REQUIREMENT FOR INCORPORATION OF VALINE AND ADENINE NUCLEOTIDE INTO RNA

Nucleotides Added	Valine Incorporation, cpm per mg RNA	AMP Incorporation cpm per mg RNA
ATP	136	578
ATP + CTP	1,800	2,845
ATP + CTP + GTP + UTP	1,740	3,045
ATP + UTP	129	494
ATP + GTP	242	550
CTP	32	...
CTP + GTP + UTP	40	...

Incorporation of valine into RNA: 0.6 ml of preincubated pH 5 fraction was incubated at 37° for 10 min. in a total volume of 1 ml in the presence of 0.2 μmole of valine-C^{14} (1.83×10^5 cpm per μmole), 3.6 μmoles of $MgCl_2$, and where indicated, 1 μmole each of CTP, UTP, or GTP and 10 μmoles of ATP.

Incorporation of adenine nucleotide into RNA: 0.6 ml of preincubated pH 5 fraction was incubated at 37° for 10 min. in a total volume of 1 ml in the presence of 3.6 μmoles of $MgCl_2$, 1 μmole of ATP-8-C^{14}, containing 3.5×10^6 cpm per μmole, and where indicated, 1 μmole each of CTP, UTP, or GTP.

The same specific nucleotide requirement is found for the incorporation of leucine into RNA.

amino acids.[2, 8, 9] This interpretation is supported by experiments of Schweet[25] in which he reports separating the S-RNA into fractions which bind specific amino acids preferentially.

TABLE 3

CTP DEPENDENCE OF AMINO ACID INCORPORATION INTO RNA

Amino Acid Added	Specific Activity of Amino Acid, cpm $\times 10^{-6}$ per μmole	RNA, cpm per mg −CTP	RNA, cpm per mg +CTP
L-valine-1-C^{14}	1.83	17	1,210
L-leucine-1-C^{14}	2.07	35	792
L-isoleucine-1-C^{14}	2.10	63	725
DL-serine-3-C^{14}	1.56	52	470
DL-lysine-1-C^{14}	1.56	15	440
DL-glutamic acid-2-C^{14}	0.55	0	79
DL-tryptophan-2-C^{14}	0.72	8	94
DL-tyrosine-2-C^{14}	0.87	48	115
DL-alanine-1-C^{14}	1.25	21	98
DL-phenylalanine-3-C^{14}	1.72	5	97
DL-aspartic acid-4-C^{14}	1.16	1	47
L-methionine methyl-C^{14}	0.90	2	72
DL-histidine-2-C^{14}	0.78	13	26
Glycine-1-C^{14}	2.37	10	196
Amino acid analogues			
L-alloisoleucine-1-C^{14}	11.7	70	1,710
L-norvaline-1-C^{14}	11.7	21	614
D-norvaline-1-C^{14}	11.7	0	36

The incubation was carried out for 15 min. at 37° in a total volume of 1 ml. The incubation mixture contained: 0.7 ml of preincubated pH 5 fraction (containing 200 μg RNA), 180 μg. of RNA prepared from a preincubated pH 5 fraction of rat liver, 10 μmoles of ATP, and 0.06 to 0.07 μmole of DL-amino acids or 0.03 to 0.04 μmole of L-amino acids. 0.1 μmole of CTP was added to the samples listed in the column marked "+CTP." The counts per minute per mg RNA are reported for each amino acid in the presence and absence of CTP.

Under the above conditions, CTP (tested in concentrations equimolar with those of ATP) cannot replace ATP and does not enhance the activity of ATP in the amino acid activation reaction, as determined by P^{32}-labeled inorganic pyrophosphate exchange with ATP in the presence of amino acids. This evidence supports the previous conclusion[26] that CTP does not participate in the initial step of activation of amino acids.

Comparison of CTP requirement for incorporation of amino acids into S-RNA and for formation of the nucleotide end grouping on the S-RNA: The concentration of

CTP required for optimal incorporation of valine into S-RNA and for the incorporation of the cytosine and adenine nucleotides into S-RNA is the same, as is shown in Figure 6. Deoxy-CTP cannot replace CTP in this reaction.

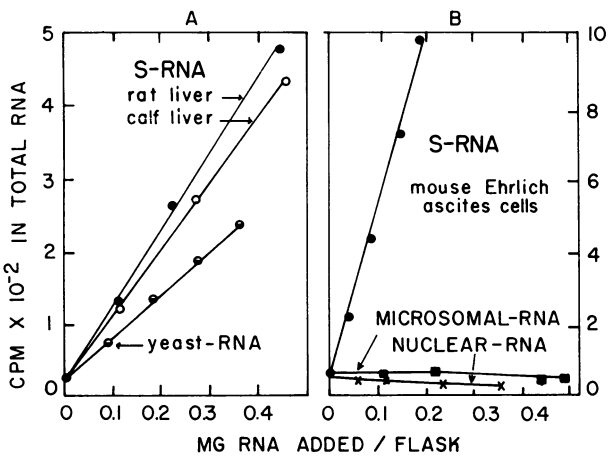

FIG. 4.—*Lack of species specificity for source of soluble RNA.* 0.3 ml of S_3 was incubated at 37° for 20 minutes in the presence of 10 mM ATP, 0.05 mM CTP, 4 mM $MgCl_2$, 0.1 mM leucine-C^{14} (1.82×10^6 cpm per μmole) in part A, 0.1 mM valine-C^{14} (4.29×10^6 cpm per μmole) in part B, and increasing amounts of RNA from the indicated sources, in a total volume of 1 ml. At the end of the incubation, the samples were chilled, 0.4 M perchloric acid was added, and to each series of flasks RNA was added to give a constant amount of RNA. The radioactivity incorporated into the RNA is reported.

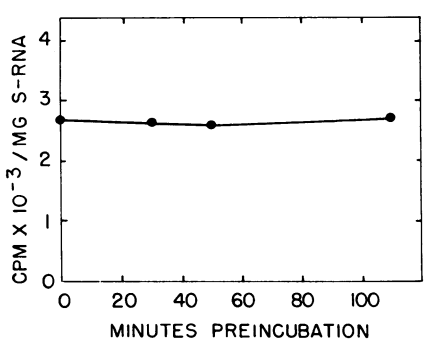

FIG. 5.—*Stability of enzymes involved in binding of leucine to soluble RNA.* Individual flasks contained 0.3 ml of S_3, 0.3 ml. of medium A, 10 μmoles of ATP, 10 μmoles of phosphopyruvate, and 10 μg of pyruvate kinase in a total volume of 0.82 ml. After preincubation at 37° for a definite period, 0.2 μmole of leucine-C^{14} (1.82×10^6 cpm per μmole) and 180 μg of S-RNA prepared from the pH 5 fraction of rat liver were added (final volume 1 ml), the incubation was continued for 10 minutes at 37°, and the radioactivity incorporated into S-RNA was determined.

Conditions required for optimal incorporation of nucleotides into RNA: Earlier work[11-16] has demonstrated the incorporation of the adenine and cytosine nucleotides into S-RNA. It has been shown that ATP and CTP are the immediate precursors in this reaction. The pH optima of the incorporation reactions are shown in Figure 7. The substrate requirements for ATP and CTP have been found to be much lower for the preincubated pH 5 fraction than for the untreated pH 5 fraction. Figure 6 shows that maximal incorporation of the cytosine nucleotide takes place when CTP is present at concentrations as low as 0.05 mM. Similarly, maximal addition of the adenine nucleotide occurs with as little as 0.05 mM ATP. This concentration of ATP is much lower than the 10 mM ATP required for the amino acid activation and transfer to S-RNA in the absence of an ATP-generating system. This leads us to suggest that the adenine nucleotide incorporation is independent of the incorporation of amino acids into S-RNA. This suggestion is supported by the data in Table 4, A and B, which demonstrate that the presence or absence of amino acids does not affect the incorporation of the nucleotides into S-RNA.

Previously it has been demonstrated that cytosine and adenine nucleotides are added to S-RNA in sequence, and that the adenine nucleotide is predominantly terminal.[11-16] Near their pH optima, the cytosine and adenine nucleotides are in-

corporated into the S-RNA at a ratio of about 2:1 (Figs. 6 and 7). When S-RNA, labeled with C^{14} in the end group cytosine and adenine nucleotides is treated with snake venom diesterase (20 units per ml incubated with 200 μg S-RNA at 37° for 30 min) the radioactivity is released quantitatively as 5'-CMP-C^{14} and 5'-AMP-C^{14}. Under these conditions, less than 5 per cent of the S-RNA was degraded. These observations are in agreement with the following end group structure: RNA-pCpCpA.[‖]

Time study of the loss of nucleotides and amino acids from the S-RNA of the pH 5 fraction: Figure 8 demonstrates the loss of nucleotides and amino acids from the end groups of the S-RNA of the pH 5 fraction, under conditions which approximate those of the preincubation. The end groups of the S-RNA of the pH 5 fraction are labeled by appropriate incubation of the pH 5 fraction at 37° for 10 min with CTP-C^{14}, CTP and ATP-C^{14}, or CTP, ATP, and valine-C^{14}, which yield RNA-pC*-pC*, RNA-pCpCpA*, and RNA-pCpCpA-val*, respectively.[¶] The reaction is stopped by chilling followed immediately by dilution of the incubation mixture with 10 volumes of ice-cold distilled water. The pH 5 fractions are freed of reactants by two precipitations of the pH 5 fraction at pH 5.2. The pH 5 fractions containing the labeled RNA are incubated at 37° and pH 7.6 for varying periods of time in the absence of added nucleoside triphosphates or amino acids, during which period there is a progressive loss of the labeled nucleotides or valine.

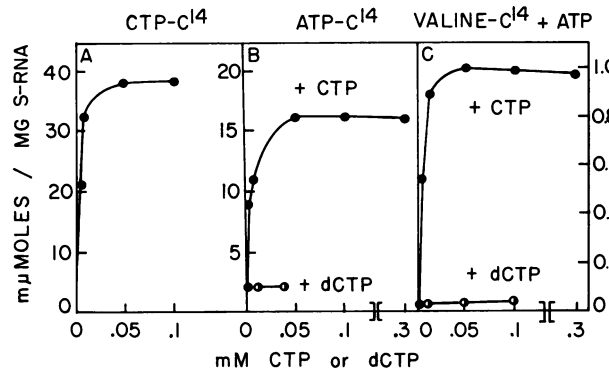

FIG. 6.—*CTP requirement for incorporation of the cytosine and adenine nucleotides and valine into RNA.* The incubation was carried out for 10 minutes at 37° in a total volume of 1 ml.

A. 0.75 ml of preincubated pH 5 fraction was incubated with increasing amounts of CTP-C^{14} (100,000 cpm per μmole).

B. 0.75 ml of preincubated pH 5 fraction was incubated with 0.1 mM ATP-8-C^{14} (346,000 cpm per μmole) and increasing amounts of CTP ● or dCTP ◑.

C. 0.75 ml. of preincubated pH 5 fraction was incubated with 10 mM ATP, 0.1 mM valine-C^{14} (1.7×10^6 cpm per μmole) and increasing amounts of CTP ● or dCTP ◑.

The incorporation of valine-C^{14} into S-RNA in the presence of CTP (0.03 mM or 0.09 mM) is the same as in the presence of CTP plus an equimolar quantity of dCTP (0.03 mM or 0.09 mM) indicating that dCTP does not compete with CTP in this reaction.

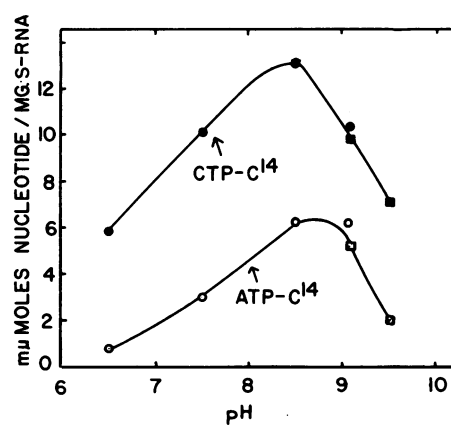

FIG. 7.—*pH curve for incorporation of cytosine and adenine nucleotide into RNA.* The preincubated pH 5 fraction was dissolved in medium A (minus buffer) by adjustment of the pH to 7 with dilute KOH. Samples of 0.60 ml preincubated pH 5 fraction were incubated with 0.20 mM CTP-C^{14} (86,000 cpm per μmole) (solid figures), or 0.20 mM CTP plus 1.0 mM ATP-8-C^{14} (350,000 cpm per μmole) (open figures), and 0.05 M Tris-maleate ●○, or 0.05 M sodium carbonate-sodium bicarbonate ■□ buffer at the indicated pH. The final volume was 1 ml. The incubation was carried out for 10 minutes at 37°.

TABLE 4

ADDITION OF TERMINAL GROUPS TO SOLUBLE RNA UNDER VARIOUS CONDITIONS

RNA-Product	Initial Incubation Additions		Subsequent Incubation Additions		RNA, cpm per mg	Exchange,[a] per cent
	A. Incorporation of Cytosine Nucleotide					
RNA-pC*pC*	RNA- + C^{14}-CTP	(30′)	None		2,100	
	RNA- + C^{12}-CTP	(20′)	+ C^{14}-CTP	(10′)	714	34
RNA-pC*pC*pA	RNA- + C^{14}-CTP + ATP	(10′)	None		2,530	
	RNA- + C^{14}-CTP + ATP	(30′)	None		2,410	
	RNA- + C^{12}-CTP + ATP	(20′)	+ C^{14}-CTP	(10′)	318	13
RNA-pC*pC*pA-aa	RNA- + C^{14}-CTP + ATP + aa	(10′)	None		2,520	
	RNA- + C^{14}-CTP + ATP + aa	(30′)	None		2,675	
	RNA- + C^{12}-CTP + ATP + aa	(20′)	+ C^{14}-CTP	(10′)	113	4
	B. Incorporation of Adenine Nucleotide					
RNA-pCpCpA*	RNA- + C^{14}-ATP + CTP	(10′)	None		2,700	
	RNA- + C^{14}-ATP + CTP	(20′)	None		2,690	
	RNA- + C^{14}-ATP	(10′)	None		720	
	RNA- + C^{14}-ATP	(10′)	+ CTP	(10′)	2,675	
	RNA- + C^{12}-ATP + CTP	(10′)	+ C^{14}-ATP	(10′)	2,150	80
RNA-pCpCpA*-aa	RNA- + C^{14}-ATP + CTP + aa	(10′)	None		2,680	
	RNA- + C^{14}-ATP + CTP + aa	(20′)	None		2,700	
	RNA- + C^{12}-ATP + CTP + aa	(10′)	+ C^{14}-ATP	(10′)	618	21
	C. Incorporation of Valine					
RNA-pCpCpA valine*	RNA- + C^{14}-valine + ATP + CTP	(10′)	None		1,690	
	RNA- + C^{14}-valine + ATP + CTP	(20′)	None		1,640	
	RNA- + C^{14}-valine + ATP	(10′)	None		26	
	RNA- + C^{14}-valine + ATP	(10′)	+ CTP	(10′)	1,873	
	RNA- + ATP + CTP	(10′)	+ C^{14}-valine	(10′)	1,755	
	RNA- + C^{12}-valine + ATP + CTP	(10′)	+ C^{14}-valine	(10′)	258	15

[a] To calculate the per cent exchange: the cpm per mg RNA obtained when C^{12}-nucleotide (or valine) was present during the initial incubation and C^{14}-nucleotide (or valine) was added during the subsequent incubation is divided by the cpm per mg RNA obtained when the C^{14}-nucleotide (or valine) was present during the entire incubation period. This value is multiplied by 100.

A. The incorporation of the cytosine nucleotide was determined by incubation at 37° of 0.7 ml of the preincubated pH 5 fraction with 5 μmoles of phosphopyruvate, 10 μg of pyruvate kinase, and 0.1 μmole of CTP-C^{14} (63,500 cpm per μmole). Further additions of 1 μmole of ATP and 0.1 μmole each of 18 amino acids were made, as indicated. The final volume was 1 ml. The minutes of incubation are given in parentheses.

The exchange reaction was studied as follows: 0.7 ml of the preincubated pH 5 fraction was incubated with 0.05 μmole of CTP, 5 μmoles of phosphopyruvate, and 10 μg of pyruvate kinase. Further additions of 1 μmole of ATP and 0.1 μmole each of 18 amino acids were made as indicated. The total volume was 0.95 ml. After 20 min. of incubation at 37°, 0.05 μmole of CTP-C^{14} was added (final specific activity = 63,500 cpm per μmole), and the incubation was continued for 10 min.

B. The incorporation of the adenine nucleotide was determined during incubation at 37° of 0.7 ml of preincubated pH 5 fraction with 3 μmoles of phosphopyruvate, 10 μg of pyruvate kinase, 2 μmoles of ATP-8-C^{14} (150,000 cpm per μmole). Further additions of 0.2 μmole of CTP and 0.1 μmole each of 18 amino acids were made as indicated. The final volume was 1 ml. The minutes of incubation are given in parentheses.

The exchange reaction was set up as follows: 0.7 ml of preincubated pH 5 fraction was incubated in the presence of 3 μmoles of phosphopyruvate, 10 μg of pyruvate kinase, 0.2 μmole of CTP, 1 μmole of ATP and 0.1 μmole each of 18 amino acids, when indicated, in a total volume of 0.9 ml. After 10 min. 1.0 μmole of ATP-8-C^{14} (0.1 ml) was added (final specific activity of ATP = 150,000 cpm per μmole) and the incubation was continued The specific activity of the RNA is reported.

C. The valine incorporation was studied at 37° with 0.75 ml. of preincubated pH 5 fraction, 1 μmole of ATP, 0.2 μmole of CTP, and 0.04 μmole of valine C^{14} (1.7×10^6 cpm per μmole), as indicated, and 3 μmoles of phosphopyruvate and 10 μg of pyruvate kinase in a total volume of 1 ml. The minutes of incubation are given in parentheses.

The exchange reaction was studied as follows: C^{12}-valine, 0.02 μmole, was incubated with 0.75 ml of preincubated pH 5 fraction in the presence of 1 μmole of ATP, 0.2 μmole of CTP, 3 μmoles of phosphopyruvate and 10 μg of pyruvate kinase in a total volume of 0.95 ml. After 10 min. of incubation at 37°, 0.02 μmole of C^{14}-valine (final valine specific activity = 1.7×10^6 cpm per μmole) was added and the incubation was continued for 10 min. The specific activity of the RNA is reported.

This loss can be reversed at any time by the addition of the appropriate nucleotides and valine. The loss of the nucleotides is almost completely dependent on the addition of inorganic pyrophosphate provided that the pH 5 fraction has been preincubated and reprecipitated at pH 5.2 several times before the above experiment is carried out. Similarly the loss of amino acids is enhanced by the addition of inorganic pyrophosphate plus AMP in such a system. This latter observation is in agreement with previous findings.[2, 5, 8, 27]

Addition of amino acids to isolated S-RNA with incomplete and complete end groupings: RNA lacking the cytosine and adenine nucleotide end groupings can be isolated by the phenol extraction method[2, 20, 21] from the preincubated pH 5 fraction. Such RNA (RNA-), when added to an incubation system containing ATP and valine, does not accept valine unless CTP is present. RNA containing the cytosine nucleotide group (RNA-pCpC) or the complete adenine and cytosine nucleotide group (RNA-pCpCpA) can be prepared from a pH 5 fraction, which has been preincubated and then incubated in the presence of CTP, or CTP plus ATP, respectively. Neither RNA-pCpC nor RNA-pCpCpA added to the above incubation system requires CTP for amino acid binding, as is shown in Figure 9. These data indicate that while CTP is required for the formation of the functional nucleotide end grouping of the RNA which serves as amino acid acceptor, CTP is not a cofactor involved in the transfer of the amino acids to the RNA.

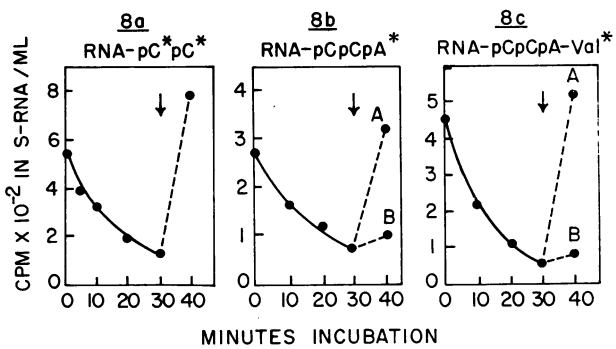

FIG. 8.—*Time curve of the loss of RNA end group nucleotides and amino acids.* The RNA of the pH 5 fraction was labeled during a 10 minute incubation at 37°. 4.5 ml of the untreated pH 5 fraction were incubated with (*a*) 0.01 mM CTP-C^{14} (100,000 cpm per μmole); (*b*) 0.01 mM CTP plus 0.1 mM ATP-8-C^{14} (346,000 cpm per μmole); and (*c*) 0.01 mM CTP, 10 mM ATP and 0.01 mM valine-C^{14} (1.7×10^6 cpm per μmole) in a total volume of 6 ml. After incubation the mixtures were chilled, diluted with 10 volumes of ice-cold distilled water and the pH was adjusted to 5.2 with 1 M acetic acid. The precipitate was washed with distilled water and dissolved in medium A. The precipitation was repeated once more. The precipitate was dissolved in 4.5 ml of medium A and the volume was made up to 6 ml with distilled water. 1 ml aliquots of each labeled pH 5 fraction were incubated for the indicated time intervals and the specific activity of the RNA was determined. In each series one sample received the following additions at 30 minutes (indicated by arrows) and the samples were incubated for 10 minutes longer: (*a*) 0.01 mM CTP-C^{14}; (*b*) A—0.01 mM CTP plus 0.1 mM ATP-C^{14}, B—0.1 mM ATP-C^{14}; and (*c*) A—0.01 mM CTP, 10 mM ATP plus 0.01 mM valine-C^{14}, B—10 mM ATP plus 0.01 mM valine-C^{14}. The specific activity of the RNA was determined. During the incubation of the pH 5 fraction the amount of RNA per ml remained constant.

Protection of the nucleotide end grouping of the RNA by amino acids: The incorporation of the adenine and cytosine nucleotides into RNA-, which has been freed of its

end grouping by preincubation, is independent of the presence of amino acids, as is shown in Table 4, A and B. The binding of the amino acids to the RNA-, however, protects the terminal nucleotides from degradation and replacement, as shown by the lower exchanges in the presence of amino acids. The incorporation of valine into RNA, chosen as an example of the amino acid binding, is the same whether the RNA nucleotide end grouping is synthesized before the addition of valine or in the presence of valine. When valine is bound to the RNA of the preincubated pH 5 fraction in the presence of CTP and ATP it exchanges with free valine to only a small extent, as is shown in Table 4C. The low level of exchange under these conditions is quite possibly due to the presence of a high concentration of ATP and valine and a very low concentration of AMP and inorganic pyrophosphate. This agrees with the previously mentioned finding that incubation of the preincubated pH 5 fraction containing RNA-pCpCpA-val* requires PP and AMP to effect maximal loss of valine from this labeled RNA.

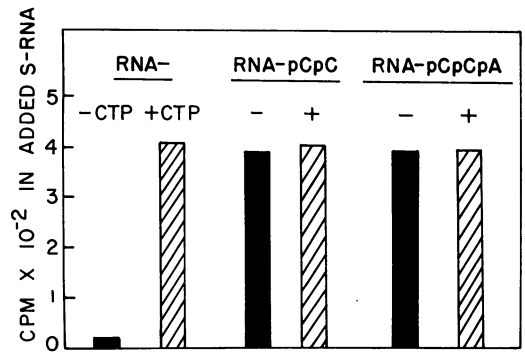

FIG. 9.—*Incorporation of valine into RNA samples containing different end groups.* The three types of RNA were isolated by the phenol extraction method from the pH 5 fraction of ascites cells, which has been treated in the following manner. RNA− was isolated from the preincubated pH 5 fraction. RNA-pCpC was prepared enzymatically by incubation of the preincubated pH 5 fraction with 0.05 mM CTP at 37° for 10 minutes. After the incubation the sample was diluted 10-fold with distilled water and the pH was adjusted to 5.2 with 1 M acetic acid. The precipitate was washed with distilled water and was dissolved in medium A. The RNA-pCpC was extracted immediately. RNA-pCpCpA was prepared by incubation of the preincubated pH 5 fraction with 0.05 mM CTP and 0.1 mM ATP at 37° for 10 minutes. The incubation mixture was treated, as above, and the RNA-pCpCpA was extracted. The basic incubation mixture contained 0.7 ml. preincubated pH 5 fraction containing 140 μg of RNA−, 10 mM ATP, 0.1 mM valine-C^{14} (1.83×10^6 cpm per μmole) in 1 ml., and 0.2 mM CTP was present in the samples marked + CTP or +. A control pair of flasks was incubated under these conditions, and to the remaining flasks 150 μg of RNA−, RNA-pCpC, or RNA-pCpCpA were added. The incubations were carried out at 37° for 10 minutes. In all cases the radioactivity incorporated into the total RNA in each flask was determined. The data reported are the cpm incorporated into the different types of *added* RNA. These values were obtained by subtraction of the cpm in RNA found in the control flasks from the cpm in the total RNA found in the flasks containing added RNA in the presence or absence of CTP, respectively.

These data suggest that the amino acids are transferred to an acceptor RNA which possesses intact nucleotide end groupings (RNA-pCpCpA), and that they are bound to the terminal adenine nucleotide, thereby preventing the replacement of the latter with mononucleotide derived from precursor ATP.

Evidence for linkage of amino acids to the 2′ or 3′ hydroxyl group of the terminal adenine nucleotide of RNA: The presence of a carboxyl phosphoanhydride linkage is unlikely, since RNA-leucine or valine is quite stable to heating at 100° in 10 per cent NaCl, pH 4 to 5,[2] and since RNA-leucine, in contrast to leucyl-adenylate, reacts only slowly with hydroxylamine at pH 5.5 at 0°.[18] The unreactivity of RNA-valine with ammonia to yield valine amide[23] under conditions where valyladenylate is converted quantitatively, as shown in Table 5, also suggests another type of linkage.

Linkage of the amino acids to the 2′ or 3′ hydroxyl group of the terminal adenine

TABLE 5

VALINE AMIDE FORMATION IN THE PRESENCE OF VARIABLE QUANTITIES OF VALINE-RNA

Incubation	Valine-Amide, cpm per ml	Valine-RNA, cpm per ml
Complete system	138	1,800
-CTP	143	20
-ATP	20	7
+ 110 μg RNA	195	2,960
+ 230 μg RNA	223	4,380
+ 110 μg RNA, -CTP	147	92

The complete system contained: 1.5 ml of preincubated pH 5 fraction (containing 220 μg RNA per 1.5 ml), 10 mM ATP, 0.2 mM CTP, 0.01 mM valine-C^{14} (11.7×10^6 cpm per μmole) in a total volume of 2 ml. Omissions of CTP, ATP, and addition of RNA prepared from a preincubated pH 5 fraction of rat liver were made as shown. The incubation was carried out at 37° for 15 min. The reaction mixture was then chilled and one aliquot from each flask was pipetted rapidly into ammonia-ethanol at −78° for the determination of valine amide. The total cpm in RNA per ml of incubation mixture were determined in a second aliquot. After addition of perchloric acid, RNA was added to the flasks to give a constant quantity of RNA per flask.

nucleotide is suggested by the demonstration of inhibition of the transfer of the amino acid to RNA-pCpCpA by borate ions, as shown in Table 6. At pH 8.5 borate ions complex with 2′ and 3′ hydroxyl groups of ribose[28] and thereby might be expected to prevent the linkage of the amino acid and of the mononucleotides to this site on the RNA. It is also possible that the borate inhibition may occur in some other way.

TABLE 6

INHIBITION OF VALINE AND NUCLEOTIDE INCORPORATION INTO RNA BY BORATE IONS

	Incubation System	Buffer System, cpm per mg RNA Tris-maleate	Sodium borate	Inhibition, per cent
I.	CTP-C^{14} + RNA-	554	145	73
II.	ATP-C^{14} + RNA-pCpC	998	240	76
III.	Valine-C^{14} + ATP + RNA-pCpCpA	5,880	1,100	81

I. Cytosine nucleotide incorporation into RNA of the preincubated pH 5 fraction was measured in the presence of pH 8.5 0.15 *M* Tris-maleate or 0.15 *M* sodium borate buffers. 0.7 ml of preincubated pH 5 fraction (containing 40 μg RNA) was incubated with 80 μg RNA-(isolated from a preincubated pH 5 fraction of rat liver) in these buffers for 10 min. at 37°. 0.01 μmole of CTP-C^{14} was then added and the incubation was continued for 10 min. at 37°. The final volume was 1 ml.

II. Adenine nucleotide incorporation into RNA-pCpC:RNA-pCpC was formed during a 10-min. incubation at 37° of 0.7 ml preincubated pH 5 fraction, 80 μg RNA-(isolated from a preincubated pH 5 rat liver fraction), plus 0.2 μmole of CTP, in a total volume of 0.8 ml. Thereafter Tris-maleate or sodium borate buffer was added, as above, and the samples were kept at 37° for 10 min. 0.5 μmole of ATP-C^{14} was then added and the incubation was continued for 10 min. The final volume was 1 ml.

III. Valine incorporation into RNA-pCpCpA:0.7 ml of preincubated pH 5 fraction was incubated for 10 min. at 37° with 80 μg RNA-(isolated from the preincubated pH 5 fraction of rat liver), 0.2μ mole of CTP, and 1 μmole of ATP to form RNA-pCpCpA. Tris-maleate or sodium borate buffer was then added and the samples were kept at 37° for 10 min. 0.1 μmole of valine-C^{14} and 9 μmoles of ATP were then added and the incubation was continued for 10 min.

The specific activities of the RNA and the percentage inhibition in the presence of borate ions are reported.

Evidence for the linkage of the amino acids to the 2′ or 3′ hydroxyl group of the ribose of the terminal adenine nucleotide of RNA is the reduced reactivity of this group with periodate in the presence of the amino acids. Whitfield[29] has shown that after oxidation with periodate, the terminal nucleoside of a di- or trinucleotide is readily split off at pH 10 while ordinary phosphodiester linkages are stable. RNA having a labeled terminal adenine nucleotide carrying amino acids was prepared (RNA-pCpCpA*-amino acid). One-half of this was treated with dilute alkali to release the amino acids, thus forming RNA-pCpCpA*. Both types of RNA were allowed to react with sodium periodate, and the resultant products were hydrolyzed with 0.05 *M* glycine buffer at pH 10 for 30 hrs. The hydrolyzates were chromatographed on Dowex-1-formate resin. RNA was held on the column, and the adenine split product was eluted with 0.05 *M* HCOOH. The data in Table 7 show that a larger

percentage of terminal adenine nucleotides was oxidized and hydrolyzed from the RNA sample which had been treated with alkali to free it of amino acids, thus indicating that in the presence of amino acid, some of the 2′ or 3′ hydroxyl groups of the terminal adenine are inaccessible to oxidation by periodate. Under ideal conditions, RNA-pCpCpA-amino acid should not react with periodate, whereas RNA-pCpCpA should react quantitatively. In the above experiment, the release of amino acids from RNA by alkali is apparently incomplete since only 71 per cent of the counts were released from the alkali treated RNA by periodate oxidation instead of the theoretical 100. In addition the RNA-pCpCpA-amino acid apparently was not fully saturated with amino acids since some terminal 2′ or 3′ hydroxyl groups (38 per cent) were available to react with periodate. Under the experimental conditions used for the isolation of RNA-pCpCpA-amino acid some amino acids may well be lost from the RNA. Nevertheless, the experiment adds evidence that the presence of the amino acids makes a number of the 2′ or 3′ hydroxyl groups of the terminal adenine nucleotide of the RNA inaccessible to oxidation by periodate.

TABLE 7

COMPARISON OF THE EXTENT OF OXIDATION OF ADENINE-LABELED RNA BY PERIODATE IN THE PRESENCE AND ABSENCE OF BOUND AMINO ACIDS

C^{14}-Adenine Labeled RNA	Splitting of RNA with Glycine Buffer, pH 10, per cent of total cpm released: Control	After Oxidation by Periodate
Alkali-treated RNA	5	71
RNA-containing amino acid	6	38

The labeled RNA containing amino acids was formed during 30 min. incubation at 37° of 10 ml S_3 and 5 ml of pH 5 fraction with 3 mg RNA, isolated from rat liver pH 5 fraction, in the presence of 1 mM ATP-8-C^{14} (349,000 cpm per μmole), 0.1 mM CTP, 10 mM phosphopyruvate, 200 μg pyruvate kinase, and 0.4 mM of each of 18 amino acids in a total volume of 20 ml. After the incubation the mixture was chilled, and precipitated at pH 5.2, the precipitate was dissolved in medium A and reprecipitated at pH 5.2 and these steps were repeated once more. RNA was isolated as described, and dialyzed against 10 per cent NaCl for 8 hrs, and against 3 changes of distilled water for 2 hrs. One-half of the RNA was treated with 0.005 *N* NaOH at 37° for 15 min. to release the amino acids,[2] and after chilling the sample was neutralized with HCl. Aliquots of untreated and alkali-treated RNA were taken as control samples at this stage. The untreated and alkali-treated RNA were then treated with sodium periodate (final concentration 0.5 *M*) at room temperature for 2 hrs. The excess periodate was removed by addition of excess glucose. The RNA was then dialyzed for 12 hrs. against 6 changes of 2 liters of distilled water at 2°. The RNA was then hydrolyzed at 37° for 30 hrs. with 0.05 *M* glycine buffer, pH 10. The RNA samples were placed on Dowex-1 × 10 formate resin (9 × 0.7 cm) and the adenine split product was eluted with 0.05 *N* HCOOH. 5 ml fractions were collected until the radioactive peak had been eluted. The total radioactivity found in this fraction was determined, and the percentage of the radioactivity released from the RNA is reported. The samples treated with periodate each contained 3,300 cpm when placed on the columns. Under these conditions of periodate oxidation, RNA-pCpCpA-Val* is stable and less than 5 per cent of free valine is formed.

Discussion.—The RNA isolated from the freshly prepared pH 5 fraction of the supernatant fraction generally contains the cytosine and adenine nucleotide end group and it is at least partially saturated with amino acids. Nucleotides and amino acids are lost from the RNA on incubation of the pH 5 fraction in the absence of CTP, ATP, and amino acids, and in the presence of inorganic pyrophosphate and AMP. The demonstration of the separate steps involved in the linkage of amino acids to the S-RNA can thus be shown best after the S-RNA has been freed of these end groups. Under the conditions described, three separate steps can be defined. The first is the addition of one or two cytosine nucleotide end groupings to the RNA. This addition can occur in the absence of amino acids. Second, the adenine nucleotide can be added to the RNA-pCpC when ATP is present at a concentration which

is insufficient for the activation and transfer of amino acids to S-RNA. The third step is the linkage of an activated amino acid to RNA-pCpCpA. Berg and Ofengand[8] report that this last reaction may be catalyzed by the same enzyme that activates the amino acid, since the relative activities of methionine activation and transfer to RNA remain constant during a 100-fold purification procedure.

The site of amino acid linkage to RNA appears to be the 2′ or 3′ hydroxyl group of the terminal adenine nucleotide of the supernatant RNA, as is indicated in this communication and by the complementary direct finding of Zachau *et al.*[18] who describe the release of 2′ or 3′ leucyladenosine from RNA-leucine by ribonuclease. The mode of the amino acid linkage to S-RNA is probably the same for all amino acids, since all of the 14 amino acids tested required the presence of the same nucleotide end group. Different RNA molecules, each possessing the same nucleotide end grouping, appear to exist, since the individual amino acids are incorporated into S-RNA additively and since there is no competition for sites on the S-RNA between natural amino acids. Partial separation of RNA fractions specific for attachment of particular amino acids has been reported by Schweet *et al.*[25] The number of nucleotide end groups attached to the S-RNA is of the right order of magnitude to accommodate all the amino acids on the terminal adenine nucleotides of specific S-RNA molecules. For example, Figure 6 shows that under optimum conditions 38 and 17 mμmoles of the cytosine and adenine nucleotide, respectively, are incorporated per mg of S-RNA, while the maximum incorporation of valine into S-RNA is 1 mμmole per mg S-RNA.

Since all of the amino acids examined have required the -pCpCpA terminal grouping on S-RNA, it is obvious that the specificity or coding does not reside in those nucleotides proximal to an activated amino acid.

Under these conditions, the lack of competition among natural amino acids, the low (and physiological) concentrations of the substrates required,[23] and the fastidious conditions for the incorporation of amino acids into preincubated RNA support the opinion that these are reactions involved in the biosynthesis of protein. The soluble RNA molecules thus appear to be carriers of activated amino acids, each RNA molecule coded in some fashion for a specific amino acid, and in turn perhaps coded for a specific, complementary site on the ribonucleic acid portion of the ribonucleoprotein particle which serves as a protein synthesizing template. In this connection there is some indication for the transfer of at least a part of the S-RNA to the RNA of the ribonucleoprotein particle.[30–32]

Summary.—The functional unit of the soluble ribonucleic acid which is required for the binding of amino acids to ribonucleic acid is a specific nucleotide end grouping. Under the conditions described, this consists of two cytosine nucleotides followed by a terminal adenine nucleotide. These nucleotides can be added to ribonucleic acid reversibly. Of 14 amino acids tested, all are incorporated into soluble ribonucleic acid to a much greater extent when the ribonucleic acid possesses this specific trinucleotide end grouping.

Evidence is presented, which indicates that the amino acids are bound in ester linkage to the 2′ or 3′ hydroxyl group of the terminal adenine nucleotide of the soluble ribonucleic acid.

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‡ Similar findings are also reported currently by J. Preiss, P. Berg, E. J. Ofengand, F. H. Bergmann and M. Dieckmann, PROC. NAT. ACAD. SCI., **45**, 319–328 (1959).

§ Tris, tris(hydroxymethyl)aminomethane; PP, inorganic pyrophosphate.

‖ Under other experimental circumstances, Herbert finds one cytosine nucleotide and a terminal adenine nucleotide (Herbert, E. B., personal communication).

¶ The asterisk* designates the location of radioactivity.

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