

A SOLUBLE RIBONUCLEIC ACID INTERMEDIATE IN PROTEIN SYNTHESIS*†

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The cell-free rat liver system in which C^{14} -amino acids are incorporated irreversibly into α -peptide linkage in protein has been used in our laboratories for a number of years as a measure of protein synthesis. The essential components of this system are the microsomal ribonucleoprotein particles, certain enzymes derived from the soluble protein fraction, adenosine triphosphate, guanosine di- or triphosphate, and a nucleoside triphosphate-generating system (1-3). The ribonucleoprotein particles of the microsomes appear to be the actual site of peptide condensation. The soluble enzymes and ATP¹ have been found to effect the initial carboxyl activation of the amino acids (4). The role of GTP is not yet understood, although the present paper sheds light on its probable locus of action.

Much evidence has accumulated in the past 8 years, beginning with the studies of Caspersson (5) and Brachet (6), implicating a role for cellular RNA in protein synthesis. The intermediate stages between amino acid activation and final incorporation into protein in the rat liver *in vitro* system offered us unexplored regions in which to seek more direct evidence for a chemical association of RNA and amino acids. A preliminary report of such an association has recently been presented by us (7). There it was shown that the RNA of a particular fraction of the cytoplasm hitherto uncharacterized became labeled with C^{14} -amino acids in the presence of ATP and the amino acid-activating enzymes, and that this labeled RNA subsequently was able to transfer the amino acid to microsomal protein

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¹ The abbreviations used in this paper are as follows: RNA, ribonucleic acid; pH 5 RNA, ribonucleic acid derived from the enzyme pH 5 fraction; AMP, adenosine 5'-phosphate; ATP, GTP, CTP, UTP, the triphosphates of adenosine, guanosine, cytosine, and uridine; PP, inorganic pyrophosphate; PPase, inorganic pyrophosphatase; PEP, phosphoenol pyruvate; Tris, tris(hydroxymethyl)aminomethane; and ECTEOLA, cellulose treated with epichlorohydrin and triethanolamine.

in the presence of GTP and a nucleoside triphosphate-generating system. This paper is a more definitive report on these studies.

Materials and Methods

Cellular fractions (microsomes and pH 5 enzymes) of rat liver and mouse Ehrlich ascites tumor were prepared by methods previously described (2, 3). Microsomes were generally sedimented at $105,000 \times g$ for 90 to 120 minutes instead of the usual 60 minutes in order to insure more complete sedimentation of microsome-like particles. pH 5 enzymes were precipitated from the resulting supernatant fraction by adjusting the pH to 5.2.

Preparation of Labeled pH 5 Enzyme Fraction—The labeling of the pH 5 enzyme fraction was carried out by incubating 10 ml. of pH 5 enzyme preparation (containing 100 to 200 mg. of protein) dissolved in buffered medium (2) with 4.0 μ moles of C^{14} -L-leucine (containing 7.2×10^6 c.p.m.) and 200 μ moles of ATP in a final volume of 20 ml. for 10 minutes at 37° . The reaction mixture was then chilled to 0° , diluted 3-fold with cold water, and the enzyme precipitated by addition of 1.0 M acetic acid to bring the pH to 5.2. The precipitate was redissolved in 5 to 10 ml. of buffered medium, diluted again (to 60 ml.) with water, and the enzyme reprecipitated at pH 5.2 with M acetic acid. This final precipitate was washed with water and dissolved in 5.0 to 10.0 ml. of the cold buffered medium.

Isolation of pH 5 RNA—Isolation of pH 5 RNA was carried out by a minor modification of the method of Gierer and Schramm (8) and Kirby (9). The labeled pH 5 enzyme solution as prepared above was shaken in a mechanical shaker at room temperature for 1 hour with an equal volume of 90 per cent phenol, followed by centrifugation at $15,000 \times g$ for 10 minutes. The top aqueous layer containing the RNA-leucine- C^{14} was removed with a syringe, more water was added, and, after thorough mixing, the centrifugation and withdrawal of the aqueous solution were repeated. Phenol was removed from the pooled aqueous solutions by three successive ether extractions. 0.1 volume of 20 per cent potassium acetate (pH 5) was then added, and the RNA was precipitated with 60 per cent ethanol at -10° , redissolved in water, and again precipitated from 60 per cent ethanol. The final precipitate was dissolved in a small volume of water and dialyzed against water for 4 hours in the cold. This method of extraction was used as a preparative procedure and yielded 50 to 70 per cent of the RNA initially present in the enzyme preparation, and was also used to prepare microsomal and unlabeled pH 5 RNA.

For analysis of pH 5 RNA-leucine- C^{14} in smaller incubations, NaCl was used to extract the RNA. To the incubation mixture (usually a

volume of 2.0 ml.), 10 volumes of cold 0.4 N perchloric acid were added. The resulting acid-insoluble precipitate, containing RNA and protein, was washed four times with cold 0.2 N perchloric acid, once with 5:1 ethanol-0.2 N perchloric acid, once with ethanol in the cold, and once with 3:1 ethanol-ether at 50°. The RNA was then extracted with 10 per cent NaCl at 100° for 30 minutes. (During this extraction, the pH drops to around 2 to 3 and it is essential to permit this to occur; if the pH is held above 6, the isolated RNA contains little or no radioactivity.) The RNA was precipitated from the NaCl extract with 60 per cent ethanol at -10°, and was dissolved in water and again precipitated with ethanol. The final ethanol suspension was filtered by suction onto disks of No. 50 Whatman paper. The dried RNA was counted by using a Nuclear micromil window gas flow counter, was then eluted from the paper with 0.005 N alkali, and the concentration determined by measuring the absorption at 260 m μ in a Beckman spectrophotometer by using an extinction coefficient of 34.2 mg.⁻¹ cm.² (10). This extraction procedure yielded 30 to 35 per cent of the RNA originally present in the incubation mixture. In experiments in which total counts are recorded, the specific activity of this NaCl-extracted RNA was multiplied by the total quantity of RNA initially added as determined by the method of Scott *et al.* (10). This was based on the assumption that the RNA extracted was a representative sample of the total.

For the determination of the specific activity of the protein, the methods described previously (1, 2) were employed.

The nucleoside triphosphate preparations, the triphosphate-generating system, and the C¹⁴-amino acids used in these studies were the same as those used in other recent work reported from this laboratory (2). 1 μ mole of Mg⁺⁺ was added per micromole of nucleoside triphosphate in all cases.

Results

Labeling of RNA Cellular Fractions with Amino Acids—In the complete system required for incorporation of C¹⁴-amino acids into protein (microsomes, pH 5 enzymes, ATP, GTP, nucleoside triphosphate-generating system, and C¹⁴-amino acids), the RNA subsequently isolated was found to be labeled with C¹⁴-amino acids. Incubation of the (pH 5) enzyme fraction without microsomes under these conditions resulted in substantially more RNA labeling than in the complete system. Little labeling of RNA was observed when microsomes were incubated alone under the above conditions. Further analysis of the requirements for labeling of pH 5 RNA revealed that ATP alone was sufficient and that GTP and the generating system were not necessary. A survey of the extent of labeling

of the RNA of various isolated liver cellular fractions with leucine is shown in Fig. 1, which shows that pH 5 RNA has the highest specific activity.

Fig. 2 shows the dependence of labeling of pH 5 RNA upon leucine concentration. Glycine- C^{14} , valine- C^{14} , or alanine- C^{14} gave about the same

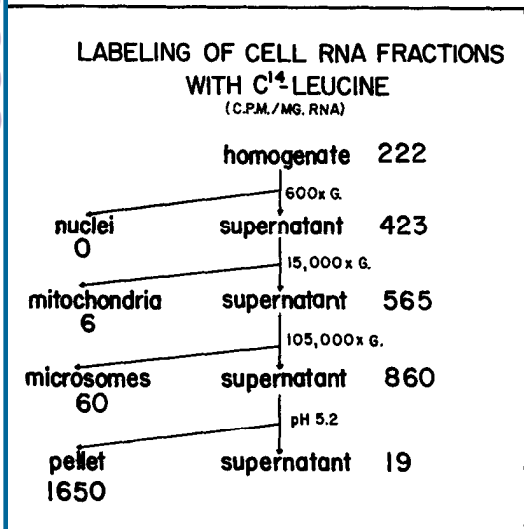


FIG. 1

FIG. 1. Cellular fractions were prepared from rat liver and resuspended or dissolved in buffered medium (2). 0.75 ml. of each fraction was then incubated at 37° for 10 minutes with 10 μ moles of ATP, 0.1 μ mole of L-leucine- C^{14} containing 180,000 c.p.m., 5 μ moles of PEP, and 0.04 mg. of pyruvate kinase in a final volume of 1.0 ml. 4 μ moles of potassium fumarate, 10 μ moles of potassium glutamate, and 10.0 μ moles of orthophosphate were added to the incubation mixtures containing the original homogenate, the 600 X g supernatant fraction, and the mitochondrial fraction, and these were shaken in an atmosphere of 95 per cent oxygen-5 per cent CO_2 during the incubation.

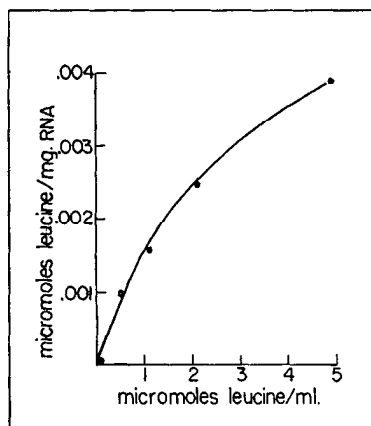


FIG. 2

FIG. 2. Leucine concentration curve for labeling of pH 5 RNA. 1.2 ml. of rat liver pH 5 enzyme preparation (approximately 13 mg. of protein), in buffered medium were incubated for 10 minutes at 37° with 20 μ moles of ATP, L-leucine- C^{14} containing 3.6×10^5 c.p.m. at the concentrations indicated, in a final volume of 2.0 ml.

extent of labeling as leucine- C^{14} when each was present at a concentration of 0.007 M. When these amino acids were combined (0.007 M each), the labeling was approximately additive. The addition of a mixture containing fifteen C^{12} -amino acids (lacking leucine) did not affect the extent of labeling with leucine- C^{14} . Maximal labeling of 0.04 μ mole of leucine per mg. of RNA was attained with the most active liver preparations by using 0.005 M leucine and 0.01 M ATP.

ATP was necessary for the labeling of the RNA with amino acids, and the extent of labeling depended upon the concentration of ATP (Fig. 3).

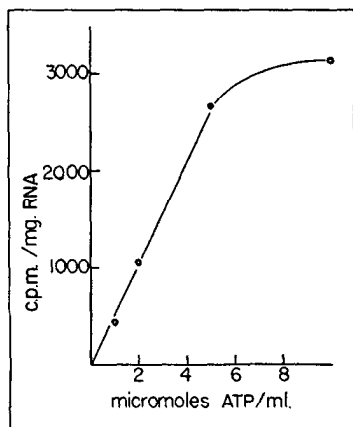


FIG. 3

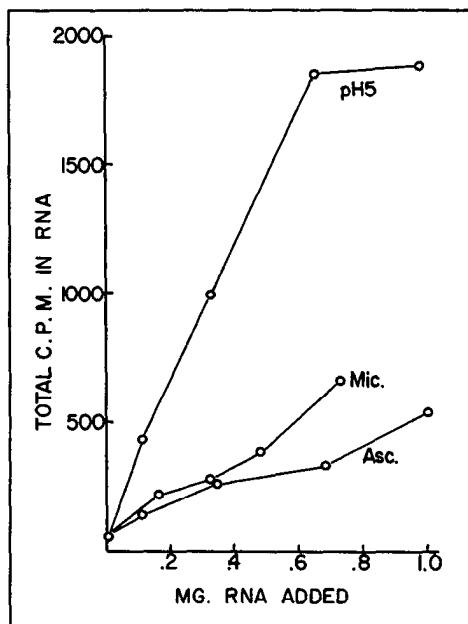


FIG. 4

FIG. 3. ATP concentration curve for labeling of pH 5 RNA. 1.8 ml. of rat liver pH 5 enzyme preparation (20 mg. of protein) in buffered medium were incubated for 20 minutes at 37° with 0.2 μ mole of L-leucine- C^{14} containing 3.6×10^5 c.p.m. and ATP at the concentrations indicated, in a final volume of 2.0 ml.

FIG. 4. Effect of addition of certain RNA preparations upon the labeling of pH 5 RNA with leucine- C^{14} . RNA was prepared from the pH 5 enzyme (pH 5) and microsome (Mic.) fractions of rat liver and from whole ascites cells (Asc.) by the phenol method. The quantities of RNA indicated, dissolved in 0.02 M Tris buffer, pH 7.6 (0.5 μ mole of $MgCl_2$ added per mg. of RNA), were incubated at 37° for 10 minutes in 1.0 ml. volumes with pH 5 enzyme obtained from Ehrlich ascites cells (containing 0.12 mg. of RNA), and the following final concentrations of additions: 0.01 M ATP; 0.01 M PEP; 0.0038 M $MgCl_2$; 0.038 M Tris, pH 7.6; 0.018 M KCl; and 0.04 mg. of pyruvate kinase. The total radioactivity in RNA plotted was calculated as the product of the specific activity of the RNA isolated by extraction by the NaCl method and the total quantity of RNA present at the end of the incubation period. Addition of liver pH 5 RNA to the liver pH 5 enzyme fraction produces a similar enhancement of incorporation to that shown here with the tumor enzyme.

Maximal incorporation was reached at about 0.01 M. A similarly shaped ATP concentration curve had earlier been found for the amino acid activation reaction, as measured by hydroxamic acid formation (4). Also, as

in the activation reaction, the requirement for ATP was specific since GTP, CTP, and UTP did not replace this nucleotide. GTP, which is necessary for the over-all incorporation of amino acids into protein, did not affect the rate of labeling of pH 5 RNA by the pH 5 enzyme fraction, in the presence or absence of a mixture containing fifteen C¹²-amino acids (lacking leucine).

The amino acid labeling of RNA was sensitive to ribonuclease. 10 γ per ml. of Worthington crystalline ribonuclease gave 40 per cent inhibition, and 40 γ gave 90 per cent inhibition in the presence of 10 mg. of enzyme protein per ml. This inhibition was accompanied by a smaller loss of absorbance at 260 m μ in the acid-precipitable fraction. It is worth recalling at this point that the activation reaction, as measured by PP³²-ATP exchange and hydroxamic acid formation, is *not* affected by ribonuclease (4).

The extent of incorporation of leucine-C¹⁴ into pH 5 RNA was markedly stimulated by the addition of isolated pH 5 RNA as may be seen in Fig. 4. The enhancement of labeling was relatively specific for this particular RNA, rat liver microsomal RNA and mouse ascites whole cell RNA being of low activity. The small amount of stimulation by microsomal RNA shown in Fig. 4 may well be due to contamination of microsomes with the supernatant fraction, since the microsomes were centrifuged from the undiluted 15,000 $\times g$ supernatant fluid of a concentrated (30 per cent) homogenate.

The labeling reaction proceeded linearly with time for 3 minutes and was complete in 10 minutes. In those preparations in which precaution was taken to minimize contamination with microsomes (by preparing pH 5 enzymes from a 105,000 $\times g$ supernatant fraction obtained after a centrifugation for 90 to 120 minutes), there was no loss of leucine-C¹⁴ for a period of 20 minutes after maximal labeling had been reached. Slight microsomal contamination, however, resulted in a loss of leucine from RNA after maximal labeling had been reached.

After incubation of the (pH 5) enzyme fraction with leucine-C¹⁴ and ATP, these latter compounds could be largely removed by reprecipitation of the enzymes at pH 5 from dilute solution, as described. Upon subsequent incubation of this reprecipitated fraction, the leucine label was rapidly lost from the RNA unless ATP was added (Table I). The equivalent effect of a nucleoside triphosphate-generating system (PEP and pyruvate kinase), also shown in Table I, was probably mediated through the presence of very small amounts of adenylates which coprecipitate with the pH 5 enzyme. PP, on the other hand, increased the extent of loss of label from the RNA. These findings suggested that the labeling process might be *reversible*. This possibility was rendered more probable by the

finding that, in the presence of added ATP, the addition of leucine-C¹² produced a dilution of the leucine-C¹⁴ labeling, as shown in Experiment 2, Table I. This would be expected if the following reactions were occurring:



The loss of label in the absence of added ATP would depend upon the presence of small amounts of indigenous PP. The failure of leucine-C¹²

TABLE I

Effect of Various Additions upon Loss of Leucine-C¹⁴ from Labeled pH 5 Enzyme

Experiment No.	Addition	Amount	Per cent initial specific activity lost
		<i>M</i>	
1	None	0	75
	ATP	0.001	44
	"	0.005	35
	" + AMP	0.005 each	37
	PP	0.01	96
	PEP	0.01	24
2	None	0	79
	Leucine-C ¹²	0.01	84
	ATP	0.01	39
	" + leucine-C ¹²	0.01 each	68

L-Leucine-C¹⁴-labeled pH 5 enzyme (0.4 ml.) was incubated at 37° for 7 minutes in a volume of 2.0 ml. with the concentrations of additions indicated. A concentration of MgCl₂ equal to that of PP was added with the latter. Pyruvate kinase (0.04 mg. per ml.) was added with the PEP. The initial specific activities of the RNA which were isolated from the pH 5 enzymes labeled during the preincubation were: Experiment 1, 770 c.p.m. per mg.; Experiment 2, 440 c.p.m. per mg.

to effect a dilution in the absence of added ATP would be anticipated since, due to the high ATPase activity of the preparation and the absence of a generating system, the ATP concentration would be effectively zero and the reaction would proceed rapidly to the left. It is of interest in this connection that Holley (11) has described an alanine-dependent, ribonuclease-sensitive incorporation of C¹⁴-AMP into ATP catalyzed by the pH 5 enzyme preparation. This would suggest a reversal of an ATP-dependent reaction between alanine and RNA. However, other amino acids have not been found to stimulate such an exchange, suggesting that AMP is generally not a free product of reaction (2). The possibility must still be entertained, however, that ATP has some stabilizing effect upon the pH 5 RNA-amino acid bond not related to mass action.

A high concentration of NH_2OH such as 1.2 M, which was used to obtain amino acid hydroxamic acid formation with this preparation (4), also inhibits (90 per cent) the labeling of RNA with leucine.

Some Properties of pH 5 RNA-Leucine- C^{14} —The RNA of the enzyme (pH 5) fraction of rat liver represents 2 per cent of the total RNA of the cell and only 20 per cent of the RNA of the $105,000 \times g$ 2 hour supernatant fraction. It is present in a concentration of 3 mg. of RNA per 100 mg. of protein. In the mouse ascites tumor essentially all the RNA of the $105,000 \times g$ supernatant fraction precipitates at pH 5.2 and amounts to 20 per cent of the total RNA of the cell.

The active component of the pH 5 enzyme fraction does not sediment at $105,000 \times g$ in 3 hours. If one compares the activity and RNA content of the pH 5 enzyme prepared from a supernatant fraction obtained after 1 hour or 3 hour centrifugations at $105,000 \times g$, one finds that the latter preparation contains only 50 per cent as much RNA as the former. The amount of leucine incorporated into the RNA of both preparations is, however, the same, suggesting that the RNA sedimented during the additional centrifugation time is not active.

RNA-leucine- C^{14} gave a mean sedimentation constant of 1.85 $s_{20,w}$ at a concentration of 0.003 per cent in 0.15 M NaCl, 0.015 M citrate, pH 6.8.² Preliminary studies indicate that this value is lower when effort is made to remove magnesium ion first by dialysis against citrate buffer. The material does not appear homogeneous, however, and probably represents a range of molecular sizes. Preliminary results with paper electrophoresis suggest at least two major components.

A sample of pH 5 RNA-leucine- C^{14} extracted by the phenol method was fractionated on ECTEOLA (12). 1 mg. of RNA, dissolved in 0.01 M phosphate buffer at pH 7 and containing 4040 c.p.m. as leucine- C^{14} , was placed on a column 0.2 cm. in diameter containing 50 mg. of ECTEOLA-SF (0.16 meq. of N per gm.).³ Elution was carried out with a gradient of NaCl in 0.01 M phosphate buffer at pH 7, which was established by feeding buffer containing 2.5 M NaCl into a 500 ml. mixing flask. 1.5 ml. fractions were collected at a flow rate of 1.8 ml. per hour. The NaCl gradient was continued until the molarity of the effluent was about 2. In accordance with the general procedure of Bradley and Rich (12), the gradient was discontinued, the column washed with water, and 10 ml. of 1 N NaOH were run through. Three fractions emerged: Fraction 1 failed to adhere to the exchanger and contained 14 per cent of the ultraviolet absorbance and 8 per cent of the radioactivity (free leucine, if present

² We wish to thank Dr. J. Fresco and Dr. P. Doty of Harvard University for performing these analyses.

³ Kindly furnished by Dr. Alexander Rich.

would have been found in this fraction); Fraction 2 emerged at a mean molarity of 0.15 NaCl and contained 48 per cent of the absorbance and 2 per cent of the radioactivity; and Fraction 3 was eluted with NaOH and contained 36 per cent of the absorbance and 68 per cent of the radioactivity. The final recovery amounted to 98 per cent of the ultraviolet absorbance and 78 per cent of the radioactivity. The low recovery of the radioactivity is most likely due to self-absorption in the NaOH-eluted fractions when plated for counting. These results, compared with those published by Bradley and Rich, suggest that at least 68 per cent of the leucine is bound to 36 per cent of the RNA of high sedimentation coefficient relative to that of the bulk of the sample.

pH 5 RNA-leucine- C^{14} isolated from the pH 5 enzyme fraction by both the phenol and NaCl methods was readily bound by Dowex 1 and charcoal at neutral pH value. However, when the RNA-leucine- C^{14} was associated with pH 5 enzyme protein in the natural state, these agents did not take up the RNA. The isolated RNA-leucine- C^{14} was non-dialyzable and stable against water, 10 per cent NaCl, or 8 M urea. There was no detectable acid-precipitable protein in the RNA extracted by the phenol method (1 per cent contamination could have been detected (13)).

The leucine was completely released from the pH 5 RNA by 0.01 N KOH in 20 minutes at room temperature. At pH 4 to 6, it was relatively stable and the labeled material as prepared by the phenol method could be kept some weeks in the frozen state. The leucine appeared to be covalently linked to the RNA, as judged from the following indirect evidence. Treatment of the RNA-leucine- C^{14} with the ninhydrin reagent indicated the absence of free leucine, although leucine is slowly released from the RNA during the course of the ninhydrin procedure. Treatment with anhydrous hydroxylamine, followed by chromatography of the products on paper (75 per cent secondary butanol, 15 per cent formic acid, 10 per cent water), resulted in a spot corresponding to leucine hydroxamic acid which contained all the radioactivity originally bound to the RNA. (A control of this experiment, in which the RNA-leucine- C^{14} bond was first hydrolyzed in 0.01 N alkali, gave no radioactivity associated with the leucine hydroxamic acid spot.)

Labeling of RNA with Leucine- C^{14} in Intact Cell—If pH 5 RNA were on the pathway of protein synthesis, it would be reasonable to expect that in the intact cell it would become labeled with leucine- C^{14} earlier than microsome protein. Previous studies in this laboratory by Littlefield and Keller (3) had shown that treatment of mouse ascites tumor microsomes with 0.5 M sodium chloride facilitates the centrifugal separation of ribonucleoprotein particles rich in RNA (about 50 per cent RNA, 50 per cent protein). The protein moiety of these "sodium chloride-insoluble" par-

ticles was found to be the most highly labeled protein fraction after incorporation of leucine- C^{14} by intact cells. A preliminary experiment in the rat showed that, at the earliest time point which it was possible to obtain after injection of leucine- C^{14} (1 minute), both RNA of the pH 5 fraction and the protein of the ribonucleoprotein particles of the microsomes were already maximally labeled. By use of mouse ascites tumor cells, it was possible to slow down the reaction by reducing the temperature of incubation. After incubation of these cells with leucine- C^{14} at 25° , the cells were washed and lysed, and concentrated solutions were added to give a final concentration of 0.5 M NaCl, 0.005 M $MgCl_2$, and 0.01 M Tris buffer, pH 7.6 (3). "NaCl-insoluble" (NaCl particles) and "soluble" fractions of a 10 minute $15,000 \times g$ supernatant fraction were separated by centrifugation at $78,000 \times g$ for 2 hours. Both the protein and RNA were isolated. Since almost all of the RNA present in the soluble fraction of the ascites cells precipitates at pH 5, the RNA of this fraction may be considered pH 5 RNA. The proteins of the soluble fraction represent the proteins of the NaCl-soluble components of the microsomes and the soluble cell proteins. Littlefield and Keller (3) have shown that these two fractions become labeled at a slow rate and therefore they were not separated. The results of this experiment are shown in Fig. 5. Soluble and particle RNA became labeled maximally in 2 minutes and remained so as if a steady state had been reached, while the protein of the ribonucleoprotein particles continued to acquire new amino acid content throughout the incubation period. Incorporation into the other cell proteins started after an initial lag period and proceeded at the slowest rate. The rate of labeling of the pH 5 RNA is so rapid that it occurs to some extent at 0° and no satisfactory rate curve for this labeling process could be obtained, since the reaction is proceeding even during centrifugal separation of the fractions. Similar results were obtained when the cell fractions were prepared from a sucrose homogenate and the pH 5 RNA and the protein of the deoxycholate-soluble and -insoluble fractions of the microsomes were isolated. These data suggest that the pH 5 RNA-amino acid compound *could* be an intermediate in the incorporation of amino acids into the proteins of the ribonucleoprotein particles of the microsomes.

Transfer of Leucine- C^{14} from Labeled pH 5 Enzyme Fraction to Microsomal Protein—We have reported (7) that the leucine- C^{14} -labeled enzyme fraction at pH 5, freed from ATP and leucine- C^{14} by reprecipitation at pH 5.1 from dilute solution, will transfer the RNA-bound leucine- C^{14} to microsomal protein upon subsequent incubation with microsomes, a nucleoside triphosphate-generating system, and GTP (Table II). The other nucleoside triphosphates, including ATP, would not replace GTP in this reaction; ATP also failed to stimulate the transfer in the presence of GTP.

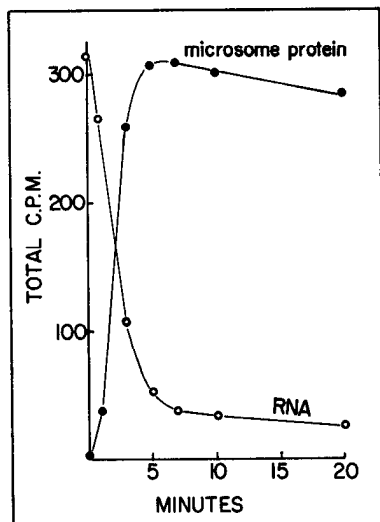


FIG. 6

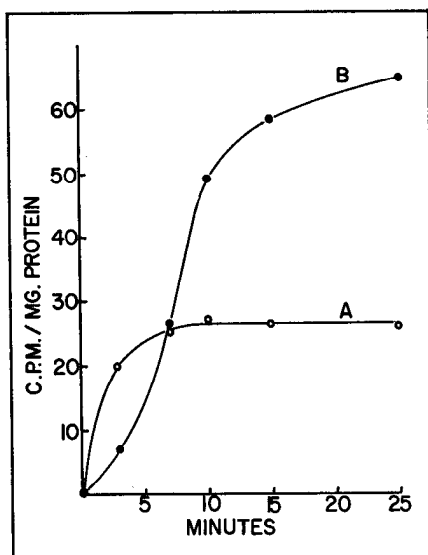


FIG. 7

FIG. 6. Time-curve of transfer of leucine- C^{14} from prelabeled pH 5 enzyme fraction to microsome protein. 4.0 ml. of a leucine- C^{14} -labeled pH 5 enzyme preparation (containing 2.6 mg. of RNA), and 6.0 ml. of microsomes (21 mg. of RNA) were incubated at 37° with 20 μ moles of GTP, 200 μ moles of PEP, and 0.8 mg. of pyruvate kinase in a volume of 20.0 ml. 2.5 ml. aliquots were taken at the time points shown. These were chilled, diluted to 12.5 ml., and centrifuged at $105,000 \times g$ for 60 minutes in the cold. The RNA and protein of the supernatant fluid and of the microsomes were separated. The total counts per minute in the pH 5 RNA (O) and in the microsome protein (●) is plotted. Since there is about 50 per cent enzymatic loss of leucine- C^{14} from pH 5 RNA during the hour's centrifugation (determined directly by centrifuging an aliquot of labeled enzyme, pH 5, of known specific activity under the same conditions), a correction for this loss was applied to the specific activity of RNA to give the final figures used.

FIG. 7. A comparison of the rates of the over-all incorporation reaction and the incorporation when starting with labeled pH 5 enzyme fraction. 40 ml. of an enzyme preparation at pH 5 were incubated for 10 minutes at 37° with 100 μ moles of ATP and 2 μ moles of leucine- C^{14} (3.6×10^6 c.p.m.) in a volume of 10 ml. An equal aliquot of the same enzyme was incubated identically with ATP and leucine- C^{14} . Both enzymes were then precipitated twice at pH 5.1 from dilute solution. An aliquot of the labeled enzyme was taken for determination of RNA content and another for determination of specific activity of RNA-leucine- C^{14} . 1.8 ml. of leucine-labeled enzyme, dissolved in buffered medium and containing 1540 c.p.m. of bound leucine- C^{14} , were then incubated with 1.8 ml. of microsomes, 3 μ moles of GTP, 60 μ moles of PEP, and 0.24 mg. of pyruvate kinase in a volume of 6.0 ml. The same volume and amount of unlabeled enzyme were incubated with the same quantity of microsomes, GTP, PEP, and pyruvate kinase, plus 3 μ moles of ATP and 0.6 μ mole of leucine- C^{14} containing 1.1×10^6 c.p.m. The incubation mixtures were equilibrated at 30° for 1 minute before addition of microsomes and incubation was carried out at 30° . 1.0 ml. aliquots of each incubation were taken, each containing approximately 9 mg. of protein, at the times indicated and the protein of the samples was precipitated, washed, plated, and counted. Curve A, reaction with prelabeled pH 5 enzyme; Curve B, over-all reaction.

In the absence of GTP there was an equally rapid microsome-dependent loss of leucine- C^{14} from the intermediate, without concomitant appearance of amino acid in protein. (Rat liver microsomes contain considerable "ATPase" activity. Whether the loss of label is due to destruction of ATP still present in the system, thus permitting reversal of the reaction, or a manifestation of an uncoupling of the basic mechanism for trans-

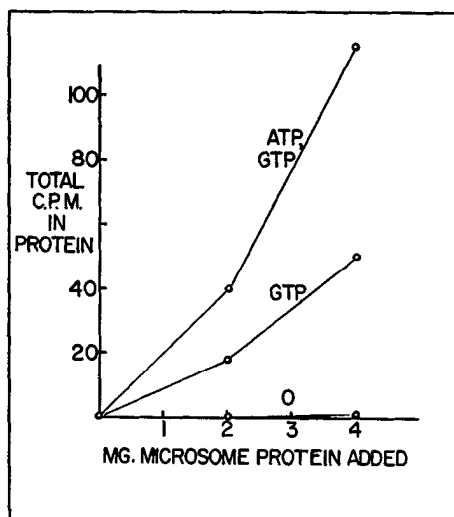


FIG. 8. Transfer of leucine- C^{14} from isolated pH 5 RNA-leucine- C^{14} to microsomal protein. 0.40 mg. of RNA, prepared by the phenol method, containing 600 c.p.m. of bound leucine- C^{14} , was incubated at 37° for 15 minutes in buffered medium (0.5 ml.) with the amount of microsomes indicated with added PEP (0.01 M), pyruvate kinase (0.04 mg.) in a volume of 1.0 ml. 0.5 μ mole of ATP and GTP was added as indicated (0 = no addition of nucleotide). ATP alone gave the same activity as with no nucleotide additions. The microsomes used were sedimented from a 15,000 $\times g$ supernatant fraction which was diluted 3.5-fold before centrifugation at 105,000 $\times g$.

ferring amino acid to microsomes is not known.) GTP had no effect on the considerably slower rate of loss of label from enzyme at pH 5 in the absence of microsomes. The addition of a relatively high concentration of leucine- C^{12} did not appreciably dilute the radioactivity transferred to protein. Fig. 6 shows a time-curve for the transfer reaction and emphasizes the high efficiency of the transfer as well as its relatively rapid rate. Fig. 7 presents a comparison of the rates of the transfer of leucine- C^{14} to microsomal protein from free leucine- C^{14} and ATP (Curve B) and from the prelabeled intermediate in the absence of free leucine and ATP (Curve A). When starting with the labeled intermediate, the lag in the initial

rate of the over-all reaction was absent, the efficiency of transfer was much greater, and the reaction attained completion at an earlier time.

Transfer of Leucine-C¹⁴ from Isolated Labeled pH 5 RNA to Microsomal Protein—pH 5 RNA-leucine-C¹⁴, extracted from labeled enzyme at pH 5 by the phenol method, precipitated twice from ethanol and dialyzed against water, will, upon incubation with microsomes, transfer leucine-C¹⁴ to microsomal protein. In seven experiments of this type, an average of 20 per cent of the leucine was transferred to protein (25 per cent maximum). In every case, pretreatment of the RNA-leucine-C¹⁴ with 0.01 N KOH at room temperature for 10 to 20 minutes resulted in lack of transfer of the leucine. Again, leucine-C¹² did not inhibit the transfer. pH 5 RNA-leucine-C¹⁴ extracted by the NaCl method was consistently found to be inactive.

GTP was again found to be necessary for this transfer, and there was no transfer in the absence of a nucleoside triphosphate-generating system. Furthermore, a partial requirement for ATP became apparent with this simplified system as shown in Fig. 8. The failure to elicit an ATP requirement for the transfer of amino acid from labeled pH 5 enzyme fraction to microsomes (previous section) was apparently due to the presence of ATP not washed free from the enzyme when reprecipitated at pH 5.

Microsomes alone appear not to react directly with pH 5 RNA-leucine but to require the mediation of enzymatic components of the pH 5 enzyme fraction. Microsomes prepared from dilute homogenates (to minimize contamination with pH 5 enzymes) were low in activity when incubated with pH 5 RNA-leucine-C¹⁴, ATP, GTP, and the generating system but activity could be restored by addition of the pH 5 enzyme fraction.

DISCUSSION

The evidence presented supports the conclusion that there occur ATP-dependent enzymatic reactions between ribonucleic acid and amino acids. These reactions are catalyzed by an enzyme preparation which is known to activate the carboxyl groups of amino acids in the presence of ATP. The product formed, an RNA or ribonucleoprotein to which amino acids are apparently covalently linked, is capable of interacting with enzymatic components of the activating enzyme preparation and with microsomes to effect the transfer of the amino acid to peptide linkage in protein. It is therefore suggested that this particular RNA fraction functions as an intermediate carrier of amino acids in protein synthesis. A growing body of evidence from other laboratories also suggests the presence of an intermediate similar to the one herein described (14, 15, 11, 16).

Since the amino acid activation reaction is insensitive to ribonuclease and since an activating enzyme has been isolated relatively free from RNA (17),

it is still necessary to invoke an initial enzymatic activation reaction as originally postulated (4), followed by a transfer of amino acid to linkage on RNA. Because of the impurity of the enzyme system at pH 5, it cannot be stated that pH 5 RNA is naturally linked to amino acid-activating enzymes or that other enzymatic steps intervene between activation and linkage to RNA. The relative specificity of the reaction of the pH 5 enzyme fraction with pH 5 RNA shown in Fig. 4, does, however, emphasize the uniqueness of this particular RNA fraction in regard to ATP-dependent amino acid binding.

The present data suggest that the pH 5 RNA molecules, when associated with protein in the natural state, are considerably lower in average sedimentation coefficient than are the ribonucleoprotein particles of the microsomes. The latter are probably of the order of 80 S (18), while the former appear to be much lower. Furthermore, the results of other experiments from this laboratory, in which pH 5 RNA is enzymatically terminally labeled with the nucleoside monophosphate moieties of nucleoside triphosphates (19), suggest that the average molecular weight of the RNA is not likely to exceed 20,000 (based on maximal labeling, and assuming no branching). The sedimentation constant of 1.85 would be consistent with a molecular weight considerably lower than this.

Thus far we cannot assign a specific structure to the amino acid-RNA linkage. An attractive possibility is an acyl anhydride involving internucleotide phosphate groups or a terminal nucleotide residue. The acid stability and alkali lability of the linkage, qualitatively similar to the behavior of the synthetic amino acyl adenylates (20, 21), the formation of a hydroxamic acid, and the relative high energy of the linkage suggested by the possible reversibility of the reaction would support this type of anhydride linkage. The linkage would appear, however, to be more stable than a phosphate diester anhydride might be expected to be. We have also given thought to the possibility that internucleotide P—O bonds may be opened by reaction with an amino acyl adenylate, with resulting attachment of the amino acyl group to one of the opened ends of the nucleotide chain and adenylate to the other. Other possible linkages to be considered are carboxyl bonding to 2'-OH on ribose and bonding involving groups on the nucleotide bases themselves. It is, nevertheless, likely that, regardless of the type of bonding, amino acids are individually linked to pH 5 RNA and do not *condense* at this stage, for the amino acid may be recovered as the specific hydroxamic acid upon treatment with hydroxylamine.

The high efficiency of the GTP-dependent transfer of amino acid from intermediate to microsome protein is striking. There is no evidence that GTP is concerned either in the activation step or in the transfer of

amino acid to pH 5 RNA. Its locus of action is thus narrowed down to the area of interaction between pH 5 RNA-amino acid and microsomes. The fact that enzymatic components of the pH 5 fraction are still required for the transfer from pH 5 RNA-leucine to microsomes could mean either that a new transfer enzyme is required or that reassociation of intermediate with activating enzymes is necessary. If this latter is the case, the possibility that pH 5 RNA acts simply as a storage site for activated amino acids must be considered.

Other studies in this laboratory to be reported have shown that the same pH 5 enzyme fraction also catalyzes a rapid incorporation of the nucleotide monophosphate moieties of ATP, CTP, and UTP into pH 5 RNA. The appearance of these reactions in the same fraction which catalyzes the amino acid binding to RNA is intriguing, but thus far it has not been possible to obtain evidence for any clear direct link between the two reactions.

We have suggested elsewhere (22) a hypothetical reaction sequence for protein synthesis which accounts for the findings presented in this paper. Its central idea is that pH 5 RNA molecules, each charged with amino acids in characteristic sequence, polymerize in microsomes (in specific order determined by the complementary structure of microsomal RNA) to higher molecular weight units with resultant configurational changes which permit peptide condensation between contiguous amino acids. This working hypothesis will form the basis for further studies in these laboratories on the mechanism of protein synthesis.

SUMMARY

Evidence is presented that a soluble ribonucleic acid, residing in the same cellular fraction which activates amino acids, binds amino acids in the presence of adenosine triphosphate. Indirect evidence indicates that this reaction may be reversible. The amino acids so bound to ribonucleic acid are subsequently transferred to microsomal protein, and this transfer is dependent upon guanosine triphosphate.

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