Protein Synthesis in a Cell-Free System Derived from Rabbit Liver[1] (37215)

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(Introduced by R. E. Ritts)
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Cell-free extracts of bacteria, plants and animals have been utilized to investigate the mechanism of protein synthesis. The ability of polyuridylic acid (poly U) and other synthetic polyribonucleotides to direct specific polypeptide synthesis by these cell-free extracts has provided a tool not only for investigating the nature of the genetic code but also for elucidating the overall mechanism of protein synthesis. However, the regulation of protein synthesis at the transcriptional and translational levels has been studied in relatively few mammalian tissues (1–8). In this report we describe a subcellular protein-synthesizing system that utilizes components derived from rabbit liver tissue which is capable of incorporating amino acids into protein at levels greater than that described for other mammalian cell-free systems.

Materials and Methods. Materials. Polyuridylic acid (poly U), with 250/260 and 280/260 optical density ratios of 0.78 and 0.35, respectively, was purchased from Calbiochem (Los Angeles, CA). Uniformly labeled L-[^{14}C]-phenylalanine (414 mCi/mmole) was purchased from New England Nuclear Corp. (Waltham, MA). Crystalline phosphoenolpyruvate kinase, potassium phosphoenolpyruvate, and sodium ATP and GTP were obtained from Sigma Chemical Corp. (St. Louis, MO). Nonidet P-40 was the gift of the Shell Chemical Co. (New York).

Methods. Preparation of ribosomes. San Juan rabbits were exsanguinated, their livers were removed and rinsed 3 times in cold buffer A (0.025 *M* KCl, 0.005 *M* $MgCl_2$, 0.006 *M* 2-mercaptoethanol, and 0.05 *M* Tris, pH 7.6). The tissue was resuspended in buffer A containing 0.25 *M* sucrose (homogenizing buffer), homogenized with a motor driven loose-fitting Teflon pestle and rehomogenized by 6 strokes each in a loose- and a tight-fitting Dounce homogenizer. The homogenizations and all subsequent preparative steps were carried out at 4°. The homogenate was centrifuged at 15,000*g* for 10 min and the supernate was centrifuged at 105,000*g* for 2 hr in a type 50.1 rotor on a Spinco L2-65B ultracentrifuge. The resulting supernate (S-105) was used to prepare pH 5 enzyme and transfer factors (see below). The microsomal pellet was resuspended in buffer A containing 0.5% Nonidet P-40, carefully layered over an equal volume of 0.5 *M* sucrose in buffer A and centrifuged at 105,000*g* for 2.5 hr. The ribosomal pellet was resuspended in buffer A, dispensed into aliquots, rapidly frozen and stored at —70°. Ribosomal RNA (rRNA) was assayed by a modification of the procedure of Fleck and Munro (9).

Preparation of pH 5 enzyme. The S-105 supernatant was diluted with 2 vol of cold, sterile glass-distilled water containing 0.006 *M* 2-mercaptoethanol, made pH 5.1 with 1 *M* acetic acid and incubated at 4° for 15 min. The preparation was centrifuged at 10,000*g* for 20 min and the clear supernatant was used for the preparation of transfer factors (11). The pellet was resuspended in buffer B (0.07 *M* KCl, 0.006 *M* 2-mercaptoethanol, 0.004 *M* $MgCl_2$, 0.35 *M* sucrose and 0.05 *M* Tris, pH 7.8) and 1 ml aliquots were rapidly frozen and stored at —70°. The protein concentration of the pH 5 enzyme frac-

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tion was determined by the method of Lowry *et al.* (10).

Cell-free amino acid incorporation. The subcellular incorporating system contained in a volume of 0.6 ml: 70 m*M* KCl, 6 m*M* 2-mercaptoethanol, 5 m*M* ATP, 0.4 m*M* GTP, 20 μg pyruvate kinase, 15 m*M* $MgCl_2$, 100 nmoles of L-[^{14}C]-phenylalanine, 50 μg rRNA and 0.5 to 0.75 mg of pH 5 enzyme. Assays were performed in the absence and presence of poly U. Additional details and modifications are described in the table and figure legends. The reaction mixtures were incubated at 37° for 45 min and the reaction was stopped by the addition of 2 ml of cold 8% trichloracetic acid containing 1% Casamino acids. The reaction mixtures were incubated for 10 min at room temperature, 30 min at 90° and 30 min at 4°. The precipitates were collected on Whatman GF/C filters, washed 3 times with cold 5% trichloracetic acid containing 1% Casamino acids and placed in scintillation vials. The protein was removed from the filters with 0.7 ml of formic acid and then 10 ml of scintillation fluid containing 1 part 2-methoxyethanol and 1 part toluene in Liquifluor (New England Nuclear Corp., Waltham, MA) was added. The incorporated radioactivity was counted in a Beckman LS-100 liquid scintillation counter to 2% error. Reagent controls contained the same filtered precipitates plus an aliquot of the [^{14}C]-phenylalanine-containing media to determine the radioactivity input, specific activity and degree of quenching in each experiment. All results are corrected for the zero time control. Net stimulation refers to the amount of phenylalanine incorporated in the presence of poly U minus the amount incorporated in the absence of poly U.

Results and Discussion. Characterization of ribosome preparations. The ribosome preparations obtained as described in Materials and Methods were found to consist of single 80S ribosomes when analyzed by sucrose gradient centrifugation. The ribosome preparations were resistant to breakdown after rapid freeze-thawing and storage at −70° for up to 8 mo as demonstrated by their specific activity and analysis on sucrose gradients. The absorption ratios of A_{260}:A_{280} and A_{260}:A_{230} for various ribosome preparations were 1.40 ± 0.10 and 1.70 ± 0.10, respectively. These ratios are somewhat low suggesting that the preparations contained protein, probably transfer and translocation factors (11).

Characteristics of the incorporation system. Transfer factors, important in aminoacyl-transfer RNA binding and the translocation reaction, have been reported to be present in the pH 5 supernatant from rat liver (11). Transfer factors, isolated as described by Siler and Moldave (11) from rabbit pH 5 enzyme, were generally inhibitory to phenylalanine incorporation in both the endogenous and poly U stimulated rabbit liver systems. Transfer factors active in a rat liver subcellular system were also found to be inhibitory to phenylalanine incorporation in the rabbit liver system. This inhibition was not due to chelation of essential ions or destruction of energy sources and was probably due to the presence of an inhibitory factor.

The optimal concentration of pH 5 enzyme for incorporation of phenylalanine in both the presence and absence of poly U increased until 0.5 mg/reaction system (Fig. 1). Concentrations greater than 0.5 mg protein were found to inhibit phenylalanine incorporation independent of the ribosomal

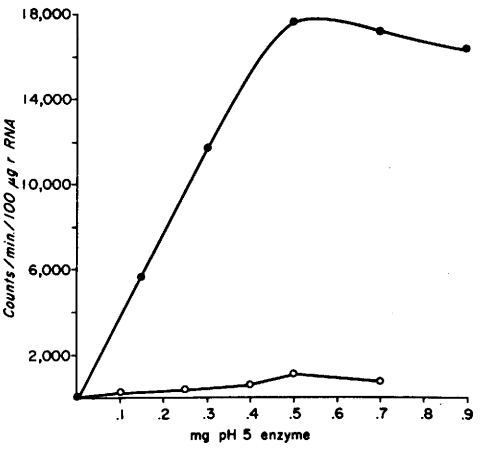

FIG. 1. The effect of varying the concentration of the pH 5 enzyme in the reaction mixture upon the incorporation of [^{14}C]-phenylalanine in the presence (●) and absence of poly U (○). The conditions of incubation were the same as described in Materials and Methods.

RNA concentration.

The total amount of phenylalanine incorporation was increased throughout the range of 0 to 200 μg of rRNA/reaction mixture in both the endogenous and poly U stimulated systems. Maximum incorporation was achieved with 50 μg rRNA/reaction mixture. Ribosomal RNA concentrations greater than 200 μg inhibited phenylalanine incorporation with varying concentrations (0.1 to 1 mg protein) of pH 5 enzyme. The reason for this is unknown. The endogenous system demonstrated maximum phenylalanine incorporation at 10 m*M* Mg^{2+} whereas the poly U stimulated system demonstrated maximum incorporation at 15 m*M* Mg^{2+} (Fig. 2). Magnesium concentrations greater than the optimum resulted in decreased activity in the poly U stimulated systems and was due to the chelation of high energy compounds as the activity could be partially restored upon the addition of ATP.

The low Mg^{2+} concentration required for the endogenous rabbit liver system was in the range found optimal for both endogenous and poly U directed phenylalanine incorporation in the rat liver and rabbit reticulocyte subcellular systems (2, 3). The significance of this higher Mg^{2+} optimum in the poly U directed rabbit liver system is not clear. The requirement for higher Mg^{2+} levels by certain types of mammalian system may be associated with the altered binding of mRNA to ribosomes and the lack of a requirement for an initiator codon in polyphenylalanine synthesis (12). Since the rabbit liver system required a higher Mg^{2+} concentration in the presence of synthetic mRNA and since sedimentation analysis revealed the absence of polyribosomes, it seems likely that the high Mg^{2+} optimum was due to the initiation of new polypeptide chains. Nevertheless, random initiation events and incomplete and out-of-frame translation, cannot be ruled out at this time.

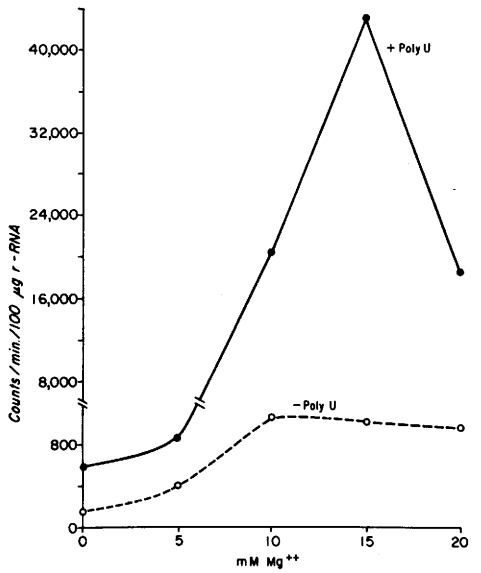

FIG. 2. The effect of varying magnesium ion concentration upon the incorporation into protein in the presence (●) and absence (○) of poly U. The conditions of incubation are the same as described in Materials and Methods except the reaction mixtures contained 50 μg ribosomal RNA and 0.5 mg pH 5 enzyme.

Table I shows that most of the components of the incorporation mixture were necessary for maximum phenylalanine incorporation in both the endogenous and poly U stimulated systems. Deletion of phosphoenolpyruvate kinase had no effect on the endogenous system and only a minimal effect on the poly U stimulated system, indicating that this enzyme is probably present in the pH 5 enzyme fraction. The addition of 2.5 to 5 m*M* phosphoenolpyruvate to the incorporation mixtures was found to be inhibitory to phenylalanine incorporation. The addition of 12.5 to 25 μmoles of the other 19 unlabeled amino acids also inhibited phenylalanine incorporation suggesting that they were present in the pH 5 enzyme fraction. However, dialysis of the pH 5 enzyme against buffer B for 24 hr at 4° did not significantly lower the activity of this fraction indicating that the amino acids, if present were bound to a nondialyzable component. The addition of varying concentrations of sucrose (0.1 to 2%) was also found to be inhibitory. Phenylalanine incorporation in both the endogenous and poly U stimulated incubation mixtures was decreased by the addition of 10 μg RNAse indicating that intact mRNA was required for maximum incorporation. The addition of 25 μg of puromycin reduced phenylalanine incorporation to less than 50% of that of the complete system.

TABLE I. Effect of Deleting Various Components of the Cell-Free System on [^{14}C]-Phenylalanine Incorporation.[a]

System	cpm/100 μg rRNA		Percentage of complete system	
	— Poly U	+ Poly U	Endogenous	Poly U
Complete	1590	34,676		
— 2-ME	1372	16,540	86.0	47.7
— ATP	328	624	20.6	1.8
— Kinase	1688	20,631	106.2	59.4
— ATP and kinase	305	520	18.9	1.5
— GTP	273	832	17.2	2.4
— pH 5 enzyme	514	624	31.9	1.8
— Ribosomes	210	768	13.1	2.2
+ 10 μg RNAse	266	728	16.7	2.1
+ 25 μg puromycin	625	16,852	39.2	48.6

[a] Each 100 cpm are equivalent to 1.14 pmoles of phenylalanine. The complete incubation mixtures had the same composition as described in Materials and Methods, except that they contained 0.5 mg pH 5 enzyme. The assay was carried out as described in Materials and Methods.

Attempts were made to "strip" the ribosomes of endogenous messenger RNA to enhance poly U stimulated activity by (a) preincubation of the complete mixture at 37° for 10–40 min and by (b) preincubation in the presence of puromycin (11). While ribosomes prepared by both procedures demonstrated reduced endogenous incorporation of phenylalanine, they also demonstrated a marked reduction in poly U stimulated activity. The inclusion of added ATP, GTP, PEP kinase or pH 5 enzyme did not increase phenylalanine incorporation in the poly U stimulated systems to levels obtained with nontreated ribosomes. Thus, these two procedures, rather than removing endogenous messenger, appeared to irreversibly alter the protein synthesizing capabilities of the rabbit liver ribosomes.

The time course of phenylalanine incorporation by this cell-free system in the presence and absence of poly U was determined (Fig. 3). Incorporation in the endogenous system was increased slowly for the duration of the reaction whereas in the poly U stimulated reaction mixtures incorporation increased rapidly for 45 min but continued until 90 min. The extended time course indicates that the system does not contain inhibitory factors such as RNAse.

The rabbit liver cell-free system reported here is superior to previously reported mammalian cell-free systems in terms of picomoles of phenylalanine incorporated per milligram of ribosomal RNA. For example, we have observed the incorporation of 4000 pmoles of phenylalanine in 45 min where the best activity reported in the literature that we are aware of was 2604 pmoles of phenylalanine in 60 min (4)/mg of rRNA. The reason for the higher activity is unknown; it

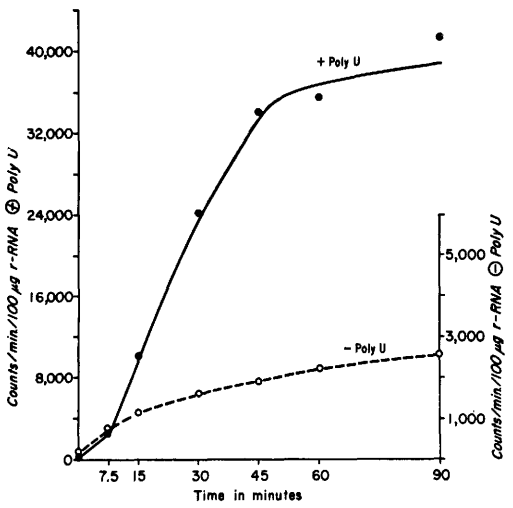

FIG. 3. The time course of [^{14}C]-phenylalanine incorporation into protein by rabbit liver ribosomes in the presence (●) and absence (○) of poly U. The conditions of incubation are the same as those described in Materials and Methods.

may be due to the use of "deprogrammed" ribosomes, the pH 5 enzyme or the nature of the system (*i.e.*, we found that use of relatively low levels of ATP and PEP and no cold amino acids to be optimal).

Summary. A cell-free amino acid incorporating system was prepared from rabbit liver tissue and shown to possess more activity than previously described mammalian cell-free systems. The active pH 5 enzyme fraction prepared from rabbit liver tissue was inhibited by added rabbit liver or rat liver transfer factors. The optimal conditions for endogenous amino acid incorporation revealed an optimal Mg^{2+} ion concentration of 10 m*M* with an extended time course of incorporation. The addition of poly U stimulated the incorporation of phenylalanine into protein by approximately 20–40-fold with an upward shift in optimal Mg^{2+} ion concentration. The incorporation of amino acids depended on the concentration of energy sources, amino acids, and an optimal pH and temperature of the incubation mixtures.

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