# A SIMPLE LIVER CELL-FREE SYSTEM SUITABLE FOR TESTING OF MESSENGER EXOGENOUS RNA

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A simple cell-free system has been prepared from rat liver suitable for testing of exogenous messenger RNA. The proteosynthesis in this system controlled by rabbit globin messenger RNA was markedly enhanced by the addition of pH 5·1 precipitate of the postmicrosomal supernatant. This enhancement can be accounted for by the presence of active ribosomal subunits. The product synthesized was identified as rabbit globin.

The biological activity of messenger RNA's isolated from eukaryotic cells is frequently tested in cell-free systems. Gurdon[1] has been able to develop a cellular system for testing the translation of exogenous messenger RNA. The simplest cell-free systems are preincubated postmitochondrial supernatants, *e.g.* from ascites cells[2,3], wheat germ[4,5], reticulocyte hemolysates[6], and bovine lens[7]. Ribosomal subunits are released after preincubation with amino acids and an energy source; these subunits subsequently are capable of translating exogenous messenger RNA. Cell-free systems consisting of isolated or purified components of the proteosynthetic apparatus from various sources[8–14] have been employed for some years.

Burke, Goldstein, and Redman[15] demonstrated that a rat liver supernatant free of polyribosomes is capable of rabbit globin synthesis directed by added messenger RNA. The supernatant was prepared to contain native ribosomal subunits. In this Laboratory this system has been enriched by active ribosomal subunits and thus its efficiency considerably increased. This enrichment has been achieved by precipitation at pH 5·1 of the rat liver supernatant after removal of polyribosomes by centrifugation.

## EXPERIMENTAL

*Materials.* L-Amino acids were from Nutritional Biochemicals, GTP, creatine phosphate and and creatine phosphokinase from Calbiochem, ATP from Boehringer, and 2-mercaptoethanol from Koch-Light. L-Leucine-[4,5 — $^3$H] was a product of The Radiochemical Centre Amersham, L-leucine-[U — $^{14}$C] was from the Institute for Research, Production, and Use of Radioisotopes, Prague. Globin messenger RNA was isolated from total RNA of rabbit reticulocyte polyribosomes[16] by affinity chromatography on a column of poly(U)-Sepharose[17].

*Preparation of postmicrosomal supernatant* containing ribosomal subunits naturally occurring in the cell. The livers of fasted Wistar rats were homogenized in a glass homogenizer with a Teflon piston in 2·5 volumes of a homogenizing medium (0·25 M sucrose, 25 mM Tris-HCl buffer, pH 7·6, 75 mM-KCl, 5 mM magnesium acetate, and 6 mM 2-mercaptoethanol). The homogenate was centrifuged (20000 *g*, 20 min, 2°C) and polyribosomes were removed from the postmitochondrial supernatant (105000 *g*, 30 min, 2°C). The postmicrosomal supernatant prepared under these conditions[15] contains both native ribosomal subunits and all the remaining factors essential for the translation of globin messenger RNA.

*Enrichment of postmicrosomal supernatant by ribosomal subunits and its analysis.* To two volumes of the postmicrosomal supernatant a few drops of 1M acetic acid were added and the precipitate was centrifuged immediately (10000 *g*, 10 min, 2°C). The sediment was suspended in one volume of the original, not enriched postmicrosomal supernatant. The pH of the suspension was adjusted to 7·6 by 1M-KOH and the suspension was homogenized by ejecting through a needle of 0·2 mm inner diameter. The enriched postmicrosomal supernatant (0·5 ml) was layered on a linear 10—40% sucrose gradient (12 ml) prepared in 25 mM Tris-HCl buffer pH 7·4, containing 5 mM magnesium acetate and 0·5 mM-KCl or alternatively 0·075M-KCl. In the former case the concentration of potassium chloride in the sample of the enriched postmicrosomal supernatant was increased to 0·5 M before the application onto the sucrose gradient. The gradients were centrifuged at 180000 *g* under the conditions described in the legends to the corresponding figures.

*The incubation mixture* contained[15] 4 mM magnesium acetate, 40 mM-KCl, 25 mM Tris-HCl buffer at pH 7·6, 6 mM 2-mercaptoethanol, 1 mM ATP, 0·1 mM-GTP, 20 amino acids, (concentration 20 μM), 12 mM creatine phosphate, 100 μg of creatine phosphokinase, and 0·5 ml of the enriched postmicrosomal supernatant in 1 ml of the cell-free system, L-leucine-$^3$[H] or L-leucine-[$^{14}$C], and rabbit globin messenger RNA. The mixture was preincubated first 5 min at 30°C; the incubation proceeded at 30°C. Samples were removed at time intervals and the radioactivity of trichloroacetic acid-precipitable protein was determined[18].

*Identification of product of protein synthesis.* A 0·6 ml sample was removed after 40 min of incubation for the identification of rabbit globin. Protein synthesis was terminated by the addition of 10 μg of pancreatic ribonuclease and incubation was allowed to proceed for additional 20 min. Carrier rabbit hemoglobin (50 mg) was added and globin was then precipitated by acetone-hydrochloric acid[19] containing 50 mM 2-mercaptoethanol. The α- and β-chains of globin were separated by the modified method of Dintzis[20] on a CM-cellulose column (1·2 × 28 cm, Whatman CM-52). The column was eluted by a linear gradient developed with 250 ml of 0·24M-HCOOH-0·024M pyridine and 250 ml of 2M-HCOOH-0·2M pyridine. In the aliquots (0·5 ml) of the effluent, the volatile buffer was evaporated and the radioactivity of L-leucine-[4,5-$^3$H] incorporated was determined in SLD-31 scintillation mixture in Mark II Nuclear Chicago scintillation counter. The efficiency of the counting of tritium was 31% and of radiocarbon 82%.

## RESULTS AND DISCUSSION

The incorporation of radioactive leucine into protein in a cell-free system, containing the enriched postmicrosomal supernatant, is much higher than in the presence of the postmicrosomal supernatant which has not been enriched (Fig. 1). This fact can be explained by the enrichment of the cell-free system by active ribosomal subunits since the addition of the fraction of pH 5 enzymes only, prepared from the high-speed centrifugation supernatant[21] containing no ribosomal subunits, did not enhance

protein synthesis[15]. In addition to aminoacylating enzymes, transfer RNA's, and certain protein factors, all ribonucleoprotein[22] is precipitated at pH 5·1. Native ribosomal subunits, occurring in the cell as components of the ribosomal cycle in the process of protein synthesis, are precipitated in the rat liver postmicrosomal supernatant at pH 5·1. These subunits represent about 10% of the total ribosome population in all the cytoplasm of cells active in proteosynthesis[23]. The enrichment of the cell-free system by active ribosomal subunits is indicated by the fact that a higher concentration of messenger RNA is necessary for the saturation of the enriched cell-free system (Fig. 2).

The enriched postmicrosomal supernatant was analyzed on a sucrose density gradient at a high concentration of potassium chloride (0·5M). Polyribosomes iso-

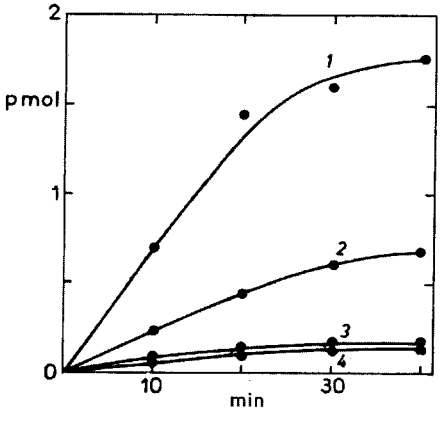


FIG. 1

Incorporation of Leucine-[$^3$H] into Protein Directed by Rabbit Globin Messenger RNA in Cell-Free System

The cell-free system contained 210 μCi of L-leucine-[4,5-$^3$H] (4·6 mol) in 1 ml. Aliquots (20 μl) of the incubation mixture were withdrawn for determination of radioactivity incorporated into trichloroacetic acid-precipitable protein. *1* globin messenger RNA (10 μg/ml), enriched postmicrosomal supernatant; 2 globin messenger RNA (10 μg/ml), postmicrosomal supernatant; *3* enriched postmicrosomal supernatant, in absence of messenger RNA; *4* postmicrosomal supernatant, in absence of messenger RNA. Time of incubation in min, level of incorporation in pmol of leucine incorporated.

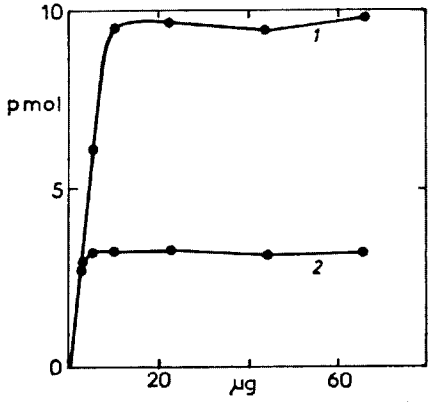


FIG. 2

Saturation of Cell-Free System with Globin Messenger RNA

The cell-free system was incubated with L-leucine-[U-$^{14}$C] (12·5 μCi/67·5 nmol) in 1 ml and with various amounts of rabbit globin RNA. Determination of radioactivity incorporated into trichloroacetic acid-precipitable proteins was carried out after 20 min of incubation. *1* enriched postmicrosomal supernatant; postmicrosomal supernatant. The amount of 9 S RNA is given in μg per ml, the level of incorporation in pmol of leucine incorporated.

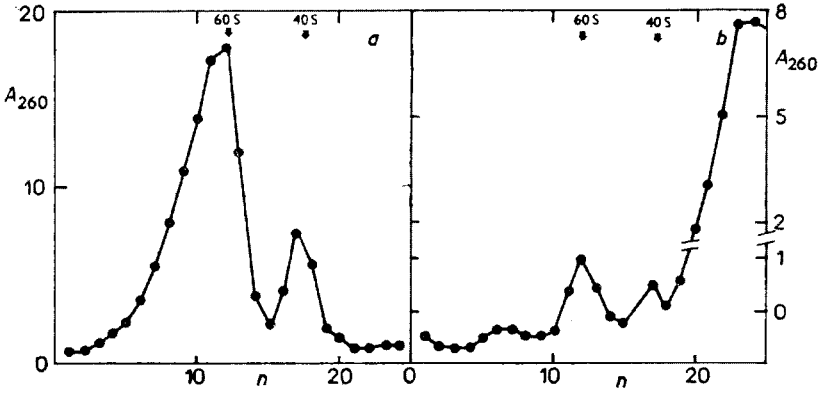


FIG. 3

Sedimentation Profile of Enriched Postmicrosomal Supernatant in Sucrose Density Gradient Containing 0·5M-KCl

The samples were analyzed by centrifugation in linear 10—40% sucrose density gradient (180000 *g*, 3 h, 2°C). (*a*) Polyribosomes degraded to subunits under identical conditions were centrifuged as a control; (*b*) enriched postmicrosomal supernatant. *n* fraction number; the concentration of subunits is given as absorbance at 260 nm.

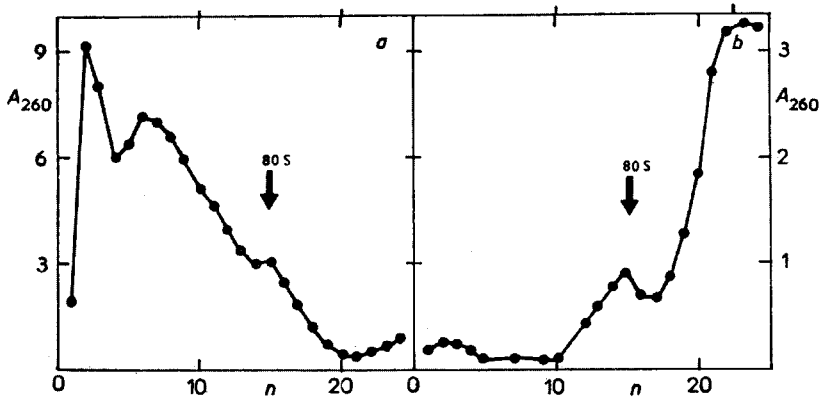


FIG. 4

Sedimentation Profile of Enriched Postmicrosomal Supernatant in Sucrose Density Gradient Containing 0·075M-KCl

The samples were centrifuged on linear 10—40% sucrose density gradient (180000 *g*, 4 h, 10°C). (*a*) Rat liver polyribosomes centrifuged as a control; (*b*) enriched postmicrosomal supernatant. *n* fraction number; the concentration of subunits is given as absorbance at 260 nm.

lated from rat liver and degraded to subunits were centrifuged at the same concentration of potassium chloride as a control (Fig. 3*a*). The postmicrosomal supernatant contained 40 and 60 S ribosomal subunits under these conditions (Fig. 3*b*). These subunits spontaneously associated to 80 S ribosomes during centrifugation in the succrose gradient in 0·075M-KCl (Fig. 4*b*). Polyribosomes from rat liver served as a control (Fig. 4*a*).

The synthesis of the α- and β-chains of rabbit globin takes place in the cell-free system containing the enriched postmicrosomal supernatant from rat liver (Fig. 5). In addition to rabbit globin messenger RNA (9 S RNA) we tested in this system also other RNA's prepared from rabbit reticulocytes, *i.e.* 4, 18, and 28 S RNA's. These RNA's had a slight effect on protein synthesis, compared to the 9 S RNA (Table I). Hemin added to the cell-free system at a $2 \cdot 10^{-5} - 1 \cdot 10^{-4}$M concentration showed no effect on the quantity of rabbit globin synthetized. The synthesis of rabbit globin controlled by the exogenous messenger RNA proceeded linearly for as long as 20 min of incubation at 30°C. The quantity of leucine incorporated into protein during 20 min in various experiments was 180–300 pmol per 1 mg ribosomal RNA (ref.[24]).

The advantage of the cell-free system used rests in the simple preparation and a relatively high efficiency. The cell-free system with the enriched postmicrosomal supernatant is suitable for testing the biological activity of eukaryotic messenger

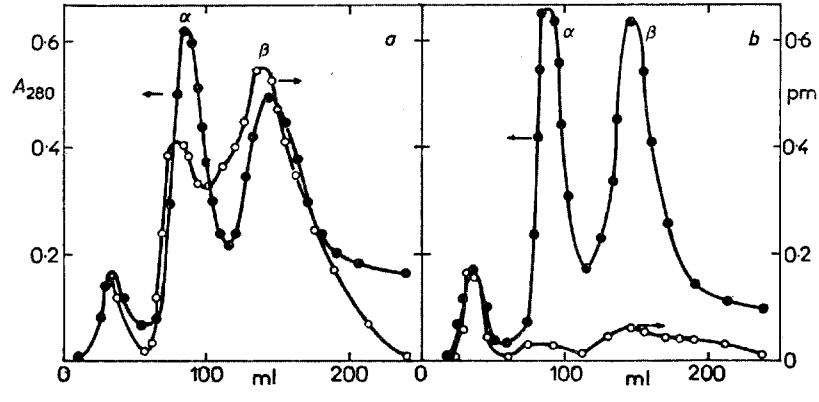


FIG. 5

Chromatography on CM-cellulose of Rat Globin Synthetized in Cell-Free System

The composition of the cell-free system containing the enriched postmicrosomal supernatant and L-leucine-[4,5-$^3$H] is the same as that described in the legend to Fig. 1. A 0·6-ml sample of the cell-free system was removed after 40 min and treated as described under Experimental. (*a*) Rat globin messenger RNA (10 μg/ml of cell-free system); (*b*) in the absence of messenger RNA. The effluent volume is given in ml, protein concentration is presented as absorbance at 280 nm, and the level of protein synthesis is given in pmol of leucine incorporated.

TABLE I

Effect of Various RNA's Isolated from Rabbit Reticulocytes on Stimulation of Protein Synthesis in Cell-free System

The cell-free system (0·1 ml) with the enriched postmicrosomal supernatant was incubated with L-leucine-[$U^{14}C$] (1·25 μCi/6·75 nmol) and the RNA indicated. The radioactivity incorporated into the protein was determined after 30 min of incubation and is given in pmol of leucine per ml of cell-free system.

| RNA | Concentration of RNA μg/ml | Incorporation pmol/ml | RNA | Concentration of RNA μg/ml | Incorporation pmol/ml |
|---|---|---|---|---|---|
| — | — | 14·2 | 18 S | 76 | 16·2 |
| 4 S | 70 | 18·6 | 28 S | 71 | 17·9 |
| 9 S | 60 | 96·8 | | | |

RNA preparations. In studies on fundamental mechanisms of protein synthesis, however, systems containing isolated or even purified components of the protein synthesis apparatus should be used.

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