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Protein Synthesis in Cell-Free Reticulocyte Lysates on Multi-Hour Incubation

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ABSTRACT

Cell-free protein synthesis in rabbit reticulocyte lysate translation mixtures has been studied during multi-hour incubations. In an impaired lysate obtained from cells stored at 0°C before lysis, and showing a low initial rate of synthesis, translation could be stimulated by a factor of 4 by including RNase inhibitor and additional ATP and GTP. In translation mixtures prepared from normal lysates, protein synthesis could be improved by ~50% by the addition of excess GTP. The observed increases in protein synthesis were obtained by improved maintenance of the initial rate of synthesis.

Index Entries: Cell-free protein synthesis; cell-free translation; reticulocyte lysate, and behavior on multi-hour incubation.

INTRODUCTION

The in vitro, cell-free synthesis of proteins is a much more difficult enterprise than in vivo synthesis using recombinant DNA technology, but it is a technique of real potential utility for the synthesis of proteins containing labeled or unnatural aminoacids, and for synthesis of proteins containing certain types of unnatural amino acid sequences. Chemical methods work well for the synthesis of relatively small peptides (<40 amino acid residues in length) but lose efficiency as synthesis of larger products is pursued (*1,2*). For larger proteins (MW>20,000), chemical synthesis may never be able to rival the combination of rDNA methods coupled with fermentation. Because of our interest in the application of

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enzymes in organic synthesis (3–5) we were interested in the suitability of cell-free enzyme-containing extracts for preparative protein synthesis.

Reticulocytes, immature red blood cells, are more metabolically active than erythrocytes, mature red cells (*6,7*). Reticulocytes actively synthesize protein, of which approximately 90% is globin that combines with endogenous heme to form hemoglobin (*8–10*). Cell-free reticulocyte lysates continue to synthesize protein at ~60% of the rate of intact cells for up to 1 h after cell breakage (*11*) and have not been equaled in their high level of translational activity (*12*). Because of the high relative activity of these lysates in comparison to other cell-free systems and the abundance of research done on and with these lysates we chose the rabbit reticulocyte lysate as the system with which to begin our studies.

Although no studies of reticulocyte lysates have described the behavior of these lysates on multi-hour incubations, their properties during short-term (~1 h) incubation have been extensively characterized as a consequence of studies of the biosynthesis of proteins. The lysate is a complex, multi-component system that requires a delicate balance in the concentrations of a variety of compounds to maintain translation (*9,13,14*). A major requirement of the reticulocyte lysate is for heme in order to maintain translational activity for more than ~10 min (*11,15–19*). Heme inhibits the activation of the heme-controlled translational repressor (HCR) that inactivates initiation factor eIF-2, which is necessary for translation (*10,20–26*).

Cell-free translation systems continue to be developed (*27–30*) and used in the study of protein biosynthesis and have become a standard tool in molecular biology for studying messenger RNAs (mRNAs) and other nucleic acids (*12,14,27–45*). These systems have been used to produce isolable products, but they have been operated only on a small scale, producing radiolabeled materials (*46*). A patent describes the production of β-lipotropin precursor protein in reticulocyte lysates, but the described procedure is based solely on standard methods and is used to produce radiolabeled product that is isolated by immunoprecipitation (*47*). In all cases the methods described are similar and use incubation periods of no more than 1.5 h. Whereas the reticulocyte lysate has been described as active for "hours" (*10*), we have seen no published documentation of maintenance of activity in these lysates for greater than 2 h, and no discussion of the properties of these lysates beyond these limited time periods. Typical translation procedures use incubation periods of ~1 h, since lysate mixtures typically rapidly lose activity after this period. The objectives of the work described in this paper were to investigate the properties of the reticulocyte lysate during multi-hour incubations to determine if it was possible to maintain high translational activity in this system.

We report here the results of studying two types of lysates. First, we obtained results using an "impaired" lysate of low initial activity (corresponding to an initial rate of synthesis of .3 μg/mL-h of new protein based on the incorporation of [^{3}H]-leucine) (*48*) that displayed the typical loss of

activity after incubation at 25°C for 1 h. Supplementation of this lysate with placental RNase inhibitor (RNasin) (*49*) and other compounds prolonged activity for more than 8 h. Second, we investigated the response of a "normal" lysate (initial activity corresponding to an initial rate of synthesis of 8 μg/mL-h of new protein synthesis) to the activity-maintaining conditions developed for the impaired lysate. The response of the normal lysate was not as great as that of the impaired lysate, but further experimentation showed maintenance of activity in the normal lysate for up to 4 h on inclusion of excess GTP.

MATERIALS AND METHODS

Materials

Rabbit reticulocyte lysate was prepared essentially by the procedure of Allen and Schweet (*13*). The lysate was either frozen immediately in liquid nitrogen and stored at −80°C, or fractionated by literature methods to obtain sRNA (*50*). Female white rabbits were from Millbrook Farms, Amherst, MA. Phenylhydrazine was from Aldrich and was recrystallized as the hydrochloride from hot ethanol. Phenylhydrazine solution (2.5% w/v) was made up immediately before use, neutralized with 1 N NaOH to pH 7, and filtered through a sterile .22 μm filter (Millex GS, Millipore). Carbon monoxide was from Matheson. [^{3}H]-Leucine, Econofluor, and Protosol were from New England Nuclear. RNasin was from Promega Biotec. Additional biochemicals were from Sigma.

Translation Mixtures

Translation mixtures were formulated based on reported procedures (*14,16*) and as noted in the figure legends. All solutions were prepared with distilled deionized water. Plastic microtubes and pipet tips were handled exclusively with gloved hands. Frozen lysate was thawed by brief hand warming and vortexing and then placed on ice. Translation mixtures were made up at 0°C by combining a master mix (*16*) with thawed lysate. Radiolabeled leucine (neutralized, .9 μCi/μL, 5% v/v) was added last followed by vortexing and placement in a thermostatted (±.5°C) water bath. Incubations were at 25°C unless otherwise noted.

Varied Cell Washing Procedures in the Preparation of Lysates

Fresh reticulocyte-rich blood was collected into buffered saline (134 mM NaCl, 5 mM KCl, 7.5 mM $MgCl_2$, 10 mM HEPES, pH 7.2 with KOH) containing 5 mM glucose. The cell suspension was filtered through cheesecloth and 7×50 mL portions were centrifuged for 10 min at 2500 rpm (650 rpm, SS-34 rotor). The plasma was decanted from the packed cells. The cells in each tube were washed with a different solution as described in Table 4: The packed cells were suspended in a total volume of 40 mL, cen-

trifuged for 10 min at 2500 rpm, and the supernatant was decanted from the packed cells; this procedure was repeated twice more.

After washing, each tube contained 5 mL of packed cells. Deoxygenated ice-cold water (7.5 mL) was added to each tube followed by vortexing to lyse the cells. The lysis mixtures were centrifuged for 20 min at 15,000 g (11,000 rpm, SS-34 rotor). The lysates were divided into 1-mL and .2-mL aliquots in plastic microtubes and immediately frozen in liquid nitrogen and stored at −80°C. Each sample yielded 9 mL of lysate. Both lysates required little adjustment of the Mg^{2+} level; .25 mM GTP was added for optimal activity. Lysates 1–3 were optimally supplemented with 20 μM hemin. Lysates 4–7 did not need added hemin but 10 μM was included to ensure an adequate level. Lysates 1–7 were tested for activity over 3 h incubations. Master mix was made up and used directly for lysates 1–3. The remainder was held under nitrogen for 10 min before combining with lysates 4–7.

Sucrose Gradient Analysis (*51*)

Sucrose solutions were prepared in 10 m*M* TRIS-HCl, pH 7.4 containing 2 m*M* magnesium acetate, 50 m*M* KCl, and .1 g/L gelatin (*61*). Lysate samples (60 μL) were diluted with 2 volumes of gradient buffer and applied to linear gradients (12 mL, 15–30% sucrose w/v). Gradients were centrifuged in the 70.1 Ti rotor at 45,000 rpm for 30 min and allowed to stop with the brake off. The gradients were passed through the UV cell of an LKB Uvicord II detector equipped with a chart recorder and operating at 254 nm.

Sample Preparation for Liquid Scintillation Counting (*14*)

Aliquots (5–10 μL) were removed from an incubation mixture with a Hamilton syringe (#801N 10 μL) and blown into .5 mL of cold water in a 12×75 mm disposable culture tube. The syringe barrel was rinsed 4 or 5 times to ensure complete transfer of the sample and to clean the syringe for the next aliquot. Samples were then stored at 4°C until a set from an experiment were ready for further processing together. NaOH (1N, .5 mL) and 30% hydrogen peroxide (50 μL) were added to each tube followed by heating at 37°C for 15 min or standing at 25°C for 20–30 min. Ice-cold trichloroacetic acid (25% w/v) was added to each sample, and the samples kept at 4°C for 1h. The contents of each tube were transferred onto a 2.1 cm GF/C glass fiber filter. The sample tube was rinsed twice with 1 mL of 8% TCA and these washes were passed through the filtered precipitate. The filter was placed in a glass LSC vial so that it was lying flat on the bottom of the vial and 1 mL of ethanol/Protosol (1:1) was added. The sealed vial was heated at 55–60°C for 30 min and ethanol (.5 mL) containing 10% glacial acetic acid was added followed by 10 mL of Econofluor. Radioactivity was measured in the scintillation counter and reported as the observed counts per minute.

RESULTS

Impaired Lysate

The kinetics of incorporation of [^{3}H]-leucine into acid precipitatable protein (*13,19,52,53*) in the impaired lysate is shown in Fig. 1. The lysate showed a near linear rate of synthesis for 1 h, and then quickly lost activity. Supplementing this lysate with either RNasin or sRNA resulted in improved maintenance of the initial rate of incorporation and greater overall protein synthesis (Fig. 1). In combination there was no additional benefit, and excess sRNA proved to be inhibitory. Incorporation in the supplemented lysate was maintained at nearly the initial rate for 2 h and was

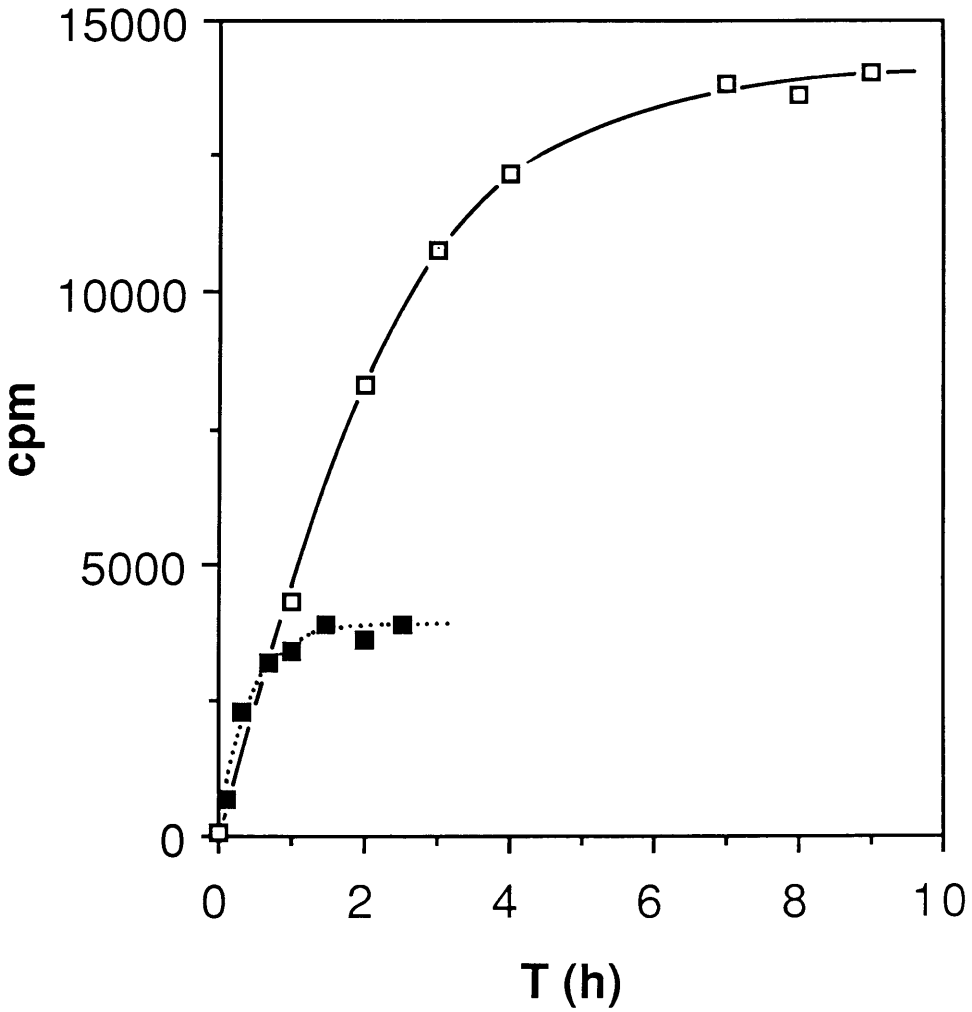

Fig. 1. Incorporation of [^{3}H]-leucine into acid precipitated material in impaired lysate translation mixtures: unsupplemented, (■); with 1 U/μL added RNasin, (□).

doubled again in the next 5 h (Fig. 1). Subsequent experiments showed that on storage the sRNA preparations tended to become inhibitory. In general, because of the equivalent activating ability of RNasin, it was used alone without sRNA.

Addition of dithiothreitol (DTT) (*54,55*) produced modest gains in incorporation but high levels were inhibitory (Table 1). Addition of extra creatine phosphate and creatine kinase afforded no benefit, nor did the addition of 1,10-phenanthroline, a known inhibitor of proteases in reticulocyte lysates (*56*). Extra ATP (1 m*M*), GTP (.2 m*M*), and Mg^{2+} (1 m*M*) (*57,58*) resulted in a modest increase in incorporation in an 8 h incubation (Fig. 2). Glucose-6-phosphate (.4 m*M*) was also modestly activating (*12,55,59,60*).

Addition of the protease inactivator phenylmethylsulfonylfluoride (PMSF) resulted in no effect on incorporation. A control experiment to evaluate the effect of ethanol, which was used as a cosolvent for PMSF, surprisingly showed activation of the lysate. Others have reported that >.3% v/v of ethanol inhibits protein synthesis in reticulocyte lysates (*61,62*). We found ethanol at up to .2% to be activating (Figs. 2, 3).

The results obtained in maintaining the activity of the impaired lysate are significant in comparison to the behavior of the unsupplemented translation mixtures. Even in the optimized translation mixture, however, the overall level of incorporation corresponds only to a level of protein synthesis of ~2 μg/mL, which is about 2% of the activity of the most active lysates reported (*12*).

Normal Lysate

This lysate displayed high initial activity for 2 h, after which activity was rapidly lost (Fig. 4). Adding extra ATP, GTP, and Mg^{2+} was modestly activating (Fig. 4). Increasing the temperature of incubation from 25°C to 30°C approximately doubled the initial rate, but shutdown of incorporation occurred at the same level of incorporation (Fig. 5).

An interesting observation came from passing carbon monoxide over a translation mixture prior to incubation. Only one-fourth of the incorporation of the control was obtained in 4 h by which time the control was

Table 1
Effects of Increasing Amounts of DTT on Incorporation in Impaired Lysate Incubation Mixtures[a]

Experiment		Cpm, 2 h	Cpm, 3 h
Control		6996	9524
DTT, 25 m*M*	1 μL	8345	8779
	2 μL	6522	10773
	5 μL	6174	11350

[a] Reaction volume, 50 μL; reaction time 3 h; 10 μL aliquots.

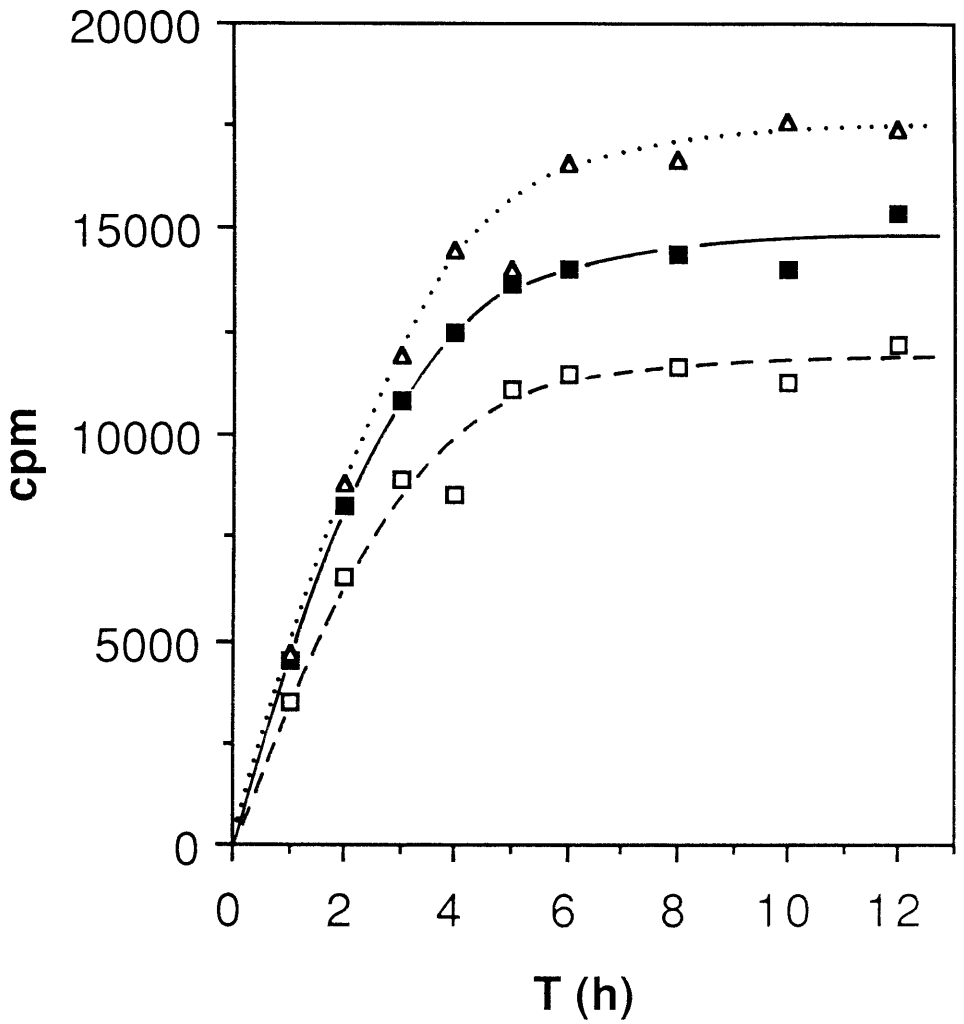

Fig. 2. Translation in the impaired lysate system: (a) control (□); (b) with .2 v/v ethanol (■); (c) control supplemented with 1 m*M* ATP, .2 m*M* GTP, and 1 m*M* $MgCl_2$ (△).

shut down. In the following 2 h after being opened to air, the level of incorporation increased to near that of the control (Fig. 6).

Concentration of normal lysate by ultrafiltration increases the "specific activity" of translation mixtures, but not by as much as the lysate is concentrated (*63*). Diluted lysate (10% v/v in a translation mixture) yielded no activity, presumably because of dilution of components of the lysate to less than optimal concentrations.

As translation proceeds in lysates, creatine phosphate is consumed and inorganic phosphate is released. To evaluate the extent to which free phosphate might inhibit translation mixtures, 0–20 m*M* sodium phosphate was added to the lysate. Increasing phosphate concentration produced a

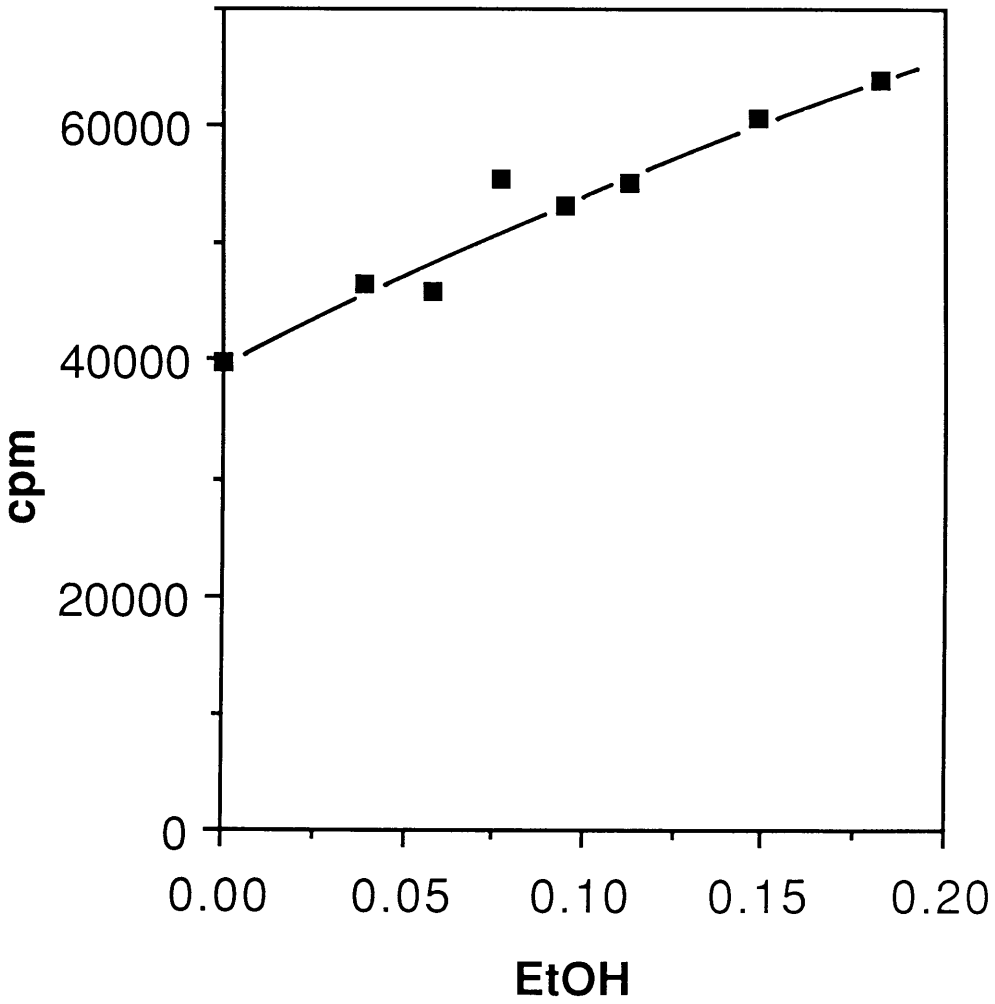

Fig. 3. Incorporation in the impaired lysate system after 1 h with varying amounts of added ethanol.

near linear decrease in the incorporation observed in a 2-h period (Fig. 7), likely in part a result of complexation of Mg^{2+}. In lysates containing 20 m*M* added phosphate, 2 m*M* added Mg^{2+} resulted in recovery of half of the initial activity.

Pyruvate kinase and phosphoenolpyruvate are a less effective phosphorylation system in whole reticulocyte lysate than creatine phosphate/creatine kinase (*11*). Whereas creatine kinase does not accept GDP as a substrate (*64*), GTP is reported not to be depleted in 1 h incubations (*20*). To test if GTP might be a factor in the loss of translational activity, several different combinations of phosphorylating potential were tried (Table 2). Consistent with previous results, mixtures containing PEP were inhibited (*11*). No activation was achieved by including PEP/PyK in incubations along with CrP/CrK. PyK alone had no effect on incorporation. Added

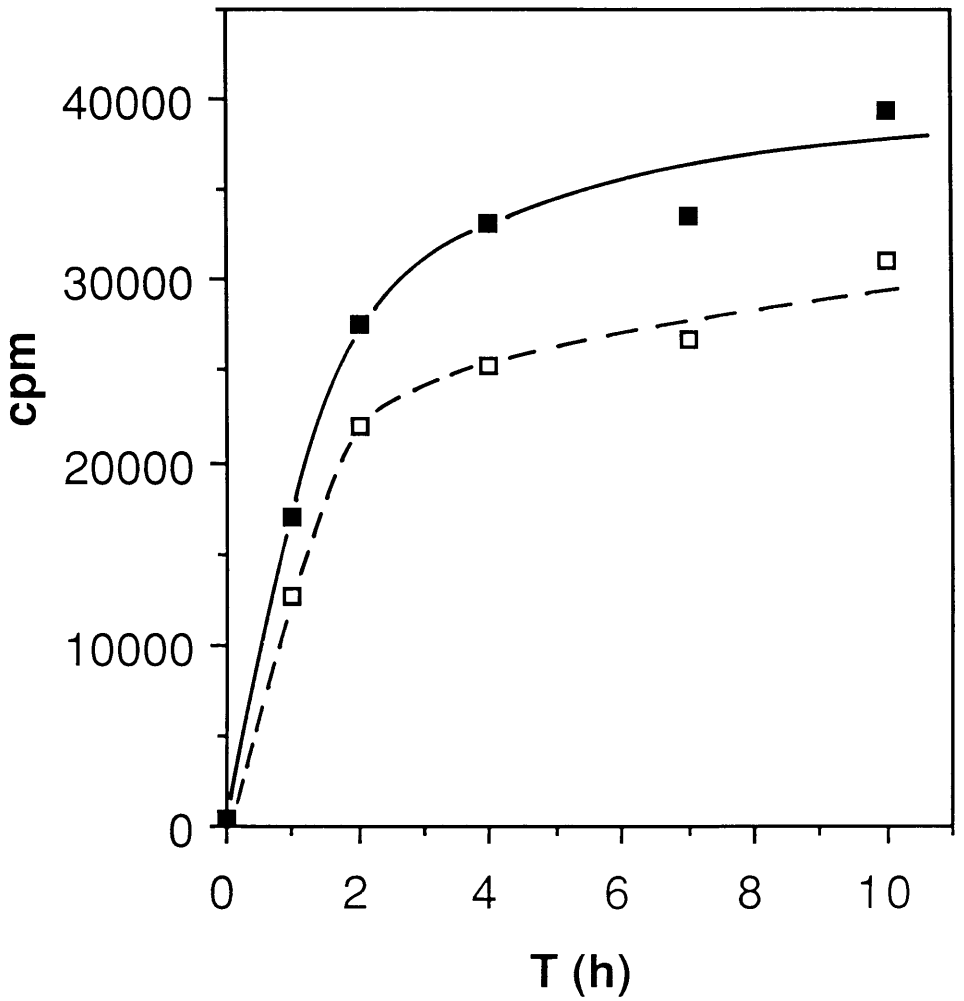

Fig. 4. Translational activity in the normal lysate system over 10 h: (a) standard translation mixture (□); (b) supplemented with 1 m*M* ATP, .2 m*M* GTP, and 1 m*M* $MgCl_2$ (■).

Table 2
Effect in Normal Lysates of Different Phosphorylating Systems[a]

				Cpm	
Experiment \ Addition	CrK + CrP	PyK	PEP	@1 h	@2 h
1, control	+	–	–	37518	46825
2	–	+	+	12339	13211
3	+	+	+	20139	29555
4	+	–	+	11713	14507
5	+	+	–	35418	42562

[a] CrK and CrP were the usual amounts if present, PyK (.5 mg/mL) and PEP (.1 *M*) were added at 4% (v/v).

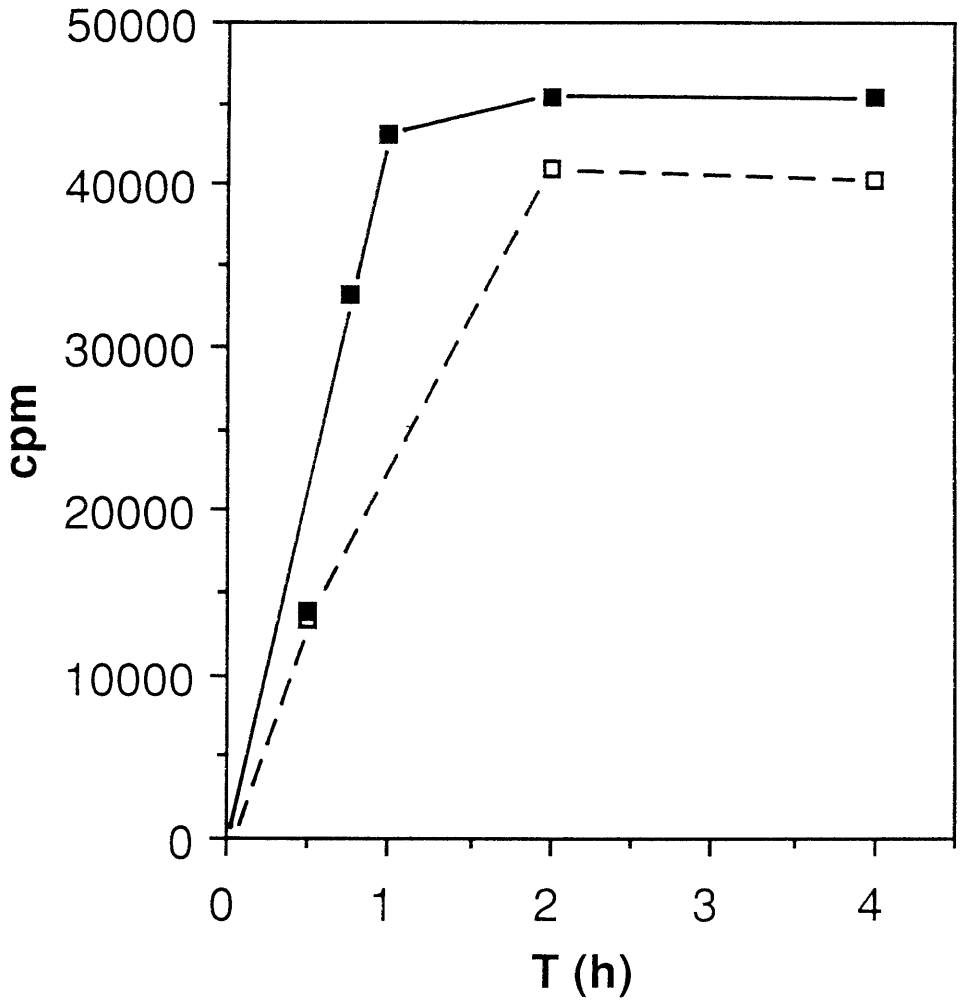

Fig. 5. Incubation of normal lysate translation mixtures at (a) 25°C (□); (b) 30°C (■).

Mg^{2+} brought the activity in the combined system up to the control level and indicated that PEP reduced Mg^{2+} activity (Table 3).

GTP is absolutely required for translation (*6,8*). Its concentration in lysates is about .1 m*M* (*14*). GTP is also capable of substituting for hemin in lysates as a blocker of the activation of HCR in preincubations in the absence of an energy system (*20*). We found that adding GTP-Mg^{2+} resulted in increased incorporation in 2 h incubations (Fig. 8). While shutdown of translation was slowed and incorporation significantly increased, the system was still nearly stopped after 2 h.

Maintenance of high activity in the reticulocyte requires the availability of either chemical or biochemical reducing agents. DTT and thioredoxin have been shown to be useful sources of reduction potential in lysates (*59,65*), one or the other required in conjunction with compounds that allow the lysate to generate NADPH such as glucose-6-phosphate. Lysates

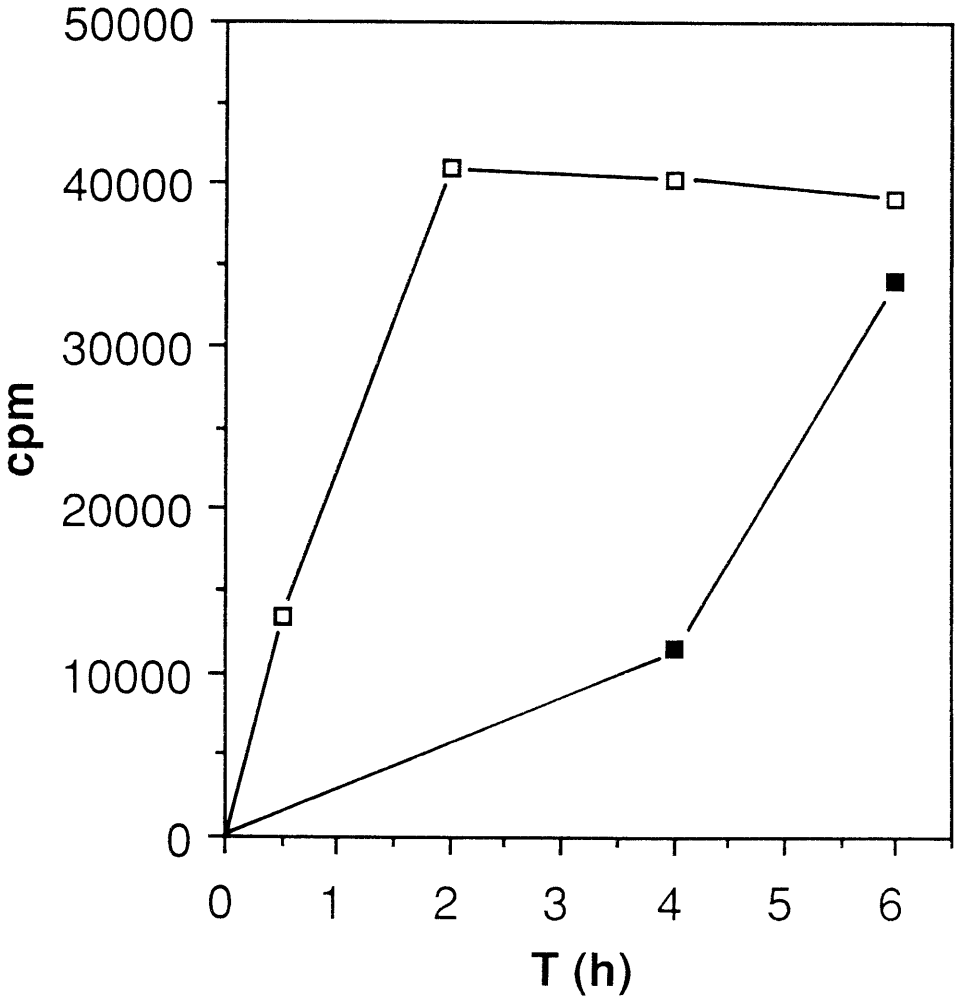

Fig. 6. Incorporation in normal lysates at 25°C showing the effect of incubation under carbon monoxide for 4 h (■) before exposure to air; control, no CO (□).

Table 3
Effect of Added $MgCl_2$ on Incorporation in Normal Lysate Mixtures Containing Pyruvate Kinase (20 μg/mL) and PEP (4 m*M*)[a]

Experiment	Cpm
control, no PyK or PEP	14158
w/PyK, PEP	
no added $MgCl_2$	7452
1 m*M* $MgCl_2$	14748
2 m*M* $MgCl_2$	10952
4 m*M* $MgCl_2$	8386
5 m*M* $MgCl_2$	5062

[a] Incubations were for 2 h.

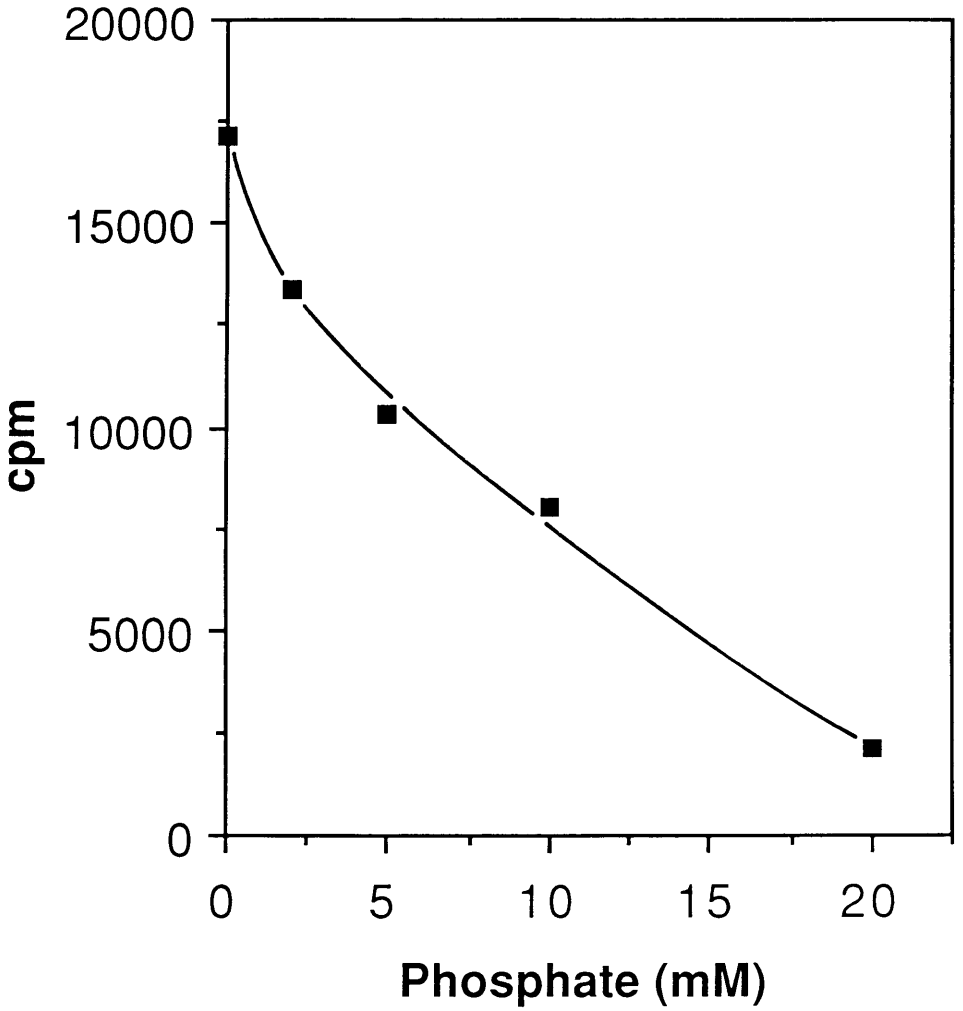

Fig. 7. Effect of added phosphate (.2 *M* sodium phosphate, pH 7.2) on incorporation in 2 h incubations of normal lysate translation mixtures.

are very sensitive to oxidized glutathione (GSSG), and addition of GSH can lead to inhibition because of small amounts of contaminating GSSG (*66,67*). There has been a caution about adding thiols to translation mixtures (*66*). Under certain conditions, however, DTT has been found to be essential for maintaining activity in lysates (*67*). GSH does not function as well as DTT as a reducing agent. In normal lysates we found DTT to be inhibitory, in contrast to the data from the impaired lysate where DTT was an important element of the improved translation mixture to insure stability of added RNasin.

An alternative to addition of thiols to maintain reduction potential in the lysate is to maintain the NADPH concentration. Added NADPH is not very efficient in this regard compared to the use of compounds to

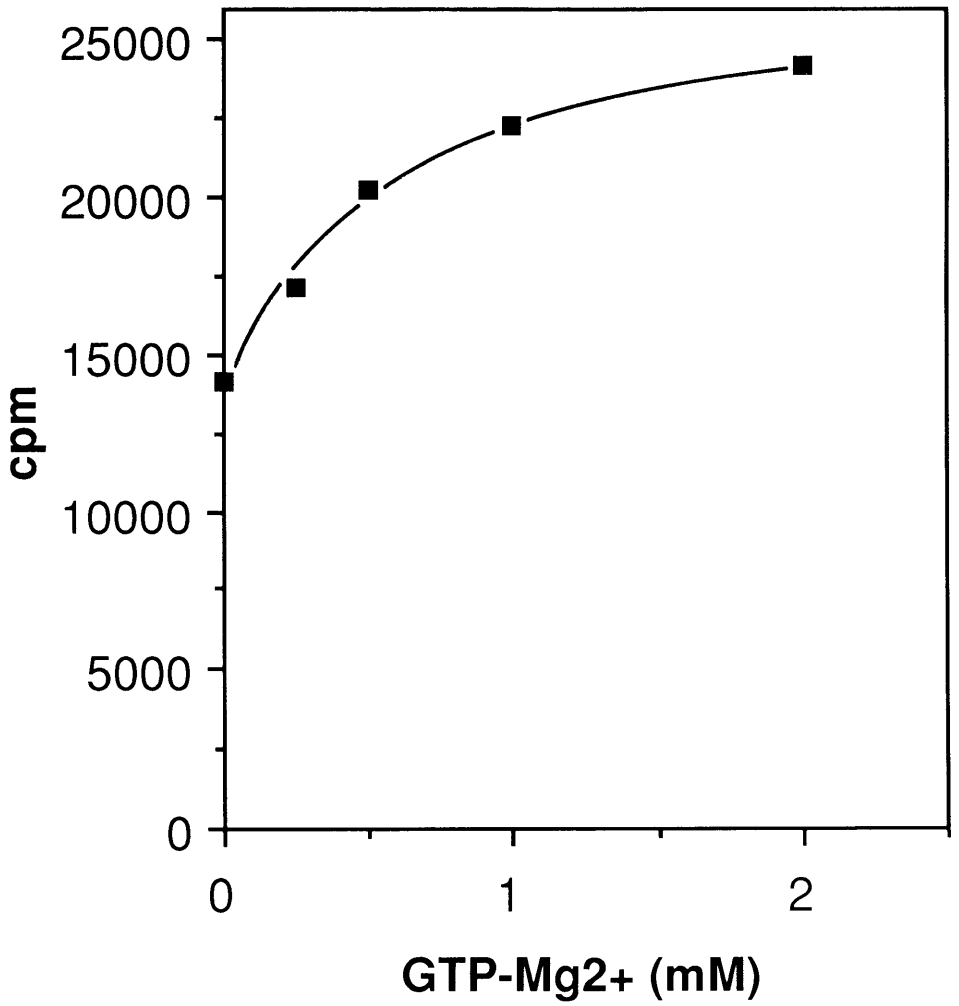

Fig. 8. Effect of added GTP-Mg^{2+} on incorporation in 2-h incubations of normal lysate mixtures.

allow regeneration of NADPH from NADP; excess NADP can inhibitively disturb the NADPH/NADP ratio (*55*). In partially fractionated lysates, .4 m*M* G6P is effective for maintaining NADPH (*55,59*). We found that added G6P over 1 m*M* was inhibitory, more so than the same amount of inorganic phosphate. Adding $MgCl_2$ did not offset this inhibition. Addition of glucose, which can function as G6P after phosphorylation by hexokinase, was inhibitory, as was NADP. This observation contrasts with the reported activating effects of both glucose and NADP in shorter incubations (*66*). Glutathione reductase was included in lysates with and without G6P and NADPH, and no advantage was observed, consistent with previous observations that GSH is not oxidized to an appreciable extent during incubations (*66,67*). The lysate pH optimum for translation has

been established as narrow at pH 7.2 (*11,59*). We found that lysate pH during incubation is constant, varying less than .1 pH units after 2 h and loss of activity.

Enzymic oxidation of G6P to recycle NADPH could ensure availability of NADPH if otherwise limited by enzymes lost in the preparation of the lysate (*68*). Inclusion of sufficient G6P-dehydrogenase to convert the available G6P to form NADPH had no effect on incorporation. Previous reports on different types of impaired lysates found that adenosine, cAMP, and related compounds could function as activators of translation (*65,67,69*). We found in the normal lysate that cAMP was very inhibitory.

Aged lysates deficient in essential factors for translation are reported to regain full activity on addition of partial volumes of lysate or ribosome-free lysate supernatant (*16,66,67,70*). Our normal lysate, after having lost activity on extended incubation, showed no further activity on the addition of fresh lysate to inactivated translation mixtures.

To probe the effect of oxygen on cells before and during lysis, a series of lysates were prepared from the same batch of cells using differently prepared cell-washing solutions (Table 4). All of these specially prepared lysates were similar in translational activity. Slightly higher activities were actually obtained from those cells washed with solutions that were not deoxygenated nor supplemented with hemin. Less active lysates have been obtained previously from cells incubated extensively under anaerobic conditions at elevated temperatures (*65,66*). The period required to wash cells several times (1–2 h), even though at 4°C, may be sufficient to lead to impaired lysates. As in previous experiments without extra GTP, incorporation proceeded well for 1 h and then was essentially stopped.

Sucrose gradient analysis of polysome, ribosome, and subunit aggregation has been extensively used in other studies of reticulocyte lysates (*11,19,22,23,55,65–67,69,71*). In studies on lysates that have been impaired

Table 4
Formulation of Cell-Washing Solutions and Extent of Incorporation in Translation Mixtures Made with Lysates from These Cell Preparations[a]

No.	Washing Solution	Cpm
1	Saline (134 m*M* NaCl, 5 m*M* KCL, 7.5 m*M* $MgCl_2$)	41680
2	Saline, buffered (10 m*M* HEPES, pH 7.2 w/KOH, 5 m*M* glucose)	41700
3	Saline, buffered, deoxygenated	38240
4	Saline, buffered, w/ 20 μM hemin	32370
5	Saline, buffered, w/ hemin, .5 m*M* adenine	34945
6	Saline, buffered, w/ hemin, 1 m*M* DTT	34340
7	Saline, buffered, w/ hemin, containing 2-mercaptopropanol	30990

[a] Incubations were for 3 h.

in a manner to cause loss of activity within several minutes, the general observation has been that active lysates show high molecular weight aggregates corresponding to polysomes that disaggregate as activity is lost resulting in the accumulation of lower molecular weight ribosomes and ribosome subunits. This general pattern is observed in instances where shutdown is believed to be caused by loss of initiation factors. In our normal lysates, after 2 h at 30°C the high molecular weight peaks are diminished in size and lower molecular weight peaks are larger indicating a state of disaggregation similar to that in lysates where initiation is inhibited (Fig. 9).

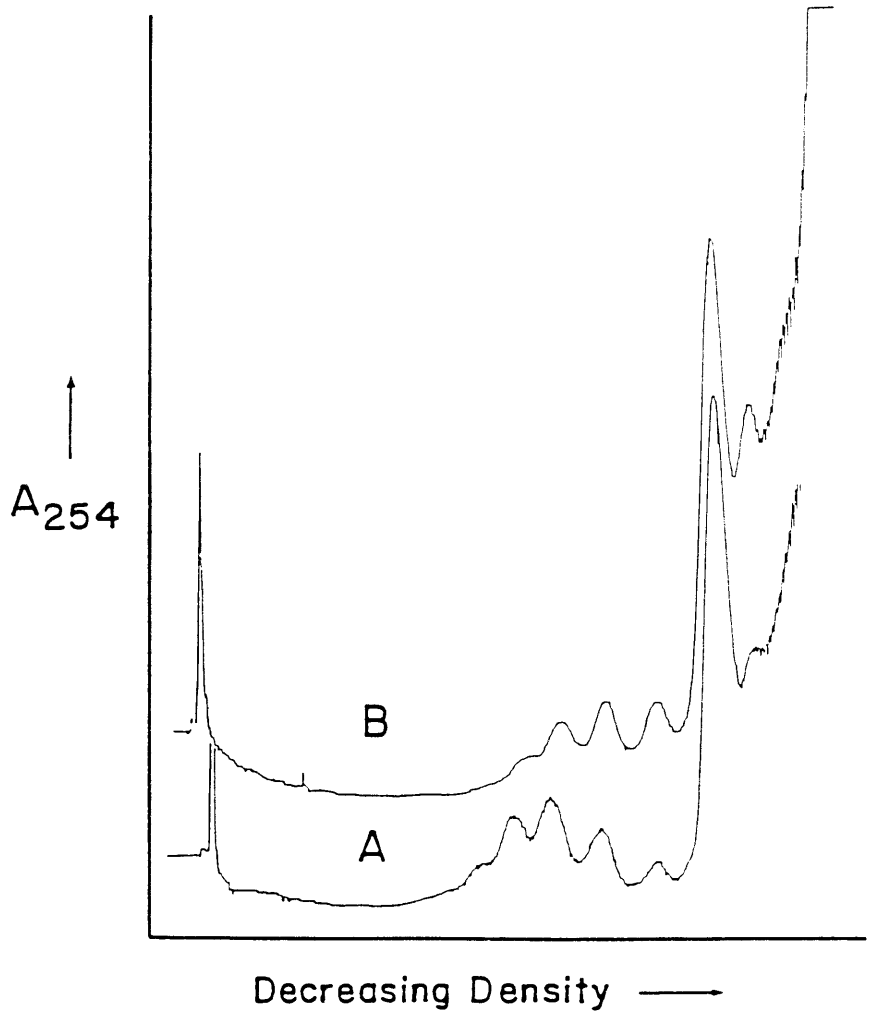

Fig. 9. Polysome profile in a 15–30% sucrose gradient: (A) translation mixture applied to the gradient after 5 min at 30°C; (B) translation mixture applied to the gradient after 2 h at 30°C. Gradients were centrifuged for 30 min at 45,000 rpm in the 70.1 Ti rotor.

When an incubation mixture was held under a stream of nitrogen gas for 10 min at 0°C before starting incubation at 30°C it showed 20% greater incorporation over that in lysate not treated with nitrogen. If the lysate was subjected to a degassing procedure with 2 freeze-thaw cycles under nitrogen and vacuum, the resulting lysate had nearly no activity.

DISCUSSION

Impaired Lysate

In optimizing the activity of cell-free translation in the reticulocyte lysate, gains in activity are only seen as a result of maintaining the initial rate of activity for a longer period of time. A higher temperature of incubation does result in an increased initial rate of synthesis but the resulting overall level of synthesis is the same as at lower temperature (*72*). The impaired lysate results are of limited use as they apply to a system with low overall activity. It is interesting, however, that translational activity in the impaired lysate can be maintained for several hours in this system. Still, with sustained activity the level of newly synthesized protein achieved in 10 h is far less than that achieved by normal lysates even with their much shorter period of sustained activity. A previous report also describes low activity lysates that maintained activity longer than usual, with lower overall synthesis (*13*).

A major limiting factor in the impaired lysate is certainly the loss of tRNA soon after the start of an incubation that can be offset by the addition of extra tRNA or RNasin. Addition of other compounds to the translation mixtures provided little further activation of protein synthesis.

The metabolic activity of red cells is significant, even at 0°C (*73*). Even though stored overnight at 0°C, the cells from which the impaired lysate was made may have become depleted in ATP. Such ATP depletion has been shown to cause inhibition of translation and a similar effect seems to have occurred in our impaired lysate.

Normal Lysate

Normal lysates do not require added RNasin or sRNA and do not exhibit enhanced translational activity under the conditions with which the impaired lysate does. The inhibitory effects of DTT in the normal lysate contrasts with activation by DTT in the impaired lysate. This observation indicates that whereas the impaired lysate has reduced biochemical reducing power, the normal lysate is still effective in maintaining reducing power. In normal lysates additional DTT affords no advantage, and in high quantities is inhibitory. In contrast to the impaired lysate, normal lysates are not activated by low levels of ethanol.

The effects of increasing temperature suggests that in a given lysate a certain amount of "metabolic activity" will lead to inactivation (*11,65*).

This suggestion is consistent with the result from incubation of the normal lysate under carbon monoxide. This incubation displayed inhibited translation, but exposure to air restored the lysate to the same level of activity as was observed for a normal incubation. Carbon monoxide apparently binds reversibly to components of the translation mixture to "freeze" the activity of the lysate. This inhibition is not related to a specific requirement by the lysate for oxygen since the lysate is modestly more active when incubated under nitrogen than when incubated under air. This result contrasts with the properties of lysates obtained from *cells* maintained under nitrogen, conditions that cause the cells to become energy starved and that result in inhibited translation in the resulting lysates (*65*).

Although the lysate is highly sensitive to the effective concentration of Mg^{2+}, inorganic phosphate is not sufficiently inhibitory to account for the loss of translational activity. Any partial inhibition by phosphate is ruled out by experiments in which small amounts of Mg^{2+} were added during incubation to offset accumulating phosphate and no activation was observed. The binding of creatine phosphate with Mg^{2+} is apparently competitive with the binding of inorganic phosphate. Thus, the effect on translational activity of accumulating inorganic phosphate from the consumption of creatine phosphate is, in effect, "buffered." In contrast, PEP has a high affinity for Mg^{2+} and this tight binding can have a deleterious effect in the lysate. As PEP is consumed the activity of Mg^{2+} increases and becomes partially inhibitory, although never sufficiently inhibitory enough to stop activity completely.

Loss of activity in these translation mixtures has been demonstrated in lysates in which HCR cannot be detected (*67*). Since HCR is catalytic and can inactivate a 100- to 1000-fold molar quantity of ribosomes, an undetectable level of the inhibitor may be sufficient to lead to loss of translational activity after 2 h. As eIF-2 is depleted the lysate may not become dependent on the concentration of this initiation factor until its concentration is so low as to result in rapid loss of activity.

The most active lysates reported synthesize new protein with an initial rate of synthesis of ~100 mg/L-h (*12*). Our normal lysates consistently had initial activities of 8 mg/L-h and produced a total of 20 μg/μL of new protein. Keeping in mind the high concentration of protein already present in the reticulocyte lysate (>50 g/L, mostly hemoglobin), it becomes apparent that isolation of a translation product could be very difficult without a selective method for removal of the product from the translation mixture, e.g., immunoprecipitation or affinity binding.

CONCLUSIONS

Protein synthesis in this cell-free system is not currently suitable for preparation of gram quantities of product. Even if a sufficient quantity of

the appropriate mRNA were available, which is feasable with the recent development of RNA splicing methods (74), only a fraction of a gram of product could be expected to be obtained from a liter-sized translation mixture that would require ~15 rabbits. For small-scale preparative applications, the reticulocyte has, however, potential useful application.

A cell-free translation system offers opportunities for the manipulation of protein structure not practical by fermentation or chemical synthesis. A cell-free system can be more efficient in the incorporation of added amino acids, important if an amino acid labeled with a stable isotope were being introduced into a protein. If it were desired to introduce an unnatural amino acid, cell-free synthesis would offer a potential route by using chemically synthesized aminoacyl-tRNA (75) containing the unnatural amino acid residue that would be accepted by the translational system and incorporated into the protein. Whereas site-specific mutagenesis can introduce an alternative natural amino acid, a fermentive system cannot usually be expected to accept an unnatural amino acid, especially if its structure is significantly different from natural amino acids.

Small-scale cell-free protein synthesis has the potential to be important as one approach to the synthesis of unnatural proteins. Although the reticulocyte lysate translation system has its limitations, it is considered the most active system for the translation of eukaryotic mRNA's (33). It may be possible to improve on the activity and cost of current cell-free translation systems by investigating systems derived from cultured cell-lines. The limited development and current usefulness of such systems has been discussed briefly (33).

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