Phosphorylated Sugars Stimulate Protein Synthesis and Met-tRNA$_f$ Binding Activity in Extracts of Mammalian Cells†

Jack R. Lenz, Gina E. Chatterjee, Patricia A. Maroney, and Corrado Baglioni*

ABSTRACT: Certain phosphorylated sugars, such as fructose 1,6-bisphosphate and glucose 6-phosphate, can stimulate protein synthesis in extracts from a variety of mammalian tumor cells or cells adapted to continuous growth in tissue culture. In rabbit reticulocyte lysates, the sugars stimulate protein synthesis if low molecular weight compounds are first removed by gel filtration. The stimulation is due to maintenance of a fast rate of protein synthesis for a longer period of time. The sugars are also effective in reactivating protein synthesis after it ceases during in vitro incubations. The decline in protein synthesis in extracts which are not supplemented with the sugars is accompanied by a drastic decrease in the amount of Met-tRNA$_f$ which binds to 40S native ribosomal subunits. Addition of the phosphorylated sugars restores the Met-tRNA$_f$ binding activity of the 40S ribosomal subunits concomitantly with the reactivation of protein synthesis. The stimulation of Met-tRNA$_f$ binding by the sugars cannot be exclusively explained by their metabolism to generate NADPH, NADH, ATP, and GTP, nor do the sugars act in a manner similar to hemin. Using ribosomes isolated from ascites cells or reticulocytes, we have found that the sugars stimulate GTP-dependent binding of Met-tRNA$_f$. The stimulation occurs at the level of the interaction of Met-tRNA$_f$ with 40S ribosomal subunits, for the sugars do not stimulate formation of the ternary complex of Met-tRNA$_f$, initiation factor eIF-2, and GTP. Possible mechanisms by which the sugars might stimulate Met-tRNA$_f$ binding to 40S subunits are discussed.

The study of translational regulation in mammalian cells has been greatly advanced by the use of cell extracts which are capable of synthesizing proteins. It is very important that these cell-free extracts be capable of initiating new polypeptides in vitro, but unfortunately most cell extracts have rather limited initiating activity (Jackson, 1975; Lodish, 1976). Some of the factors which promote polypeptide initiation have been recognized, such as the addition of hemin (Zucker and Shulman, 1968; Mathews, 1972; Weber et al., 1975). Hemin promotes protein synthesis by preventing the formation of an inhibitor (Maxwell et al., 1971). This inhibitor has protein kinase activity and blocks initiation of protein synthesis by phosphorylating the initiation factor which binds the initiator tRNA (Met-tRNA$_f$) to the small ribosomal subunit (Farrell et al., 1977; Kramer et al., 1976; Lenz and Baglioni, 1977; Levin et al., 1975). Recently, it has been shown that cell extracts synthesize the greatest amount of protein when incubated under ionic conditions close to those present in the cell cytoplasm (Weber et al., 1977).

Certain phosphorylated sugars can stimulate initiation of protein synthesis in lysates of rabbit reticulocytes prepared from cells which had been incubated anaerobically in glucose-free medium (Giloh (Freudenberg) and Mager, 1975). Such lysates are depleted of ATP, but addition of only ATP and an enzymatic system for its regeneration is not sufficient to effectively support protein synthesis. This suggests that the phosphorylated sugars may provide some other component necessary for initiation of protein synthesis.

The present investigation is directed at establishing the importance and the role of phosphorylated sugars in promoting protein synthesis in mammalian cell extracts. Our results show that phosphorylated sugars enhance protein synthesis in extracts of mammalian cells. The mechanism of action of the phosphorylated sugars has been investigated by localizing the step in protein synthesis which becomes limiting in their absence. This appears to be the formation of the Met-tRNA$_f$/40S ribosomal subunit complex. Using isolated ribosomes, we have shown that the phosphorylated sugars promote the formation of this complex.

Experimental Procedures

Growth of Cells. HeLa and L cells were grown in Joklik's modified minimum Eagle's medium (ME medium)[1] (Grand Island Biological Co.) supplemented with 7% horse serum. Mouse myeloma cells (MOPC-21, line P-3) were grown in Dulbecco's modified ME medium as described by Baglioni et al. (1971). Ascites cells were obtained from the ascitic fluid of mice injected intraperitoneally with these tumor cells as described by Mathews and Korner (1970).

Preparation of Extracts. Cells were harvested and extracts prepared as described by Weber et al. (1975). All extracts from HeLa and myeloma cells were supplemented with 0.05 mM hemin as described by Weber et al. (1975). Hemin was added to L or ascites cell extracts only where indicated.

Reticulocytes were obtained from anemic rabbits and lysates prepared as described by Weber et al. (1977). Gel-filtered reticulocyte lysates were obtained by chromatographing 5 mL of lysate on a column of Sephadex G-25 (2 × 15 cm) equilibrated with 0.1 M K(OAc), 2 mM Mg(OAc)$_2$, 20 mM Hepes–KOH (pH 7.4), and 2 mM dithiothreitol. The fractions corresponding to the excluded volume were combined and hemin was added to 0.05 mM before freezing in liquid N_2.

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[1] Abbreviations used: ME medium, minimum Eagle's medium; Fru-P$_2$, fructose 1,6-bisphosphate; ATA, aurintricarboxylic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DEAE, diethylaminoethyl; CTAB, cetyltrimethylammonium bromide; GDPCP, guanylyl β,γ-methylene diphosphonate; eIF, eukaryotic initiation factor (nomenclature adopted at the International Symposium on Protein Synthesis, Bethesda, Md., October 18–20, 1976; see (1977), *FEBS Lett. 76*, 1 10).

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Protein Synthesis Assay. The composition of the protein synthesis assays has been described in detail by Weber et al. (1977). All incubations contained 0.05 mM amino acids minus lysine and 5 μCi of [^{3}H]lysine (40 Ci/mmol) in a 0.05-mL final volume. All incubations, except those from reticulocytes, were supplemented with 120 mM K(OAc) and 2.5 mM $Mg(OAc)_2$. Incubations of reticulocyte lysates were supplemented with 120 mM K(OAc) and 0.9 mM $Mg(OAc)_2$.

Incubations of gel-filtered reticulocyte lysates contained 0.8 vol of lysate instead of the usual 0.7 vol used with other cell extracts. These incubations were supplemented with 54 mM K(OAc) and 0.4 mM $Mg(OAc)_2$. Spermidine was added to 0.3 mM final concentration, as suggested by Farrell et al. (1977). Spermidine stimulated protein synthesis 1.6-fold in these lysates.

Incubations were performed at 30 °C and 5-μL aliquots were sampled in duplicate at the times indicated. The procedure for measuring [^{3}H]lysine incorporation has been described by Weber et al. (1975). Heme was removed from aliquots of reticulocyte lysate before counting as described by Jacobs-Lorena and Baglioni (1973). Phosphorylated sugars or other compounds to be tested were added immediately before starting the incubations as 20-fold or 10-fold concentrated solutions.

Met-tRNA$_f$ Binding Assays in Cell Extracts. Stripped $tRNA_f^{Met}$ from rabbit liver was purchased from Grand Island Biological Co. and charged with [^{35}S]methionine (308 Ci/mmol) using an *Escherichia coli* aminoacyl synthetase (Smith and Henshaw, 1975). Binding of [^{35}S]Met-tRNA$_f$ in cell extracts was assayed by assembling 0.05 mL reactions as described above, except that all 20 unlabeled amino acids were included at 0.05 mM final concentration. The reactions were incubated for 2 min with 0.1 mM sparsomycin and then for an additional 2 min with 1.2 pmol of [^{35}S]Met-tRNA$_f$, diluted, and fractionated by sucrose gradient centrifugation as described by Weber et al. (1975). The fractions were counted by precipitation with CTAB as described by Weber et al. (1975).

Binding of Met-tRNA$_f$ to Isolated Ribosomes. Gel-filtered reticulocyte lysates were incubated for 30 min in the presence of all 20 unlabeled amino acids as described above, except that the volume of the incubation was increased to 1 mL. Sparsomycin was then added to 0.1 mM and the incubations were continued for 2 additional min. Ribosomes were then isolated as described by Baglioni et al. (1972), except that 2 mM dithiothreitol was included in all buffers. Approximately 25 μg of ribosomes was incubated with 1.2 pmol of [^{35}S]Met-tRNA$_f$, 1 mM GTP, 1 mM unlabeled methionine, and 0.1 mM sparsomycin in a final volume of 0.05 mL. The incubations contained 134 mM K(OAc), 3 mM $Mg(OAc)_2$, 20 mM Hepes-KOH (pH 7.4), and 1.4 mM dithiothreitol. Other additions were included as indicated. Incubations were performed at 30 °C for 7 min. Reactions were stopped by adding 0.5 mL of ice-cold buffer and fractionated by sucrose gradient centrifugation as described by Weber et al. (1975).

Binding of Met-tRNA$_f$ to Isolated Reticulocyte Initiation Factors. The procedure for preparing initiation factors has been described in detail by Merrick et al. (1974). Briefly, a 0.5 M KCl ribosomal salt wash was concentrated by precipitation with 70% $(NH_4)_2SO_4$. The precipitate was resuspended in 0.1 M KCl in buffer A (20 mM Tris (pH 7.5), 1 mM dithiothreitol, 0.1 mM EDTA (pH 7), and 10% glycerol), dialyzed against the same buffer and applied to a DEAE-cellulose column (Whatman DE-52) equilibrated with this buffer. The initiation factors were eluted with 0.4 M KCl in buffer A and the fractions containing eIF-2 activity were pooled and concentrated by precipitation with 70% $(NH_4)_2SO_4$, resuspended in 0.1 M KCl in buffer A, dialyzed against the same buffer, and frozen in liquid N_2.

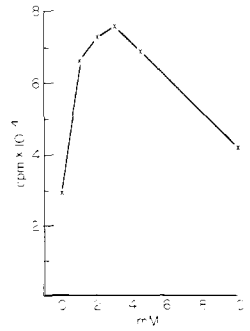

FIGURE 1: Stimulation of protein synthesis in an L cell extract by fructose 1,6-bisphosphate. The extract contained 0.05 mM hemin and was incubated for 60 min at 30 °C as described under Experimental Procedures. The final concentration of fructose 1,6-bisphosphate added is indicated on the abscissa. The [^{3}H]lysine incorporation was measured in duplicate 5-μL aliquots.

Binding of Met-tRNA$_f$ to the isolated initiation factors was performed in a volume of 0.05 mL. Incubations contained 25 μg of initiation factors, 1.2 pmol of [^{35}S]Met-tRNA$_f$, 1 mM unlabeled methionine, 134 mM K(OAc), 2 mM $Mg(OAc)_2$, 20 mM Hepes (pH 7.4), and 1.2 mM dithiothreitol. GTP and phosphorylated sugars were included in the incubations as indicated. Incubations were performed at 30 °C for 5 min and were stopped with 1 mL of the same buffer. They were filtered through nitrocellulose filters, washed twice with 1 mL of the same buffer, dried, and counted.

Results

The Effect of Fructose 1,6-Bisphosphate on Protein Synthesis. Extracts prepared from different mammalian cells were tested for in vitro protein synthesizing activity as described under Experimental Procedures. These extracts are not preincubated, translate endogenous mRNA, and are supplemented with an energy-generating system consisting of creatine phosphate and creatine phosphokinase. All the extracts tested synthesize protein initially at a relatively fast rate. Afterward, the rate slows and protein synthesis usually stops after 30 to 60 min. However, we have observed some variability in the duration of protein synthesis among different extracts from the same cell type. For example, in 16 extracts of L cells, protein synthesis was found to continue for 10 to 60 min with an average of about 30 min. In six extracts of ascites cells, protein synthesis continued for 20 to 60 min, with an average of about 45 min (data not shown). This variability suggested that some cellular constituent necessary for protein synthesis was present in different amounts in each extract and became limiting after varying times.

Giloh (Freudenberg) and Mager (1975) have recently reported that phosphorylated sugars such as fructose 1,6-bisphosphate (Fru-P_2) and glucose 6-phosphate can stimulate initiation of protein synthesis in lysates prepared from rabbit reticulocytes. Therefore, we tested whether various concentrations of Fru-P_2 could stimulate protein synthesis in L cell extracts (Figure 1). Maximal stimulation was obtained with 3 mM Fru-P_2. We next investigated whether Fru-P_2 could stimulate protein synthesis in extracts from five different types of mammalian cells (Table I). All the extracts tested showed stimulation by 3 mM Fru-P_2, except those from reticulocytes. This stimulation is due to a prolonged, high rate of protein synthesis close to that of the initial rate (Figures 2A and 2B).

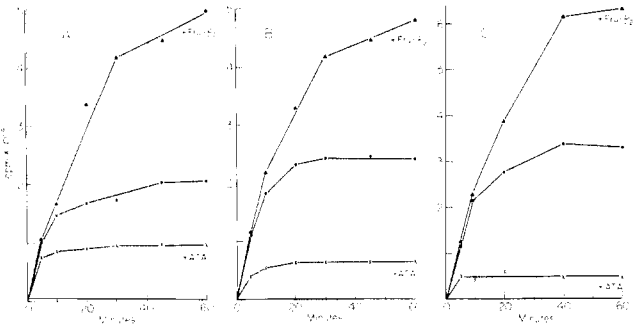

FIGURE 2: Time course of protein synthesis in an L cell extract (A), in an ascites cell extract (B), and in a gel-filtered reticulocyte lysate (C) (see Experimental Procedures for details of the preparation of the extracts). The L cell extract and the reticulocyte lysate contained 0.05 mM hemin. The extracts were incubated at 30 °C and duplicate 5-μL aliquots taken for counting as described in the Experimental Procedures. Each extract was tested without any addition (●—●), with added 3 mM fructose 1,6-bisphosphate (+Fru-P_2, ▲—▲), and with added 0.1 mM aurintricarboxylic acid (+ATA, X—X).

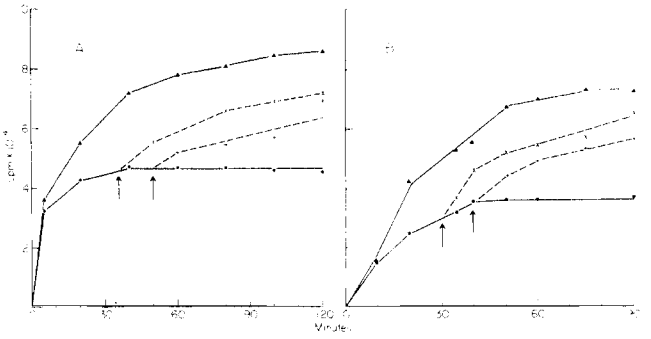

FIGURE 3: Reactivation of protein synthesis upon addition of phosphorylated sugars. (A) An ascites cell extract was incubated as described in the legend of Figure 2 without additions (●—●), with 3 mM Fru-P_2 added at time zero (▲—▲), with 3 mM Fru-P_2 added after 35 min (X- -X), and with 3 mM Fru-P_2 added after 50 min (+- -+). (B) A reticulocyte lysate obtained by gel filtration was incubated as described in Figure 2 with no additions (●—●), with 0.5 mM glucose 6-phosphate added at time zero (▲—▲), with 0.5 mM glucose 6-phosphate added after 30 min (X- -X), and 0.5 mM glucose 6-phosphate added after 40 min (+- -+).

TABLE I: Stimulation of Protein Synthesis by Fructose 1,6-Bisphosphate in Extracts from Different Types of Mammalian Cells.

Extract	[^{3}H]Lysine incorp (cpm)[a]		Stimulation (cpm + Fru-P_2)/(cpm − Fru-P_2)
	−Fru-P_2	+Fru-P_2 (3 mM)	
Ascites	24 600	48 000	2.0
	21 800	54 000	2.5
	74 900	115 000	1.5
L	32 800	72 100	2.2
	30 000	72 700	2.4
	29 800	38 500	1.3
HeLa	42 300	55 600	1.3
Myeloma	4 600	19 400	4.2
Reticulocyte	38 300	39 600	1.0
	41 200	39 400	1.0
	49 300	48 200	1.0

[a] Incubations were for 60 min at 30 °C; 5-μL aliquots were sampled for counting as described under Experimental Procedures.

The stimulation of protein synthesis by Fru-P_2 was initially demonstrated in reticulocyte lysates prepared from cells which had been incubated anaerobically in glucose-free medium (Giloh (Freudenberg) and Mager, 1975). It seemed possible, therefore, that lysates from reticulocytes treated in this way are depleted of a cellular constituent affecting protein synthesis, whereas lysates from untreated reticulocytes may already contain optimal amounts of this constituent. Therefore, we removed low molecular weight compounds from reticulocyte lysates by gel filtration (see Experimental Procedures). Protein synthesis in these gel-filtered lysates was stimulated by 3 mM Fru-P_2 in a manner similar to other mammalian cell extracts (Figure 2C).

To determine whether the stimulation of protein synthesis by Fru-P_2 is due to increased initiation, the amount of protein synthesis due to elongation of peptide chains previously initiated in intact cells prior to preparation of the extract was measured by adding the inhibitor of initiation, aurintricarboxylic acid (ATA). ATA was used at a concentration of 0.1 mM. At this concentration, it inhibits only initiation (Weber et al., 1975). With no added Fru-P_2, the extracts synthesize from two to five times the amount of protein synthesized with added ATA (Figure 2). The extracts, therefore, actively initiate synthesis of new polypeptides in vitro. With added 3 mM Fru-P_2, however, five to ten times more protein was synthesized than in incubations with added ATA (Figure 2), presumably as a result of increased initiation. Protein synthesis is exactly the same in incubations with ATA whether Fru-P_2 is added or not (data not shown).

The cessation of protein synthesis seen in extracts not supplemented with Fru-P_2 could be due to a depletion of the endogenous amounts of this or other sugars in the extracts. Therefore, we investigated whether protein synthesis could be reactivated after it had stopped, by adding Fru-P_2 at various times during the incubation. Addition of 3 mM Fru-P_2 to ascites cell extracts, which had stopped synthesizing protein, caused an immediate reactivation of translation (Figure 3A). A similar experiment was performed with an L cell extract and again Fru-P_2 addition reactivated protein synthesis after it had completely ceased (results not shown). The same effect was also demonstrated in gel-filtered reticulocyte lysates (Figure 3B), except that glucose 6-phosphate was used in this experiment instead of Fru-P_2 (see below).

We examined whether the same proteins were synthesized by ascites and L cell extracts in the presence or absence of Fru-P_2. The extracts were incubated with [^{35}S]methionine as the labeled amino acid and the proteins were analyzed by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate. The same proteins were synthesized whether the extracts were supplemented with Fru-P_2 or not (data not shown).

The Effect of Other Phosphorylated Sugars on Protein Synthesis. The effect of various concentrations of phosphorylated sugars on the reactivation of protein synthesis was determined by preincubating an ascites cell extract until protein synthesis ceased and then adding the compound to be tested together with a radioactive amino acid (see Experimental Procedures). This method provided a more sensitive measurement of the effect of the sugars on protein synthesis, by reducing the background amount of protein synthesis obtained in the absence of added sugars (Figure 3). Of all the compounds tested, Fru-P_2 gave the greatest stimulation and was maximally active at the lowest concentration, about 3 to 4 mM (Figures 4A and 4B). Fructose 6-phosphate, glucose 6-phosphate, and ribose 5-phosphate also caused significant stimulation, although at higher concentrations than Fru-P_2. Glucose, 2-deoxyglucose 6-phosphate, and glucosamine 6-

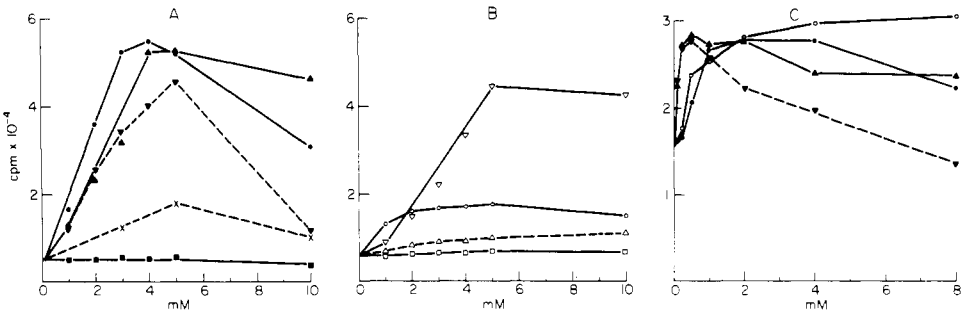

FIGURE 4: Reactivation of protein synthesis by phosphorylated sugars and other compounds in an ascites cell extract (A and B) and in a gel-filtered reticulocyte lysate (C). These were incubated as described in Figure 2 without the addition of a labeled amino acid. In addition to the 0.05 mM unlabeled amino acids, the reticulocyte incubations were supplemented with 0.023 mM unlabeled lysine. [^{3}H]Lysine (5 μCi) was added after 30 min to the ascites incubations and after 20 min to the reticulocyte incubations along with the compounds to be tested. The latter were added at the concentrations indicated on the abscissa and the incubations continued for 60 more min. Duplicate 5-μL aliquots were sampled and counted as described under Experimental Procedures. The intercept of the curves with the ordinate shows the incorporation with no addition. Additions: fructose 1,6-bisphosphate (●—●); fructose 6-phosphate (▲—▲); glucose 6-phosphate (▼- -▼); glucose (X- -X); phosphoenolpyruvate (■—■); ribose 5-phosphate (▽—▽); 2-deoxyglucose 6-phosphate (○—○); glucosamine 6-phosphate (△- -△); reduced glutathione (□—□).

phosphate gave low stimulation, while phosphoenolpyruvate had no effect. Similar results were obtained in L cell extracts (results not shown).

In gel-filtered reticulocyte lysates, glucose 6-phosphate and fructose 6-phosphate were maximally active at the lowest concentration, about 0.5 mM (Figure 4C). Fru-P_2 and 2-deoxyglucose 6-phosphate also stimulated protein synthesis significantly, but at higher concentrations. Therefore, in all subsequent experiments, we used 0.5 mM glucose 6-phosphate in reticulocyte lysates and 3 mM Fru-P_2 in ascites and L cell extracts.

The Effect of Phosphorylated Sugars on Protein Synthesis Is Different than That of Hemin. While the ascites cell extract used in the experiment shown in Figure 2 was not supplemented with hemin, the reticulocyte lysate and the L cell extract contained 50 μM hemin. We have found that this concentration of hemin maximally stimulates protein synthesis in all our cell extracts (results not shown). Although hemin is not required for active initiation in extracts from L and ascites cells (Figures 2 and 5), it can stimulate protein synthesis in these cell extracts up to twofold (Figure 5).

One possible explanation for the stimulation of protein synthesis by the phosphorylated sugars is that they act similarly to hemin. However, it seemed likely that hemin and the phosphorylated sugars stimulate protein synthesis by different mechanisms since addition of Fru-P_2 or glucose 6-phosphate to cell extracts containing hemin resulted in increased protein synthesis. To determine whether they act by different mechanisms, hemin and Fru-P_2 were tested in different combinations in ascites and L cell extracts. Addition of hemin or Fru-P_2 stimulated protein synthesis in these extracts to a variable extent. Some extracts were stimulated more by hemin (Figures 5A and 5C), while others were stimulated more by Fru-P_2 (Figures 5B and 5D). However, in every extract tested, the addition of hemin and Fru-P_2 together produced greater stimulation than either compound added separately. Furthermore, hemin and Fru-P_2 can potentiate the activity of each other for in some cases the effect of combined addition was greater than the sum of their individual effects (Figure 5D).

Additional evidence that hemin and the phosphorylated sugars stimulate protein synthesis in a different manner was obtained by incubating ascites cell extracts in the absence of added hemin until protein synthesis ceased and then determining whether the addition of 50 μM hemin or Fru-P_2 could reactivate protein synthesis. The addition of hemin did not restore protein synthesis (data not shown), whereas the addition of Fru-P_2 did (Figure 3A). The same result was obtained using L cell extracts (results not shown).

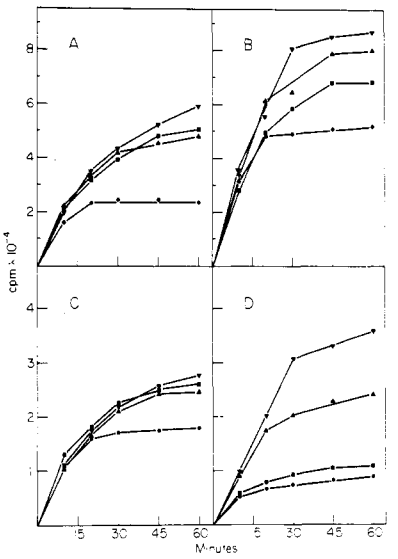

FIGURE 5: Effect of hemin and fructose 1,6-bisphosphate on protein synthesis by ascites and L cell extracts. Extracts were prepared from ascites cells (A and B) or from L cells (C and D). Each extract was divided into two aliquots. One aliquot was supplemented with hemin immediately after cell homogenization at 0.05 mM final concentration, while the other received an equal volume of H_2O. Each aliquot was tested for protein synthesis with or without added 3 mM Fru-P_2. No additions (●—●); hemin added (■—■); Fru-P_2 added (▲—▲); hemin and Fru-P_2 added (▼—▼). The extracts were incubated at 30 °C and 5-μL aliquots were taken for counting at the times indicated.

The Effect of Phosphorylated Sugars on Protein Synthesis Is Not Solely Due to Generation of NADH, NADPH, or Reduced Glutathione. In addition to hemin, several other factors are known to affect initiation of protein synthesis in vitro. Among these are reducing agents, because compounds which oxidize -SH groups, such as oxidized glutathione, are potent inhibitors of initiation (Kosower et al., 1971). One possible explanation for the effect of phosphorylated sugars on protein synthesis is that they are metabolized via the pentose phosphate pathway, thus generating NADPH, which in turn could generate reduced glutathione. Alternatively, the sugars could be metabolized via the glycolytic pathway thus generating NADH. Therefore, we tested whether the reducing agent dithiothreitol, reduced glutathione, NADPH, or NADH could

TABLE II: Stimulation of Protein Synthesis in Ascites Cell Extracts and Reticulocyte Lysates by Phosphorylated Sugars and Other Compounds.

Extract	Additions	[^{3}H]Lysine Incorp Concn (mM)	[^{3}H]Lysine Incorp In 60 min (cpm)	[^{3}H]Lysine Incorp Stimulation[a]
	Experiment 1			
Ascites	None		28 800	
	Fru-P_2	3	48 400	1.7
	NADH	1	26 400	0.9
	NADH	3	29 600	1.0
	Dithiothreitol	3	27 900	1.0
	Dithiothreitol	10	24 200	0.8
	Experiment 2			
Ascites	None		20 700	
	Fru-P_2	3	54 400	2.6
	NADPH	1	23 400	1.1
	NADPH	3	25 500	1.2
	Experiment 3			
Reticulocyte (gel-filtered)	None		3 600	
	Creatine phosphate	5	4 500	1.2
	Glucose 6-phosphate	0.5	19 800	5.5
	NADPH	0.2	3 600	1.0
	NADPH + creatine phosphate	0.2/5	3 700	1.0
	NADH	0.1	3 600	1.0
	NADH + creatine phosphate	0.1/5	3 700	1.0

[a] Stimulation is defined here as the counts/min obtained with addition of a compound divided by the counts/min obtained without this addition.

substitute for the phosphorylated sugars in stimulating protein synthesis.

None of these compounds were effective in promoting protein synthesis in ascites cell extracts or gel-filtered reticulocyte lysates (Figure 4B and Table II). The preparations of NADH and NADPH were verified to be at least 90% in the reduced form by measuring their absorbance at 259 and 339 nm. Therefore, the stimulation of protein synthesis by phosphorylated sugars cannot be explained solely by their ability to produce reduced cofactors.

The Effect of Phosphorylated Sugars on Protein Synthesis Cannot Be Explained Solely by ATP/GTP Generation. GTP and ATP are necessary for active initiation of protein synthesis. In particular, a high GTP/GDP ratio is necessary for the functioning of the initiation factor eIF-2, which binds Met-tRNA$_f$ to the 40S ribosomal subunit (Walton and Gill, 1975). Our extracts are supplemented with creatine phosphate and creatine phosphokinase to generate ATP. GTP is generated from ATP by the endogenous nucleotide diphosphokinase (Walton and Gill, 1975). It seemed possible that the supply of creatine phosphate might be exhausted or that the creatine phosphokinase might be inactivated. Thus, the phosphorylated sugars could stimulate protein synthesis by being metabolized to generate ATP.

To test whether energy generation was the factor limiting protein synthesis, a gel-filtered reticulocyte lysate and an ascites cell extract were incubated until protein synthesis ceased. Additional creatine phosphate (10 mM) and creatine phosphokinase were then added (Figure 6). The effect of phosphorylated sugars was tested in parallel incubations. Addition of the ATP-generating system had almost no effect in promoting reinitiation of protein synthesis in the reticulocyte lysate. In the ascites extract, however, it stimulated protein synthesis about half as much as 3 mM Fru-P_2. Lower concentrations of creatine phosphate gave relatively less stimulation in the ascites extract, and higher concentrations provided

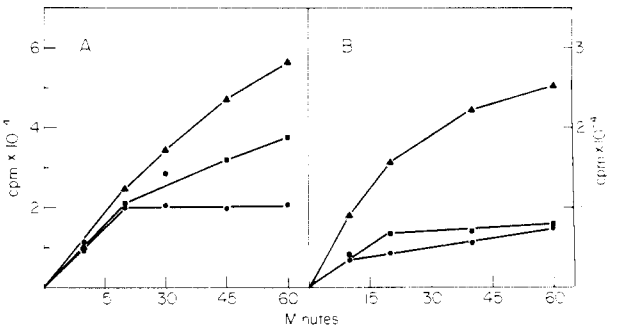

FIGURE 6: Effect of readdition of creatine phosphate and creatine phosphokinase to an ascites extract (A) or to a reticulocyte lysate (B). The cell extracts were incubated as described in the legend of Figure 4. After 20 min, [^{3}H]lysine was added to incubations with no other additions (●—●), with 10 mM creatine phosphate and two additional units of creatine phosphokinase (■—■), or with 3 mM Fru-P_2 to the ascites extract or 0.5 mM glucose 6-phosphate to the reticulocyte lysate (▲—▲).

no further stimulation (data not shown). It thus appears that energy generation may be involved in the stimulation of protein synthesis by Fru-P_2 in ascites extracts. This could explain why the concentrations of phosphorylated sugars which give the greatest stimulation are higher in ascites extracts than in reticulocyte lysates (Figure 4). Nonetheless, energy generation can only partially account for the stimulation of protein synthesis by Fru-P_2 (Figure 6A).

Moreover, ATP generation cannot explain the stimulation of protein synthesis by glucose 6-phosphate in reticulocyte lysates. Its analogue, 2-deoxyglucose 6-phosphate, is just as active in stimulating protein synthesis, although at higher concentrations (Figure 4C). This compound is not metabolized (Schmidt et al., 1974) and therefore cannot generate ATP.

Neither ATP generation nor regeneration of NADPH or NADH can individually account for the stimulation of protein

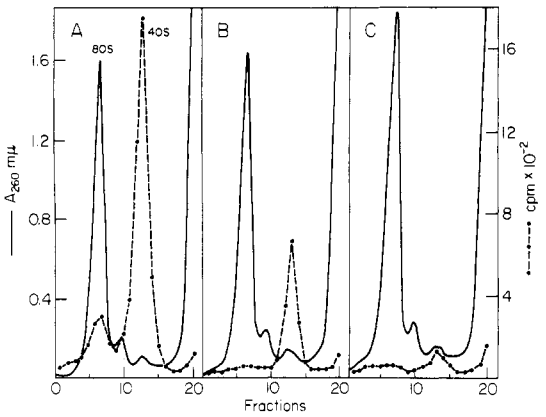

FIGURE 7: Loss of Met-tRNA$_f$ binding activity in an ascites cell extract incubated without added fructose 1,6-bisphosphate. The Met-tRNA$_f$ binding assay is described under Experimental Procedures. Three identical reaction mixtures were assembled, all containing 20 unlabeled amino acids from zero time. (A) Binding in an extract which was not preincubated. (B) Binding in an extract which was preincubated for 20 min in the presence of the 20 unlabeled amino acids and with 3 mM Fru-P$_2$ before the addition of sparsomycin. (C) Binding in an extract which was preincubated for 20 min in the presence of the 20 unlabeled amino acids, but without added Fru-P$_2$ before the addition of sparsomycin. The incubations were fractionated by sucrose gradient centrifugation and the Met-tRNA$_f$ in gradient fractions determined as described under Experimental Procedures. Solid line: A_{260} nm. Broken line: counts per min per fraction.

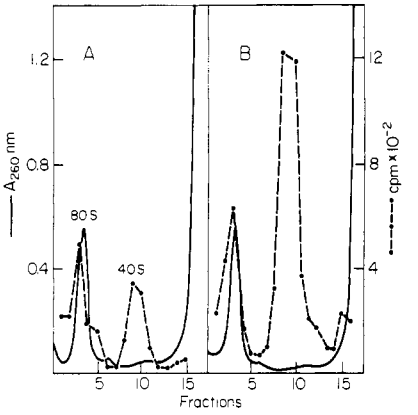

FIGURE 8: Increase in Met-tRNA$_f$ binding activity in a reticulocyte lysate upon addition of 0.5 mM glucose 6-phosphate. The reticulocyte lysate obtained by gel filtration was used for this experiment. The incubations were assembled as described under Experimental Procedures for Met-tRNA$_f$ binding assay. (A) Reticulocyte lysate incubated for 20 min prior to the binding in the presence of the 20 unlabeled amino acids but without added glucose 6-phosphate. (B) Incubated for 20 min as in A and then 0.5 mM glucose 6-phosphate added for 2 min before the binding assay. Solid line: A_{260} nm. Broken line: counts per min per gradient fraction.

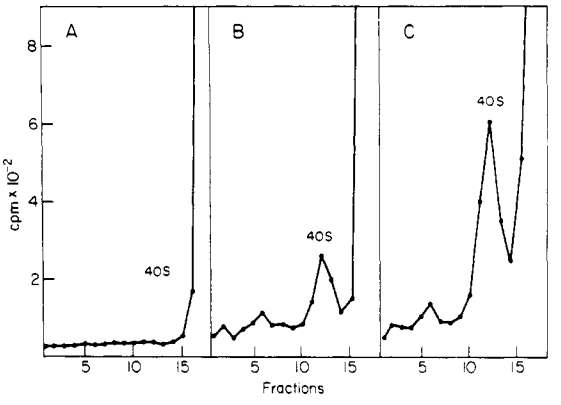

FIGURE 9: Stimulation of Met-tRNA$_f$ binding to isolated reticulocyte ribosomes by glucose 6-phosphate. Isolated reticulocyte ribosomes were incubated as described under Experimental Procedures with (A) 0.5 mM glucose 6-phosphate but no GTP, (B) 1 mM GTP but no glucose 6-phosphate, or (C) 1 mM GTP and 0.5 mM glucose 6-phosphate.

synthesis by phosphorylated sugars. We also seem to have ruled out that simultaneous generation of ATP and reduced cofactors can account for the stimulation by preincubating a reticulocyte lysate and then adding creatine phosphate and either NADPH or NADH. No stimulation of protein synthesis was observed with these additions (Table II).

Phosphorylated Sugars Stimulate Met-tRNA$_f$ Binding to 40S Ribosomal Subunits. It seemed likely that the decrease in protein synthesis in the absence of phosphorylated sugars was due to a decrease in initiation (Figure 2). To directly determine whether phosphorylated sugars promote initiation, we measured the activity of the extracts in carrying out the binding of Met-tRNA$_f$ to native 40S ribosomal subunits. This is the first step in the process of initiation at the ribosomal level (Staehelin et al., 1975). We measured the binding of Met-tRNA$_f$ (see Experimental Procedures) in unincubated ascites extracts, and in extracts incubated with added 3 mM Fru-P$_2$ or with no addition (Figure 7). The Met-tRNA$_f$ binding activity of the extracts decreased 60% upon 20 min of incubation with added Fru-P$_2$, but decreased 90% or more with no added Fru-P$_2$. An identical observation was made using a gel-filtered reticulocyte lysate incubated with added 0.5 mM glucose 6-phosphate or with no addition (results not shown).

Other known instances of inhibition of Met-tRNA$_f$ binding in mammalian cell extracts are known to cause an inhibition of protein synthesis (Darnbrough et al., 1972; Legon et al., 1973; Clemens et al., 1975). It seemed likely, therefore, that the low Met-tRNA$_f$ binding activity of cell extracts incubated without added sugars is responsible for the low amount of protein synthesis. If this were the case, it would be expected that the increase in protein synthesis observed upon addition of phosphorylated sugars would be accompanied by an increased Met-tRNA$_f$ binding activity. Therefore, we incubated an ascites extract and a gel-filtered reticulocyte lysate until protein synthesis and Met-tRNA$_f$ binding activity were drastically reduced (20 and 30 min, respectively). Addition of glucose 6-phosphate to the lysate caused an immediate three- to fourfold increase in the amount of Met-tRNA$_f$ bound to 40S subunits (Figure 8). Addition of Fru-P$_2$ to the ascites extract resulted in a similar increase in Met-tRNA$_f$ binding activity (results not shown).

To investigate the mechanism by which phosphorylated sugars stimulate the binding of initiator tRNA, we isolated ribosomes and measured their Met-tRNA$_f$ binding activity. Ribosomes were obtained from reticulocyte lysates by centrifugation through sucrose cushions to remove small molecular weight compounds. These ribosome preparations contain polysomes, 80S monosomes, and native ribosomal subunits. The ribosomes were incubated with Met-tRNA$_f$ and various additions, and the amount of Met-tRNA$_f$ bound to native 40S subunits was determined by centrifugation through sucrose gradients (see Experimental Procedures). Binding of Met-tRNA$_f$ to these ribosomes was dependent on the addition of GTP for, when GTP was omitted from the incubations, the binding was eliminated even in the presence of glucose 6-phosphate (Figure 9). This indicates that the binding of initiator tRNA is most likely via the initiation factor eIF-2, since this is the only initiation factor which has been reported to bind Met-tRNA$_f$ in a GTP-dependent fashion (Adams et al., 1975).

TABLE III: Stimulation of Binding of Met-tRNA$_f$ to Isolated Reticulocyte Ribosomes by Phosphorylated Sugars.

Nucleotide (1 mM)	Addition	Concn (mM)	Met-tRNA$_f$ bound (cpm)	Stimulation[a]
		Experiment 1		
GTP	None		610	
GTP	Glucose 6-phosphate	0.5	1530	2.5
GTP	NADPH	0.1	380	0.6
GTP	NADH	0.25	740	1.2
		Experiment 2		
GDPCP	None		320	
GDPCP	Glucose 6-phosphate	0.5	740	2.3

[a] See Table II for the definition of stimulation. the conditions of incubation and the determination of Met-tRNA$_f$ bound to native 40S ribosomal subunits are described under Experimental Procedures.

TABLE IV: Met-tRNA$_f$ Binding to Reticulocyte Initiation Factors in the Presence or Absence of Phosphorylated Sugars.

Additions	Concn (mM)	GTP (mM)[a]	Met-tRNA$_f$ bound (cpm)
None		0	5 100
Glucose 6-phosphate	0.5	0	5 800
None		1	12 800
Glucose 6-phosphate	0.5	1	12 700
Fru-P_2	3	1	13 200

[a] An additional 1 mM $Mg(OAc)_2$ was included in incubations containing GTP.

The binding of Met-tRNA$_f$ to isolated reticulocyte ribosomes was stimulated two- to threefold by the addition of 0.5 mM glucose 6-phosphate (Figure 9). A similar result was obtained with isolated ascites ribosomes using 3 mM Fru-P_2 (results not shown). Stimulation of Met-tRNA$_f$ binding occurs at any Mg^{2+} concentration between 0.3 and 5.3 mM (results not shown). This stimulation is not due to generation of NADH or NADPH on addition of glucose 6-phosphate to isolated reticulocyte ribosomes, for NADH does not significantly stimulate binding and NADPH is slightly inhibitory (Table III). Moreover, it is unlikely that phosphorylated sugars stimulate Met-tRNA$_f$ binding by generating ATP or GTP. ATP is not required for binding of Met-tRNA$_f$, and in fact, it is inhibitory (Lenz and Baglioni, 1977; Farrell et al., 1977). GTP is added to the incubations at an optimal concentration to promote binding.

Several other possible explanations for the stimulation of Met-tRNA$_f$ binding to isolated ribosomes by the phosphorylated sugars were tested. It was conceivable that the sugars could act by preventing deacylation of Met-tRNA$_f$ or by preventing GTP hydrolysis. We measured the rate of deacylation of Met-tRNA$_f$ incubated with ascites ribosomes and found no difference in the presence or absence of 3 mM Fru-P_2 (results not shown). The nonhydrolyzable GTP analogue, guanylyl β,γ-methylene diphosphonate (GDPCP), can substitute for GTP in promoting binding of Met-tRNA$_f$, although the binding occurs at lower efficiency (Levin et al., 1973). We replaced GTP with GDPCP and determined whether 0.5 mM glucose 6-phosphate stimulated Met-tRNA$_f$ binding to reticulocyte ribosomes. The sugar stimulated binding 2.3-fold, showing that stimulation occurs even when GTP hydrolysis is prevented (Table III).

We next tested whether the interaction of Met-tRNA$_f$ with eIF-2 was stimulated by phosphorylated sugars in the absence of 40S ribosomal subunits. An unfractionated preparation of reticulocyte initiation factors (see Experimental Procedures) was incubated with Met-tRNA$_f$ and GTP in the presence or absence of glucose 6-phosphate or Fru-P_2. The amount of Met-tRNA$_f$ bound was determined by retention of the ternary complex with GTP and eIF-2 on nitrocellulose filters (Schreier and Staehelin, 1973). No stimulation by the phosphorylated sugars was detected (Table IV). This suggests that the stimulation by the phosphorylated sugars occurs at the level of interaction of Met-tRNA$_f$ with 40S ribosomal subunits.

Discussion

Protein synthesis in every mammalian cell extract we have tested can be stimulated by certain phosphorylated sugars. Our observations provide a simple and effective way to increase initiation of protein synthesis in these extracts. This improvement in the activity of the extracts may be of practical use in studies of translational regulation in mammalian cells.

Stimulation by phosphorylated sugars can be shown directly in extracts from a variety of mammalian tumor cells or cells adapted to continuous growth in culture, whereas it can be demonstrated in reticulocyte lysates only after the low molecular weight compounds have been removed by gel filtration. This difference may be due to the presence of different levels of sugars in normal and tumor cells and/or a different rate of metabolism of these sugars. Cells transformed by oncogenic viruses have been shown to have a greater glycolytic flux than corresponding normal cells (Singh et al., 1975). Therefore it seems possible that the level of phosphorylated sugars may become rapidly depleted in tumor cell extracts.

Sugars that stimulate protein synthesis are either phosphorylated hexoses or pentoses, the most active being Fru-P_2, glucose 6-phosphate, fructose 6-phosphate, and ribose 5-phosphate. Our results and those of Giloh (Freudenberg) and Mager (1975) show that triose phosphates, such as phosphoenolpyruvate and 3-phosphoglycerate, do not significantly stimulate protein synthesis.

Cell extracts which are not supplemented with phosphorylated sugars lose the ability to bind Met-tRNA$_f$ to 40S ribosomal subunits and to synthesize protein. Addition of phosphorylated sugars to these extracts reverses the decline in Met-tRNA$_f$ binding activity and allows protein synthesis to resume. It is not clear, however, how the phosphorylated sugars stimulate Met-tRNA$_f$ binding.

We have eliminated some possible explanations of the effect of phosphorylated sugars on Met-tRNA$_f$ binding. Metabolism of the sugars to produce ATP, NADPH, or NADH cannot completely explain the stimulation, since ATP production with creatine phosphate/creatine phosphokinase and/or addition of the reduced cofactors does not stimulate Met-tRNA$_f$ binding or protein synthesis nearly as well as the phosphoryl-

ated sugars do. No reduction in Met-tRNA$_f$ deacylase activity has been found on addition of the phosphorylated sugars. They stimulate Met-tRNA$_f$ binding even when the nonhydrolyzable GTP analogue, GDPCP, is used in place of GTP. This suggests that the sugars do not act by preventing GTP hydrolysis. The sugars do not stimulate formation of the eIF-2/GTP/Met-tRNA$_f$ complex.

Addition of the sugars to isolated ribosomes stimulates formation of the Met-tRNA$_f$/40S subunit complex two- to threefold. We discuss here three possible mechanisms which could explain this stimulation: (i) the phosphorylated sugars may allosterically stimulate Met-tRNA$_f$ binding; (ii) they may prevent or reverse an inhibition of Met-tRNA$_f$ binding caused by a protein kinase; and (iii) they may prevent or reverse inhibition of Met-tRNA$_f$ binding caused by some metabolite which accumulates during the incubation of cell extracts.

There is no evidence for a direct allosteric effect of the phosphorylated sugars on the formation of Met-tRNA$_f$/40S complex. We are now preparing radioactively labeled sugars to test whether the sugars bind to this complex. We consider this an attractive hypothesis, since it can explain how the concentration of glycolytic intermediates may directly regulate the rate of initiation of protein synthesis in intact cells. However, it is also possible that the sugars could act via some unidentified metabolite. In search of such a compound we have tested 2,3-diphosphoglycerate and guanidine 5′-diphosphoglucose. Neither stimulated protein synthesis at concentrations up to 1 mM.

It seems unlikely that phosphorylated sugars stimulate protein synthesis by preventing the formation or action of the inhibitor which appears in lysates not supplemented with hemin (Maxwell et al., 1971). Both these processes involve protein kinases (Farrell et al., 1977; Datta et al., 1977). However, we can detect the stimulation of protein synthesis by phosphorylated sugars in cell extracts which are supplemented with hemin. Furthermore, hemin and the phosphorylated sugars stimulate protein synthesis synergistically and can act in different ways. Addition of sugars to extracts which have stopped synthesizing protein causes protein synthesis to resume, whereas hemin addition has no effect. Therefore, it seems unlikely that the sugars interfere with either the formation of an inhibitory protein kinase or that they can inhibit its activity. We have not excluded, however, the possibility that the sugars may promote the activity of a phosphatase, which reverses the effect of the inhibitory kinase.

It is possible that the loss of Met-tRNA$_f$ binding activity, which occurs in an extract which is not supplemented with a phosphorylated sugar, is due to the accumulation of some metabolite during the incubation. The sugars could conceivably act by decreasing the amount of this metabolite in the extract or by countering its effect.

The results presented indicate that phosphorylated sugars stimulate Met-tRNA$_f$/40S complex formation, but we have not tested whether they also stimulate the joining of mRNA and 60S subunits to form the 80S initiation complex. Since these processes occur subsequent to Met-tRNA$_f$/40S complex formation (Staehelin et al., 1975), it would be difficult to distinguish whether a stimulation results from a direct effect of the sugars or from their ability to stimulate Met-tRNA$_f$/40S complex formation.

The effect of phosphorylated sugars on protein synthesis has also been studied in extracts from wheat germ. These extracts are gel-filtered and dependent on the addition of exogenous mRNA for protein synthesis. No stimulation of globin mRNA translation has been observed with several concentrations of Fru-P_2 (unpublished observations). Therefore, it seems possible that the stimulation of protein synthesis by the phosphorylated sugars may be limited to mammalian cell extracts.

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