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Stimulation of Protein Synthesis and Met-tRNA$_f$ Binding by Phosphorylated Sugars: Studies on Their Mechanism of Action†

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ABSTRACT: It has been previously reported by J. R. Lenz et al. [(1978) *Biochemistry 17*, 80–87] that certain phosphorylated sugars stimulate protein synthesis in extracts of mammalian cells. This effect was found to be due to a stimulation of Met-tRNA$_f$ binding to 40S ribosomal subunits, both in whole extracts and with isolated ribosomes. However, formation of a ternary complex of Met-tRNA$_f$, initiation factor eIF-2, and GTP was not stimulated. It was also shown that the stimulation is not due solely to metabolism of the sugars. The present communication further characterizes the stimulatory effect of the sugars. They were found to prevent the inactivation of ribosomes that occurs during protein synthesis incubations. The sugars were also found to inhibit cAMP-dependent protein kinases noncompetitively. However, they stimulate Met-tRNA$_f$ binding to 40S ribosomal subunits even under conditions in which an inhibition of protein kinase has no effect. Although it has not been possible to demonstrate a direct association of the sugars with the 40S initiation complex, the evidence suggests that their effect is mediated by an interaction with one of the components involved in the formation of this complex.

The initiation of protein synthesis in mammalian cells and their extracts has been extensively studied as the main site of translational regulation (Lodish, 1976). One of the compounds known to exert an effect at this level is hemin (Zucker & Shulman, 1968; Weber et al., 1975), which acts by preventing the formation of an inhibitor of translation, known as the hemin-controlled repressor or HCR[1] (Maxwell et al., 1971; Gross & Rabinovitz, 1972). HCR is a cAMP-independent protein kinase that phosphorylates eIF-2, the initiation factor responsible for the binding of Met-tRNA$_f$ to native 40S (40S^N) ribosomal subunits (Levin et al., 1975; Kramer et al., 1976; Lenz & Baglioni, 1977; Farrel et al., 1977). Phosphorylated eIF-2 is much less active in initiation because it cannot respond

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[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DEAE, diethylaminoethyl; HCR, hemin-controlled repressor; G6P, glucose 6-phosphate; dG6P, 2-deoxyglucose 6-phosphate; FDP, fructose 1,6-bis(phosphate); 2AP, 2-aminopurine; eIF, eukaryotic initiation factor (nomenclature adopted at the International Symposium on Protein Synthesis, Bethesda, MD, October 18–20, 1976; see *FEBS Lett. 76*, 1–10 (1977); cAMP, cyclic adenosine monophosphate; 40S^N, native 40S ribosomal subunits; IF, initiation factors; Met-tRNA$_f$, initiator methionyl-tRNA which can be formylated with *E. coli* transformylase.

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to the stimulatory activity of an accessory protein, the eIF-2 stimulating protein (deHaro et al., 1978).

Other compounds which stimulate initiation of protein synthesis are certain phosphorylated sugars, primarily hexoses and pentoses. Giloh (Freudenberg) & Mager (1975) observed a stimulatory effect of these sugars on protein synthesis in reticulocytes that had been incubated anaerobically in the absence of glucose. The study of this phenomenon was extended by Lenz et al. (1978) to other mammalian cell extracts, including reticulocyte lysates that had been gel-filtered to remove small molecular weight compounds. It was found that the addition of phosphorylated sugars to cell extracts maintains a linear rate of protein synthesis for a longer time. Furthermore, when added to extracts in which protein synthesis has ceased, the sugars have a reactivating effect. Both the stimulation and reactivation of protein synthesis are due to an increased level of Met-tRNA$_f$ binding in the extracts, an effect that can also be demonstrated with isolated ribosomes. However, the sugars do not stimulate the formation of the eIF-2/GTP/Met-tRNA$_f$ ternary complex.

Some possible mechanisms that could explain the stimulatory effect of phosphorylated sugars on protein synthesis were discussed by Lenz et al. (1978). We report here the results of experiments which examine these possible mechanisms, as well as some other mechanisms not previously considered. The conclusion most strongly supported by these data is that phosphorylated sugars are interacting with one or more of the components of the Met-tRNA$_f$/40S^N initiation complex to directly stimulate its formation, although the precise mechanism of this interaction still remains to be determined.

Experimental Procedures

Preparation of Extracts. Reticulocytes were obtained from anemic rabbits and lysates were prepared as described previously (Weber et al., 1977). Gel filtration of reticulocyte lysates has also been described (Lenz et al., 1978). Unless otherwise indicated, the pooled fractions were supplemented with 0.05 mM hemin as described by Weber et al. (1975) and then frozen in liquid N_2.

Protein Synthesis Assay. The methods used for the assay of protein synthesis have been previously reported (Weber et al., 1977; Lenz et al., 1978). [^{3}H]Lysine (40 Ci/mmol) was present at 0.1 mCi/mL and the final assay volumes used are indicated in the figure legends. In preincubations in the absence of the labeled amino acid, the mixtures were supplemented with 25 μM lysine.

Preparation of Ribosomes and Supernatants. Preincubated protein synthesis mixtures were fractionated by centrifugation at 150000*g* at 4 °C for 1.5 h. The supernatants (S150) were saved and the ribosomal pellets were rinsed with a small volume of 1 mM dithiothreitol and then resuspended in a mixture of salts, amino acids minus lysine, spermidine, and an energy regenerating system at the concentrations used for protein synthesis with gel-filtered lysates (Lenz et al., 1978). [^{3}H]Lysine was added at 0.5 mCi/mL. The volume used to resuspend the pellets was equivalent to 20% of the original volume of the preincubation mixtures.

Isolated ribosomes to be used for Met-tRNA$_f$ binding experiments, glucose 6-phosphate binding experiments, or the preparation of initiation factors were prepared according to the method of Baglioni et al. (1972) by diluting 1 mL of reticulocyte lysate or protein synthesis mixture with 1 mL of 10 mM KCl, 1.5 mM $MgCl_2$, 20 mM Hepes–KOH, pH 7.4, and 2 mM dithiothreitol (buffer A). The diluted mixture was layered onto 4 mL of 15% sucrose (w/w) in buffer A and centrifuged for 3 h at 150000*g*. The ribosomal pellets were washed with a small volume of buffer A, then resuspended in 0.1 mL of the same buffer, and stored in small aliquots at −60 °C.

Ribosomes to be used for the preparation of initiation factors were prepared as described above, except that the pellets were resuspended in 1 mM dithiothreitol, 6 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, and 20 mM Hepes–KOH, pH 7.4.

Preparation of Initiation Factors. A 0.5 M KCl ribosomal wash was prepared by the method of Crystal et al. (1974). The ribosomal suspension was fractionated by centrifuging for 2 h at 150000*g*. The supernatant, containing the initiation factors (IF), was removed and either assayed immediately for Met-tRNA$_f$ binding activity or further purified by precipitation between 33% and 66% $(NH_4)_2SO_4$ according to Shafritz & Anderson (1970). Protein concentrations were determined by the method of Lowry et al. (1951).

Met-tRNA$_f$ Binding Assay. The charging of stripped rabbit liver tRNA$_f^{Met}$ with [^{35}S]methionine (308 Ci/mmol) using *E. coli* aminoacyl synthetase has been described by Smith & Henshaw (1975). The binding of [^{35}S]Met-tRNA$_f$ to isolated ribosomes was performed in 135 mM K(OAc), 3 mM $Mg(OAc)_2$, 20 mM Hepes–KOH, pH 7.4, and 1.4 mM dithiothreitol (buffer B) as described previously by Lenz et al. (1978). The binding of labeled glucose 6-phosphate to isolated ribosomes was measured under the same assay conditions, except that yeast tRNA charged with unlabeled methionine using the *E. coli* enzyme was substituted at 1.4 mg/mL for [^{35}S]Met-tRNA$_f$. Glucose 6-[^{32}P]phosphate (0.2 μCi/0.05 mL; 8.3 Ci/mmol) or [^{3}H]glucose 6-phosphate (5 μCi/0.05 mL; 18 Ci/mmol) was added to the incubation, and the bound glucose 6-phosphate was analyzed either by nitrocellulose filter binding or by sucrose density gradient centrifugation (Weber et al., 1975).

The binding of [^{35}S]Met-tRNA$_f$ was performed in buffer B containing 2 mM $Mg(OAc)_2$ as described by Lenz et al. (1978). In Table II, the IF were incubated for 7 min at 30 °C in 0.03 mL of this buffer with PK-II (see below), with ATP, and in some experiments with 2-deoxyglucose 6-phosphate; [^{35}S]Met-tRNA$_f$ and GTP were then added in 0.02 mL of buffer B, and the incubation was continued for another 7 min. The reaction was stopped by dilution with 3 mL of ice cold buffer B. For the experiments in Table V, [^{35}S]-Met-tRNA$_f$ binding to IF was measured in a single 7-min incubation. Bound [^{35}S]Met-tRNA$_f$ was determined by filtering onto nitrocellulose filters and washing three times with 3 mL of buffer B.

Preparation of Glucose 6-[^{32}P]Phosphate and [^{3}H]Glucose 6-Phosphate. The reaction was performed in 0.1 mL of 50 mM Tris-HCl, pH 7.1, 20 mM $MgCl_2$, 0.1 mM EDTA, 35 units of yeast hexokinase (Boehringer-Mannheim), and either 12.5 mM D-glucose plus 0.1 mCi of [γ-^{32}P]ATP (8.3 Ci/mmol) or 0.2 mCi of [^{3}H]glucose (18 Ci/mmol) plus 12.5 mM ATP. The mixture was incubated for 1 h at 30 °C and then diluted with 0.9 mL of water. The sample was applied to a column of Dowex I-8 formate (1 × 1.5 cm) and eluted according to the method of Bartlett (1968). The column was washed with 10-mL aliquots of water, 0.4 M ammonium formate, and 1.9 M ammonium formate; 1-mL fractions were collected and aliquots of each fraction counted. The peak fractions which eluted at 0.4 M ammonium formate, containing labeled glucose 6-phosphate, were pooled and lyophilized.

Preparation of PK-II. Crude HCR was prepared by the method of Levin et al. (1975). The fraction from the DEAE-cellulose column containing HCR activity was pre-

cipitated with 50% $(NH_4)_2SO_4$. The pellet was resuspended in 2 mL of 50 mM KCl, 20 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, and 10% glycerol and dialyzed overnight against the same buffer. This partially purified preparation of HCR is referred to as PK-II and is known to contain both HCR and cAMP-dependent protein kinase activities.

Assay of Protein Kinase Activity. Protein kinase activities were assayed by the method of Traugh & Traut (1974). The incubations contained 50 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 0.3 mg of either histone IIA (Sigma) or casein, 0.15 mM ATP, 2.1 μCi of [γ-^{32}P]ATP (3333 Ci/mmol), PK-II as described in the figure legends, and, where indicated, 1.5 μM cAMP in a final volume of 0.07 mL. The mixtures were incubated 15 min at 30 °C; then 0.05 mL was removed and spotted onto Whatman 3MM filter disks (2.1 cm). The filters were washed once in ice-cold 10% Cl_3CCOOH and then three times in 5% Cl_3CCOOH at room temperature, dried and counted.

Preparation of [γ-^{32}P]-Labeled Phosphorylated Ribosomes. Approximately 0.1 mg of isolated ribosomes was incubated in 0.1 mL of buffer B with 24 μg of PK-II, 8 μCi of [γ-^{32}P]ATP, and 0.1 mM ATP for 7 min at 30 °C. The reaction was stopped by dilution with ice-cold buffer A, and the ribosomes were reisolated by centrifuging through 1 mL of 15% sucrose in buffer A and resuspending in the same buffer.

Electrophoresis and Autoradiography. Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was performed as previously described by Lenz & Baglioni (1977) using the method of Laemmli (1970) with bis(acrylamide) concentrations from Blattler et al. (1972). Autoradiography was performed on Kodak XR-2 film at −60 °C. A Kodak X-ray intensifying screen was used to decrease the time necessary for exposure. The film was preexposed to an electronic flash through a Wratten no. 23 gelatin filter (Laskey & Mills, 1975).

Results

Lenz et al. (1978) have previously discussed three possible mechanisms by which phosphorylated sugars could stimulate protein synthesis. The sugars may prevent or reverse the inhibition of Met-tRNA$_f$ binding caused (i) by the accumulation of a metabolite or (ii) by a protein kinase or they may (iii) allosterically stimulate Met-tRNA$_f$ binding. The experiments reported here were devised to examine these mechanisms. Rabbit reticulocyte lysate was used in this study to eliminate the effect due to metabolism of the sugars observed with extracts of other mammalian cells (Lenz et al., 1978). Reticulocyte lysates do not metabolize sugars because of a deficiency of NAD^+ (Lennon et al., 1977).

Phosphorylated Sugars Do Not Act by Preventing or Reversing the Accumulation of an Inhibitory Metabolite. To test for the accumulation of an inhibitory metabolite, gel-filtered reticulocyte lysate was incubated 30 min under conditions for protein synthesis (see Figure 1). An identical control incubation was kept at 0 °C. Both samples were fractionated to yield S150 and ribosomes as described in Experimental Procedures and then remixed in the four possible combinations, and protein synthesis was assayed during a second incubation at 30 °C. Figure 1A shows that preincubated ribosomes synthesize only about half as much protein as ribosomes that have not been preincubated, regardless of the source of the S150 used to reconstitute the extract. If 0.5 mM glucose 6-phosphate (G6P) is included in the second incubation (Figure 1B), protein synthesis is stimulated approximately 1.5- to 2-fold in all the reconstituted mixtures. These results suggest that the decline in the rate of protein

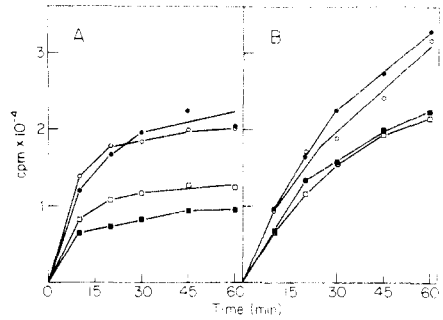

FIGURE 1: Time course of protein synthesis in reconstituted systems in the absence and presence of glucose 6-phosphate. Incubations for protein synthesis were assembled in a total volume of 0.3 mL, incubated, and fractionated as described in Experimental Procedures. The ribosomal pellets were resuspended in a volume of 0.06 mL, and 0.05-mL assay mixtures were made using 0.01 mL of ribosomes plus 0.04 mL of supernatant. Protein synthesis was assayed by removing 5-μL aliquots at the indicated times as described by Weber et al. (1975). The A_{260} of the resuspended ribosomes was determined and the cpm adjusted to correct for any differences in ribosome concentration. The reconstituted mixtures are unincubated supernatant plus unincubated ribosomes (●—●); incubated supernatant plus unincubated ribosomes (○—○); incubated supernatant plus incubated ribosomes (■—■); and unincubated supernatant plus incubated ribosomes (□—□). (A) No additions to the second incubation mixture; (B) 0.5 mM glucose 6-phosphate added to the second incubation mixture.

synthesis that occurs during the incubation of a lysate is not due to the buildup of a metabolite in the S150, since only the ribosomes and not the S150 significantly determine the level of protein synthesized. Moreover, G6P could not be reversing the accumulation of an inhibitory product, because the sugar was found to stimulate protein synthesis almost equally in all four incubations.

In a similar experiment, gel-filtered lysates were preincubated for 30 min in the presence or absence of 0.5 mM G6P, fractionated, remixed, and assayed for protein synthesis as described above. Assays reconstituted with ribosomes preincubated with G6P synthesized about twice as much protein in 60 min as those with ribosomes preincubated without the sugar (data not shown). Therefore, the sugars prevent the inactivation of ribosomal functions that occurs during the incubation of extracts under conditions of protein synthesis.

The Effects of Phosphorylated Sugars and 2-Aminopurine on Protein Synthesis Are Not Additive. Previous experiments had shown that hemin and phosphorylated sugars sometimes act synergistically to stimulate protein synthesis when added together to ascites and L cell extracts (Lenz et al., 1978). This result suggests that hemin and the sugars are acting through different mechanisms. It has been proposed that hemin exerts its effect by preventing the activation of a cAMP-dependent protein kinase that, in turn, activates a proinhibitor to form HCR (Datta et al., 1977a,b). It has also been shown that various purines inhibit the activity of cAMP-dependent protein kinases competitively with respect to ATP (Iwai et al., 1972). Similarly, Farrel et al. (1977) demonstrated that several purines, including 2-aminopurine (2AP), inhibit the protein kinase activity of HCR. Therefore, to investigate whether the sugars affect the activity of protein kinases, the effect of a phosphorylated sugar and a purine on protein synthesis was compared.

Gel-filtered lysates were preincubated for 20 min; then protein synthesis was measured in the absence or presence of 0.5 mM G6P or 5 mM 2AP (Figure 2A). When added separately to incubations, G6P stimulated protein synthesis about 2-fold and 2AP, about 2.5-fold. When added together, no greater stimulation was observed than with 2AP alone.

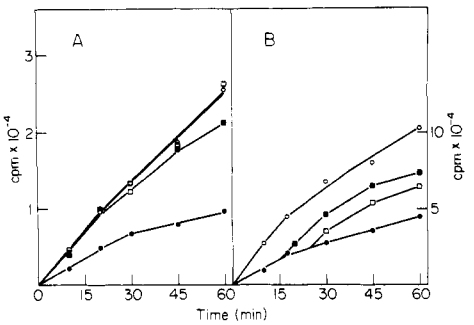

FIGURE 2: Comparison of the effects of glucose 6-phosphate and 2-aminopurine on protein synthesis. Gel-filtered reticulocyte lysates were preincubated at 30 °C under conditions of protein synthesis as described in Experimental Procedures for 20 min (A) or 30 min (B). The indicated additions were made and the mixtures were supplemented with [^{3}H]lysine. The total volume for each incubation was 0.05 mL. During the second incubation 5-μL aliquots were removed at the indicated times and treated as described in Figure 1. (A) No additions (●—●); 0.5 mM glucose 6-phosphate (■—■); 5 mM 2-aminopurine (○—○); 0.5 mM glucose 6-phosphate and 5 mM 2-aminopurine (□—□). (B) No additions (●—●); 5 mM 2-aminopurine added at zero time (○—○), at 15 min (■—■), and at 25 min (□—□).

Therefore, the effects of G6P and 2AP are not additive. These results suggest that phosphorylated sugars and purines may stimulate protein synthesis through similar mechanisms.

The 2-aminopurine also acts similarly to the sugars when added after the start of an incubation. Gel-filtered lysates were preincubated for 30 min; then protein synthesis was measured in assays to which 5 mM 2AP was added at 0, 15, and 25 min after the start of the second incubation (Figure 2B). The results indicate that, like phosphorylated sugars (Lenz et al., 1978), 2AP exerts a reactivation effect when added to lysates in which protein synthesis has leveled off.

Phosphorylated Sugars Stimulate Protein Synthesis in Reticulocyte Lysates at Low Hemin Concentrations. The results of Figure 2 raised the possibility that the sugars could affect the activity of a protein kinase. If the sugars inhibit the activation and/or activity of HCR, they should stimulate protein synthesis in nonfiltered as well as gel-filtered lysates. However, the results of experiment no. 2 in Table I do not show such an effect. We reasoned that at an optimum hemin concentration (0.05 mM), the effect of the sugars may not be detectable, either because the protective effect of hemin is much greater by comparison or because the sugars are already present at optimum concentrations. Similarly, without added hemin, the sugar effect may be obscured by the excessively large amounts of HCR that become activated. Therefore, we tested whether phosphorylated sugars stimulate protein synthesis in nonfiltered lysates at hemin concentrations that allow only limited activation of HCR. Table I shows that, in nonfiltered lysates with 0.02–0.025 mM hemin, protein synthesis was stimulated 28–35% by phosphorylated sugars. In some experiments, 2-deoxyglucose 6-phosphate (dG6P) was used because it could be added to a final concentration of up to 10 mM without inhibiting protein synthesis. G6P and other phosphorylated sugars inhibit protein synthesis when added at concentrations greater than 3–5 mM (Lenz et al., 1978). The greatest effect, almost 60% stimulation, was observed when 20 mM dG6P was added in the absence of hemin (Table I, experiment no. 5). The greatest effect observed in the presence of 0.05 mM hemin was only about 7% with 10 mM dG6P. Nevertheless, an effect of phosphorylated sugars in nonfiltered lysates could be demonstrated by manipulating the hemin concentration, suggesting that the sugars might affect HCR activity or activation.

Table I: Effect of Phosphorylated Sugars on Protein Synthesis at Different Hemin Concentrations

	hemin concn (μM)	phosphorylated sugar (mM)	[^{3}H]lys incorp at 60 min (cpm)[a]	% stimulation[b]
gel-filtered lysate				
expt 1	0	none	4 700	
	0	G6P (0.5)	4 800	2.1
	50	none	12 300	
	50	G6P (0.5)	20 400	65.9
unfiltered lysate				
expt 2	0	none	7 500	
	0	G6P (0.5)	7 600	1.3
	50	none	13 800	
	50	G6P (0.5)	14 500	5.1
expt 3	25	none	34 300	
	25	G6P (1)	43 900	28.0
	25	G6P (5)	31 100	−9.3
	25	G6P (10)	14 900	−56.6
expt 4	20	none	38 200	
	20	dG6P (5)	51 500	34.8
	25	none	55 600	
	25	dG6P (5)	59 800	7.6
	30	none	47 500	
	30	dG6P (5)	53 600	12.8
	35	none	48 200	
	35	dG6P (5)	51 200	6.2
expt 5	0	none	14 500	
	0	dG6P (10)	19 000	31.0
	0	dG6P (20)	23 000	58.6
	50	none	66 000	
	50	dG6P (10)	70 400	6.7
	50	dG6P (20)	54 100	−18.0

[a] Background [^{3}H]lysine incorporation at zero time was subtracted. [b] Negative (−) stimulation denotes percent inhibition.

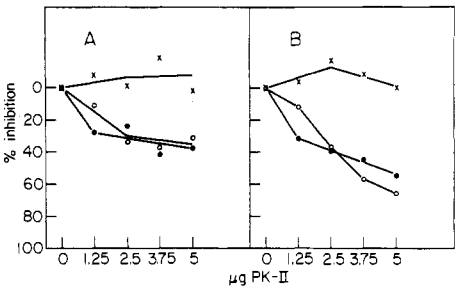

FIGURE 3: 2-Deoxyglucose 6-phosphate and 2-aminopurine protect protein synthesis in unfiltered and in gel-filtered reticulocyte lysates from inhibition by PK-II. Protein synthesis assays were performed in a final volume of 0.025 mL as described in Experimental Procedures. The incubations contained the indicated amounts of PK-II, expressed as μg of protein, and either no additions (●—●), 10 mM 2-deoxyglucose 6-phosphate (○—○), or 10 mM 2-aminopurine (X—X). Aliquots of 5 μL were removed at 0 and 60 min, and incorporation of [^{3}H]lysine was determined as described in Figure 1. The incorporation in the absence of PK-II was considered to be 0% inhibition. (A) Nonfiltered lysates; 0% inhibition was equivalent to 44 300 cpm (no additions), 51 300 cpm (2-deoxyglucose 6-phosphate), or 60 000 cpm (2-aminopurine). (B) Gel-filtered lysates; 0% inhibition equaled 6400 cpm (no additions), 14 300 cpm (2-deoxyglucose 6-phosphate), or 17 200 cpm (2-aminopurine).

Effect of Phosphorylated Sugars on the Inhibition of Protein Synthesis by PK-II and on the Protein Kinase Activities of PK-II. The stimulation of protein synthesis in nonfiltered lysates by phosphorylated sugars is relatively small. Therefore, to study the effect of the sugars on HCR more directly, 10 mM dG6P or 10 mM 2AP were added to protein synthesis assays containing nonfiltered lysate, 0.05 mM hemin, and increasing amounts of added PK-II (Figure 3A). The sugar afforded some protection against low amounts of PK-II,

Table II: Effect of 2-Deoxyglucose 6-Phosphate on Inhibition of Met-tRNA$_f$ Binding Activity in Initiation Factors by PK-II

dG6P (5 mM)	ATP (0.1 mM)	PK-II (12 μg)	[^{35}S] Met-tRNA$_f$ bound (cpm)	% binding[a]
–	–	–	8300	100
–	–	+	7400	89
–	+	–	8600	103
–	+	+	2000	24
+	–	–	8600	100
+	–	+	8400	98
+	+	–	7900	92
+	+	+	2500	29

[a] Percent binding is defined as cpm bound in each assay divided by cpm bound in the assay without ATP and PK-II × 100.

but was ineffective at higher concentrations. On the other hand, 2AP provided complete protection against all concentrations of PK-II. In experiments using different lysates, the effect of dG6P varied somewhat (data not shown), whereas 2AP always completely prevented the inhibition of protein synthesis by HCR. In a similar experiment with gel-filtered lysates (Figure 3B), the results are almost identical with those obtained with nonfiltered lysates.

These results suggest that the sugars could affect the phosphorylation of eIF-2 by HCR (Levin et al. 1975; Kramer et al., 1976; Lenz & Baglioni, 1977; Farrel et al., 1977). Therefore, phosphorylation of eIF-2 was directly examined by incubating either isolated ribosomes (Figure 4A) or IF (Figure 4B) with [γ-^{32}P]ATP and PK-II in the absence or presence of a phosphorylated sugar or 2AP. Phosphorylated proteins were analyzed by $NaDodSO_4$ gel electrophoresis and autoradiography. Figure 4A shows the phosphorylation of isolated ribosomes in the absence of PK-II (track 1) and in the presence of PK-II with (track 2) and without (track 3) 0.5 mM G6P. PK-II significantly increased the phosphorylation of only one protein, the small subunit of eIF-2 (arrow). The addition of G6P substantially decreased the phosphorylation of all the proteins (compare track 2 with tracks 1 and 3). The nonspecific reduction suggested that a loss of the ^{32}P label might be occurring via exchange between labeled ATP and unlabeled G6P. To investigate this possibility, an incubation mixture containing G6P was applied to a Dowex I-8 formate column and eluted with a linear gradient of 0–5 M ammonium formate to separate phosphorylated compounds (Bartlett, 1968). Most of the label eluted in the position of G6P, while less than 25% of the labeled material eluted with ATP (data not shown). Therefore, the decreased phosphorylation in the presence of G6P is due to a greater than 75% reduction in the specific activity of the [γ-^{32}P]ATP.

This rapid exchange of the ^{32}P label may be due to the presence of hexokinase in isolated ribosome preparations. This difficulty was overcome by using partially purified IF and 5 mM dG6P, which is a poor substrate for hexokinase (Figure 4B). Significant phosphorylation of the small subunit of eIF-2 in a purified IF preparation occurred only in the presence of PK-II (compare tracks 3 and 4). With added dG6P a slight decrease in the phosphorylation of all proteins was observed (track 2). However, this reduction is again nonspecific and can probably be attributed to some ^{32}P exchange between ATP and dG6P. The presence of 2AP in the incubation, however, almost eliminated ^{32}P incorporation into all proteins (track 1). Therefore, dG6P does not prevent the inactivation of eIF-2 by decreasing its phosphorylation by HCR.

The effect of the phosphorylated sugars on the inhibition of Met-tRNA$_f$ binding by HCR was similarly investigated.

Table III: Effect of 2-Deoxyglucose 6-Phosphate and cAMP on the Protein Kinase Activities of PK-II[a]

additions		^{32}P incorp (cpm)[b]	
1.5 μM cAMP	10 mM dG6P	histone	casein
+	–	47 000	24 000
–	–	4 500	21 600
+	+	21 100	25 300
–	+	5 000	28 600

[a] Protein kinase activities were assayed as described in Experimental Procedures; 8.4 μg of PK-II per 0.07 mL of assay. [b] Background incorporation in the absence of PK-II was subtracted: histone, 6100 cpm; casein, 6200 cpm.

Table IV: Effect of Various Phosphorylated Sugars on the Protein Kinase Activities of PK-II[a]

	^{32}P incorp (cpm)[b]			
	histone[c]		casein	
additions	cpm	% inhibn[d]	cpm	% inhibn[d]
none	88 400		28 800	
3 mM glucose 6-phosphate	80 200	9.3	30 700	0
2.5 mM fructose 6-phosphate	80 400	9.0	30 800	0
3 mM fructose 1,6-bis(phosphate)	53 000	40.0	31 600	0
2.5 mM ribose 5-phosphate	79 200	10.4	30 900	0

[a] 8.4 μg of PK-II/0.07 mL assay. [b] Background incorporation in the absence of PK-II was subtracted: histone, 6000; casein, 4700. [c] 1.5 μM cAMP was included in the assay only when histone was the substrate. [d] % inhibition was calculated as 100 × [cpm (no additions) – cpm (with addition)]/cpm (no additions).

Initiation factors were incubated with [^{35}S]Met-tRNA$_f$ as described under Experimental Procedures in the presence or absence of unlabeled ATP, PK-II, and dG6P (Table II). Significant inhibition of Met-tRNA$_f$ binding occurred only when both ATP and PK-II were present, and this inhibition was not reduced by addition of dG6P. These data, together with those from Figure 4A, suggest that phosphorylated sugars do not inhibit the activity of HCR.

The possibility that the sugars stimulate a phosphatase that dephosphorylates eIF-2 was also investigated. Reticulocyte ribosomes were incubated with [γ-^{32}P]ATP and PK-II and then reisolated and incubated with or without 0.5 mM G6P. The ^{32}P-labeled polypeptides are shown in Figure 4A. Incubation with dG6P did not significantly affect the phosphorylation of the small subunit of eIF-2 (arrow). Apparently, the sugars do not reverse the phosphorylation of eIF-2 by stimulating a phosphatase.

Since the PK-II preparation used in these experiments was known to contain both HCR (cAMP-independent) protein kinase activity and cAMP-dependent protein kinase activity (Levin et al., 1976), the effect of phosphorylated sugars on each type of activity was studied by using specific protein substrates. cAMP-dependent kinases phosphorylate histones well, but use casein poorly; cAMP-independent kinases utilize casein preferentially over histones (Traugh & Traut, 1974). The results are shown in Tables III and IV.

The data in Table III confirm the validity of using the protein substrates histone and casein to differentially measure cAMP-dependent and cAMP-independent protein kinases, respectively. When histone was the substrate, ^{32}P incorporation increased tenfold in the presence of 1.5 μM cAMP. In

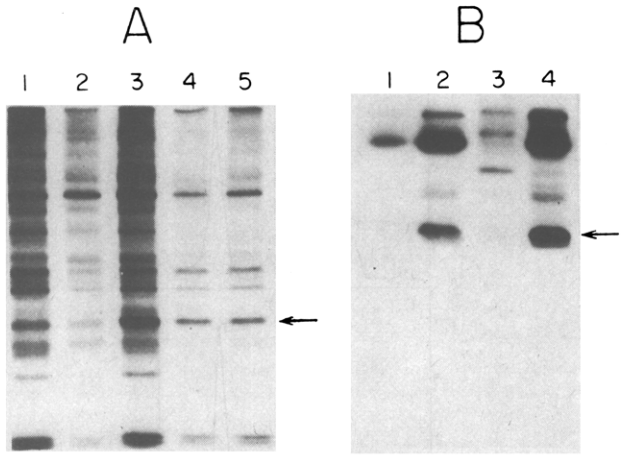

FIGURE 4: The effect of phosphorylated sugars and 2-aminopurine on the phosphorylation of ribosomes and initiation factors. (A) Tracks 1–3: Approximately 25 μg of ribosomes was incubated as described in Experimental Procedures with 4 μCi of [γ-^{32}P]ATP, 0.1 mM unlabeled ATP, and other additions as indicated for 7 min at 30 °C in a final volume of 0.03 mL. Tracks 4 and 5: Approximately 50 μg of ^{32}P-phosphorylated ribosomes was incubated (see Experimental Procedures) in the absence or presence of 0.5 mM glucose 6-phosphate in a final volume of 0.05 mL for 7 min at 30 °C. Tracks: (1) and (5) no additions; (2) 12 μg of PK-II and 0.5 mM glucose 6-phosphate; (3) 12 μg of PK-II; (4) 0.5 mM glucose 6-phosphate. (B) Approximately 6 μg of initiation factors (prepared by $(NH_4)_2SO_4$ precipitation) was incubated with 4 μCi of [γ-^{32}P]ATP, 0.1 mM unlabeled ATP, and other additions as indicated for 7 min at 30 °C in a final volume of 0.03 mL. Tracks: (1) 12 μg of PK-II and 5 mM 2-aminopurine; (2) 12 μg of PK-II and 5 mM 2-deoxyglucose 6-phosphate; (3) no additions; (4) 12 μg of PK-II. All reactions were stopped by the addition of an equal volume of 2× sample buffer, then fractionated by electrophoresis on 12.5% polyacrylamide–sodium dodecyl sulfate gels, and autoradiographed as described in Experimental Procedures. The arrows indicate the position of the 38 000 mol wt subunit of eIF-2. The gel shown in B was run for a shorter length of time than the one shown in A.

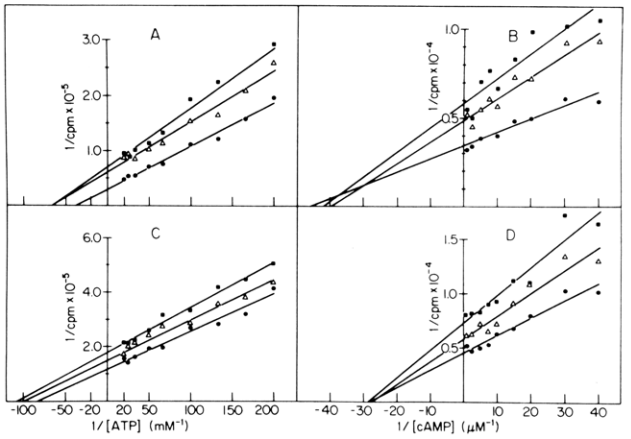

FIGURE 5: The kinetics of inhibition of beef heart protein kinase and HCR activities by fructose 1,6-bis(phosphate). Phosphorylation of histones by beef heart protein kinase (A and C) and by partially purified HCR (B and D) was assayed as described in Experimental Procedures. The assays contained 2 μg of beef heart protein kinase or 4.9 μg of PK-II. (A and B) ATP concentration varied from 0.005 to 0.05 mM; cAMP concentration was 1.5 μM. (C and D) cAMP concentration varied from 0.025 to 1.0 μM; ATP concentration was 0.15 mM. Fructose 1,6-bis(phosphate) concentrations were (A) 0 mM (●), 2.5 mM (△), and 10 mM (■); (B and D) 0 (●), 1 mM (△), and 2.5 mM (■); (C) 0 (●), 2.5 mM (△), and 5 mM (■). The substrate concentrations at which half-maximal activity was observed as determined at zero concentration of fructose 1,6-bis(phosphate) are: beef heart protein kinase, cAMP 2.16×10^{-8} M, and ATP 2.67×10^{-5} M; PK-II, cAMP 3.75×10^{-8} M, and ATP 1.22×10^{-5} M. The lines giving the best fit to the data were determined by least-squares analysis.

contrast, little or no increase was observed when casein was the substrate. dG6P, 10 mM, reduced the phosphorylation of histone by over 50% but had no effect on casein phosphorylation. Therefore, dG6P inhibits cAMP-dependent protein kinase.

The effects of various phosphorylated sugars on both types of protein kinase activity were tested at concentrations optimal for their stimulation of protein synthesis (Lenz et al., 1978). All the sugars tested inhibited the cAMP-dependent protein kinase activity (Table IV). Fructose 1,6-bis(phosphate) (FDP) gave the greatest inhibition, 40%, while the other sugars inhibited by approximately 10%. None were found to inhibit the cAMP-independent protein kinase; in fact, a slight stimulation of this activity by the sugars was consistently observed (see also Table III).

Phosphorylated Sugars Inhibit a cAMP-Dependent Protein Kinase Noncompetitively with Respect to Both cAMP and ATP. Phosphorylated sugars could inhibit cAMP-dependent protein kinases by two possible mechanisms: (i) they could prevent activation of the kinase by cAMP; (ii) they could inhibit the kinase activity of the free catalytic subunit (see Rubin & Rosen, 1975). To distinguish between these two possibilities, protein kinase activity was measured in the presence of different FDP concentrations (including zero) under two sets of conditions: (i) constant cAMP, variable ATP concentration; and (ii) constant ATP, variable cAMP concentration. Beef heart protein kinase, a cAMP-dependent enzyme, was also studied for comparison. The kinetics of inhibition were determined by expressing the data as double-reciprocal plots (see Figure 5).

Figure 5A,C shows the kinetics of inhibition of the beef heart kinase and partially purified HCR (PK-II), respectively, when the variable is ATP concentration. The inhibition of both enzymes by FDP is noncompetitive. Therefore, the sugars are not acting as ATP analogues, as do 2AP and other purines. The kinetics of inhibition of the beef heart protein kinase (Figure 5B) and the PK-II preparation (Figure 5D) is also noncompetitive with respect to cAMP. The kinetic constants determined from these plots are reported in the legend of Figure 5.

Loss of Met-tRNA$_f$ Binding Activity in Hemin-Supplemented Lysates Is Not Due to Inactivation of eIF-2. The inhibition of a cAMP-dependent protein kinase by phosphorylated sugars suggests that they may prevent the activation of HCR (Datta et al., 1977a). For the sugars to act through this mechanism, the decreased Met-tRNA$_f$ binding activity observed in incubated, hemin-supplemented lysates (Lenz et al., 1978) must result from reduced eIF-2 activity. The addition of sugars at the start of an incubation would then prevent HCR activation and, thus, the inactivation of eIF-2. This inactivation can be shown by measuring Met-tRNA$_f$ binding with crude IF preparations (Datta et al., 1977a).

To examine this possibility, gel-filtered, hemin-supplemented lysates, either unincubated or incubated under conditions for protein synthesis in the absence or presence of 0.5 mM G6P, were used for the preparation of IF. Met-tRNA$_f$ binding activity was then assayed as described in Experimental Procedures (Table V). During a 30-min incubation in the absence of G6P, the Met-tRNA$_f$ binding activity of IF decreased by only 4%. Similar results have been obtained using nonfiltered, hemin-supplemented lysates (data not shown). After a 30-min incubation of the lysate in the presence of the sugar, the Met-tRNA$_f$ binding activity of IF was essentially the same as for lysates incubated in its absence. Thus, the significant reduction in Met-tRNA$_f$ binding observed in lysates during an incubation (Lenz et al., 1978) is not due to a loss

Table V: Met-tRNA$_f$ Binding to Initiation Factors from Lysates Subjected to Different Incubation Conditions

conditions of incubation of lysates					
time (min)	G6P (0.5 mM)	GTP (1 mM)	[^{35}S]Met-tRNA$_f$ bound (cpm)[a]	GTP-dependent binding (cpm/μg)[b]	% binding[c]
0	−	−	345		
0	−	+	1320	20.2	100
30	−	−	260		
30	−	+	1315	19.4	96
30	+	−	245		
30	+	+	1125	19.1	95

[a] Average of duplicate assays. [b] GTP-dependent binding was calculated as [cpm with GTP − cpm without GTP]/μg of initiation factors. Approximately 45–55 μg of initiation factors was added per 0.1-mL assay. [c] % binding was calculated as 100 × GTP-dependent binding of each initiation factor preparation divided by the GTP-dependent binding of initiation factors prepared from unincubated lysates without glucose 6-phosphate.

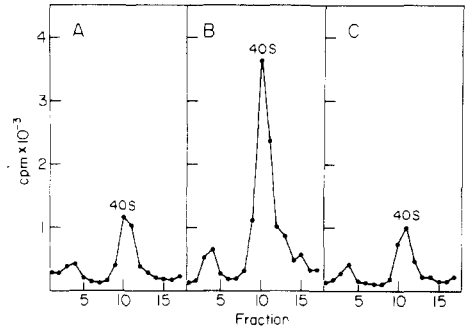

FIGURE 6: The effect of 2-deoxyglucose 6-phosphate and 2-aminopurine on Met-tRNA$_f$ binding to ribosomes. [^{35}S]Met-tRNA$_f$ binding to isolated ribosomes was determined as described in Experimental Procedures using 50 μg of ribosomes per assay. The incubations were analyzed on sucrose gradients. (A) No additions; (B) 5 mM 2-deoxyglucose 6-phosphate; (C) 5 mM 2-aminopurine.

of eIF-2 activity. Therefore, the sugars do not stimulate protein synthesis by preventing an inactivation of eIF-2, since no such inactivation occurs.

Phosphorylated Sugars Directly Stimulate Met-tRNA$_f$ Binding to Native 40S Ribosomal Subunits. Although evidence has been obtained to show that the sugars affect the activity of a protein kinase, this might not be the only, or even the most physiologically important, mechanism by which phosphorylated sugars stimulate protein synthesis. Earlier work has established that phosphorylated sugars can directly stimulate Met-tRNA$_f$ binding to native 40S ribosomal subunits (40S^N) (Lenz et al., 1978). Figure 6 shows the results of a similar experiment in which the effects of 5 mM dG6P and 5 mM 2AP on Met-tRNA$_f$ binding were compared. The [^{35}S]Met-tRNA$_f$ bound to 40S^N was greatly increased by the presence of the sugar (compare Figure 6A,B). In contrast, 2AP did not stimulate the binding of [^{35}S]Met-tRNA$_f$ (Figure 6C). These data indicate that the sugar stimulates Met-tRNA$_f$ binding via a mechanism that does not involve inhibition of a protein kinase.

The sugars have no effect on ternary complex formation (Lenz et al., 1978). However, they might stabilize the binding of ternary complex (eIF-2/GTP/Met-tRNA$_f$) to 40S^N, which has been shown to dissociate during sucrose gradient centrifugation (Smith et al., 1976). To test this possibility, 0.1% glutaraldehyde was used to cross-link [^{35}S]Met-tRNA$_f$ to 40S^N in the absence or presence of 5 mM dG6P before analysis on sucrose gradients (Hunter et al., 1977). If the sugars stabilize

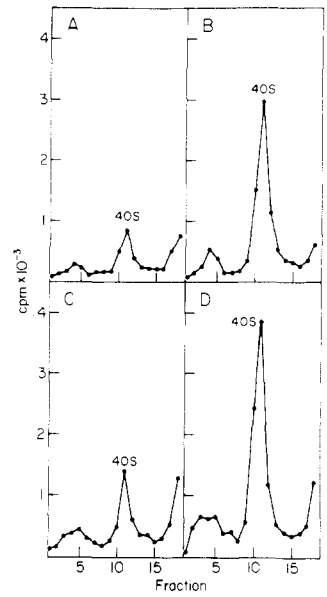

FIGURE 7: The stimulation of Met-tRNA$_f$ binding by 2-deoxyglucose 6-phosphate in the presence of glutaraldehyde. [^{35}S]Met-tRNA$_f$ binding to isolated ribosomes was performed as described in Figure 6 and Experimental Procedures, except that the buffer used to stop the reactions in C and D contained 0.1% glutaraldehyde. These mixtures were allowed to stand on ice for 5 min to ensure cross-linking before analysis on sucrose gradients. (A and C) No additions; (B and D) 5 mM 2-deoxyglucose 6-phosphate.

ternary complex association with 40S^N, the ratio of [^{35}S]-Met-tRNA$_f$ bound in the presence of the sugar to that bound in its absence would decrease in the presence of the cross-linking reagent. However, dG6P stimulated [^{35}S]Met-tRNA$_f$ binding equally, whether or not the assays were treated with 0.1% glutaraldehyde (Figure 7). Cross-linking with 1% glutaraldehyde did not increase the amount of Met-tRNA$_f$ bound in either the presence or absence of the sugar (data not shown). Therefore, phosphorylated sugars do not stimulate Met-tRNA$_f$ binding by stabilizing the association of the ternary complex with 40S^N to sucrose gradient centrifugation.

Phosphorylated Sugars Do Not Appear to Associate Allosterically with 40S^N. The possibility that the sugars directly stimulate Met-tRNA$_f$ binding by allosterically associating with 40S^N was investigated using ^{32}P-labeled and ^{3}H-labeled G6P. The labeled sugar was incubated with isolated ribosomes under conditions for Met-tRNA$_f$ binding and then analyzed by either sucrose gradient centrifugation or nitrocellulose filter binding. Regardless of the label used, G6P was not bound to polysomes, monosomes, or free 40S or 60S subunits displayed by sucrose gradients, nor was it bound to nitrocellulose filters by isolated ribosomes incubated as described under Experimental Procedures. Thus, we have not been able to show that phosphorylated sugars allosterically associate with 40S^N to stimulate Met-tRNA$_f$ binding.

Discussion

We have previously discussed three possible mechanisms to explain the stimulatory effect of some phosphorylated sugars on protein synthesis and Met-tRNA$_f$ binding in various mammalian cell extracts (Lenz et al., 1978). The results of further studies are reported here, and, although none of the three mechanisms can yet be completely discarded, the data presented support only one of these, a direct effect on Met-tRNA$_f$ binding.

Experiments in which protein synthesis was measured in mixtures reconstituted from ribosomes and S150s preincubated under different conditions established three points: (1) the

diminished activity of incubated extracts for protein synthesis and Met-tRNA$_f$ binding is not due to accumulation of an inhibitory metabolite in the S150, but is instead due to some change occurring at the level of the ribosome; (2) this inhibitory event is largely prevented from occurring in ribosomes incubated with a phosphorylated sugar; and (3) the inhibition can be reversed or circumvented when the sugar is added to ribosomes previously incubated in its absence. However, these experiments do not eliminate the possibility that an inhibitor is produced and accumulated only at the ribosome level and that the sugars prevent or reverse its accumulation.

The possibility that phosphorylated sugars affect protein kinases was first suggested by the observation that the sugars and 2AP do not potentiate each other's effects on protein synthesis. The initial hypothesis was that they work via similar mechanisms and the sugars therefore inhibit protein kinase activities. Gel filtration of lysates removes low molecular weight compounds, including ATP and phosphorylated sugars. Thus the ATP concentration is significantly lower in incubations with gel-filtered lysate than with nonfiltered lysate. However, the concentration of phosphorylated sugars is substantially greater in incubations containing gel-filtered lysate and sugars relative to incubations with nonfiltered lysate, since the sugar concentrations added are ten times those found in reticulocyte lysates by Lennon et al. (1977). The addition of phosphorylated sugars to nonfiltered lysates does not stimulate protein synthesis (see Table I, experiment no. 2). Thus, the presence of high concentrations of phosphorylated sugars and low concentrations of ATP determine whether protein synthesis is stimulated. These results suggested that the sugars inhibit protein kinases by competing with ATP. This possibility was discarded, however, when the sugars were found to inhibit a cAMP-dependent protein kinase noncompetitively with respect to ATP.

A stimulatory effect of phosphorylated sugars was also observed in nonfiltered lysates when protein synthesis was inhibited by partial activation of endogenous HCR at low hemin concentrations. However, the evidence presented shows that the sugars neither inhibit the phosphorylation of eIF-2 by HCR nor prevent HCR inactivation of Met-tRNA$_f$ binding. In other words, they do not inhibit HCR activity.

The most direct evidence for an effect on protein kinases is that the sugars inhibit a cAMP-dependent protein kinase activity in the preparation of HCR used for these studies. Inhibition by fructose 1,6-bis(phosphate) of a similar enzyme from pig liver has recently been reported (Berglund et al., 1977). Datta et al. (1977a,b) have proposed that activation of HCR involves the action of a cAMP-dependent protein kinase on the proinhibitor. According to their model, hemin prevents activation of this kinase by cAMP noncompetitively (Datta et al., 1978). In the absence of hemin, the Met-tRNA$_f$ binding activity of IF is rapidly lost. A similar mechanism could be envisioned for the phosphorylated sugars. Although both protein synthesis and Met-tRNA$_f$ binding to 40S^N in hemin-supplemented lysates decrease during incubation (Lenz et al., 1978), the Met-tRNA$_f$ binding activity of IF from incubated lysates does not. The conclusion is that a mechanism of action for phosphorylated sugars similar to that proposed for hemin is unlikely.

We have not yet examined the possibility that the sugars inhibit the phosphorylation of a 40S ribosomal subunit structural protein. A protein with mol wt of approximately 31 000 may be involved in the regulation of protein synthesis (Kramer et al., 1977). This protein is phosphorylated in vivo in rabbit reticulocytes (Cawthon et al., 1974; Traugh & Porter, 1976), and the degree of phosphorylation is increased by raising cellular cAMP levels (Cawthon et al., 1974). In vitro phosphorylation of the same or a similar protein results in an inhibition of Met-tRNA$_f$ binding to 40S^N, even when eIF-2 is not phosphorylated (Kramer et al., 1977).

The inhibition of a cAMP-dependent protein kinase by phosphorylated sugars is probably not involved in their effect on protein synthesis. This is supported by the fact that sugars stimulate Met-tRNA$_f$ binding to isolated ribosomes, while 2-aminopurine does not, indicating that inhibition of a protein kinase cannot be the explanation for this stimulation. This effect is also not due to stabilization of Met-tRNA$_f$ on 40S^N to sucrose gradient centrifugation, since cross-linking with glutaraldehyde increases Met-tRNA$_f$ binding by the same proportion both with and without the sugar. It was shown previously that phosphorylated sugars do not prevent either Met-tRNA$_f$ deacylation or GTP hydrolysis (Lenz et al., 1978).

We were unable to show association of radioactively labeled glucose 6-phosphate with either ribosomal subunits or ribosomes, but we cannot exclude that such association may occur. Binding of the ternary complex eIF-2/GTP/Met-tRNA$_f$ to 40S^N may be at least partially unstable to sucrose gradient centrifugation (Smith et al., 1976). The possibility exists that this may also be true for a phosphorylated sugars/40S complex. No association of labeled sugar with isolated ribosomes in a nitrocellulose filter binding assay could be detected. However, a complex of one molecule of G6P per 40S^N may not have been detected because of the low specific activity of G6P used. Therefore, allosteric association of phosphorylated sugars with 40S^N remains as a possible explanation for their stimulation of protein synthesis. We have not yet investigated alternative allosteric binding sites for the sugars. Although they do not stimulate ternary complex formation (Lenz et al., 1978), it is still possible that the sugars bind to eIF-2 and increase the affinity of the ternary complex for the 40S subunit.

Although the mechanism by which phosphorylated sugars stimulate protein synthesis in mammalian cell extracts remains to be determined, a number of possible explanations can be ruled out. They do not prevent or reverse the accumulation of an inhibitory metabolite in the supernatant, nor do they stimulate a phosphatase activity. The sugars do not inhibit either HCR activity or activation. They do inhibit a cAMP-dependent protein kinase, but the relationship of this effect to their stimulation of protein synthesis is uncertain. They do not stabilize Met-tRNA$_f$ binding on 40S^N, but instead appear to directly stimulate formation of the Met-tRNA$_f$/40S^N complex without affecting ternary complex formation. The data presented support the possibility that phosphorylated sugars interact with one of the components involved in formation of the 40S initiation complex, although the identity of this component and the mechanism of stimulation remain to be elucidated.

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Reaction of Bisulfite with the 5-Hydroxymethyl Group in Pyrimidines and in Phage DNAs†

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ABSTRACT: 5-Hydroxymethylcytosine reacted with bisulfite and, instead of undergoing usual deamination process, gave cytosine 5-methylenesulfonate as the product. The conversion was rapid and quantitative, and the optimum pH was 4.5. The product was isolated as crystals and characterized. Cytosine 5-methylenesulfonate was only very slowly deaminated by treatment with bisulfite. 5-Hydroxymethyl-2′-deoxycytidine 5′-phosphate reacted with bisulfite in the same way as 5-hydroxymethylcytosine. Residues of 5-hydroxymethylcytosine in native as well as denatured T2 DNA were convertible to those of cytosine 5-methylenesulfonate by treatment of the DNA with bisulfite. While it is known that the 5-hydroxymethyl groups of T-even bacteriophage DNA can be enzymatically glucosylated, this observation offers chemical evidence that the 5-hydroxymethyl groups in DNA are situated in such a way that they can readily react with external agents. 5-Hydroxymethyluracil gave uracil 5-methylenesulfonate on treatment with bisulfite. This reaction was much slower than that of 5-hydroxymethylcytosine, and the optimum pH was between 6 and 7.

Bisulfite adds reversibly to the 5,6 double bond of cytosine and uracil, and the cytosine–bisulfite adduct undergoes easy deamination to give the uracil–bisulfite adduct. The bisulfite modification of these pyrimidine bases has been widely used in nucleic acid research (Hayatsu, 1976; Shapiro, 1977).

There are many naturally occurring, as well as synthetic, 5-substituted pyrimidine nucleoside derivatives, and they are subjects of current, chemical and biochemical studies (Bradshaw & Hutchinson, 1977). We have been investigating the reactivities of 5-substituted pyrimidine nucleosides to bisulfite (Shiragami et al., 1975; Hayatsu et al., 1975) and have now encountered an abnormal case for 5-hydroxymethylcytosine (hm^5Cyt)[1] and 5-hydroxymethyluracil

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