Effect of Nucleoside Triphosphate and Magnesium Ion Concentration on the Stability and Function of Rat Liver Polysomes *in vitro*

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The effects of different concentrations of ATP, GTP, UTP and CTP on polysome stability and function in a cell-free protein-synthesizing system prepared from rat liver were studied. Increasing the concentration of ATP in the incubation medium to 15 mM resulted in progressive disaggregation of the polysomes; at ATP concentrations above 2 mM their capacity to incorporate amino acids into peptide chains diminished. The same disaggregation phenomenon could be produced by incubating polysomes in a buffered medium containing 5 mM-Mg²⁺ and increasing concentrations of ATP. Although the disaggregating action of ATP could be prevented by increasing Mg²⁺ concentration, the amino acid incorporation in the cell-free protein-synthesizing system remained impaired. The effects of different concentrations of GTP, UTP and CTP on polysome stability were similar to those of ATP. Increasing the concentrations of each nucleoside triphosphate also inhibited the hydrolysis of GTP in the cell-free protein-synthesizing system.

Amino acids attached to t-RNA* can be incorporated into protein by polysome preparations incubated in the presence of aminoacyltransferases and GTP (Gasior & Moldave, 1965). Since the amino acids do not need to be activated in this system, there is no requirement for ATP. We have observed that addition of ATP to this system causes decreased incorporation of amino acids from t-RNA. This effect is due to disaggregation of polysomes. Other nucleoside triphosphates have a similar action. Further studies with protein-synthesizing systems prepared from liver polysomes show that the synthesis of peptides *in vitro* from free amino acids and the accompanying hydrolysis of GTP are both affected by variations in the ATP or Mg²⁺ content of the medium.

EXPERIMENTAL

Materials. A synthetic mixture of ¹⁴C-labelled L-amino acids (1 µC/mg. of uniformly labelled amino acid mixture) and L-[U-¹⁴C]leucine (specific radioactivity 250 mc/m-mole) were obtained from the New England Nuclear Corp. (Boston, Mass., U.S.A.). Rat liver t-RNA stripped of amino acids was purchased from General Biochemicals Corp. (Chagrin Falls, Ohio, U.S.A.). International Chemical and Nuclear Corp. (Boston, Mass., U.S.A.) provided [γ -³²P]GTP (specific radioactivity 2.2 mc/µmole), which was further purified by chromatography on DEAE-cellulose (Gordon, 1967). Other chemicals were reagent grade. Most of the media

used were made up in TKM buffer (25 mM-KCl-5 mM-MgCl₂-50 mM-tris-HCl, pH 7.6).

For the preparation of radioactive aminoacyl-t-RNA, activating enzymes prepared in the form of the pH 5 fraction from rat liver cell sap were allowed to react with the ¹⁴C-labelled amino acid mixture in the presence of 10 mM-ATP, 10 mM-MgCl₂, 10 mM-GSH and 0.1 M-tris-HCl buffer, pH 7.6. The aminoacyl-t-RNA was then isolated by the method of Moldave (1963). The final product had a specific radioactivity of 350 000 counts/min./mg. of RNA.

Preparation of liver polysomes and enzymes. Starved male albino rats weighing 150 g. were used for preparing polysomes (C-ribosomes) by the method of Wettstein, Staehelin & Noll (1963). Livers were homogenized in 2 vol. of 0.375 M-sucrose made up in TKM buffer. The post-mitochondrial supernatant fraction was made 1% (w/v) with respect to sodium deoxycholate, and the C-ribosome fraction was obtained by layering a portion of this solution on to a discontinuous gradient consisting of 0.5 M-sucrose and 2 M-sucrose, each made up in TKM buffer (Fleck, Shepherd & Munro, 1965). The polysome pellets were sedimented by centrifugation for 4 hr. at 105 000 g in rotor 50 of the Spinco L2 ultracentrifuge. The pellet was washed twice with TKM buffer and gently suspended in 0.3 ml. of the same buffer.

A mixture of amino acid-activating enzymes and aminoacyltransferases was obtained from the post-mitochondrial supernatant by first removing the microsomes by spinning them down at 105 000 g for 3 hr. The microsome-free cell sap was then passed through Sephadex G-25 to remove inhibitors of protein synthesis (Munro, Jackson & Korner, 1964) and subsequently treated with protamine sulphate to remove endogenous t-RNA, the enzyme mixture being finally isolated by (NH₄)₂SO₄ fractionation (Lin, Mosteller & Hardesty, 1966). For incorporation of amino acids from

* Abbreviation: t-RNA, transfer RNA.

aminoacyl-t-RNA into polysomes, unfractionated aminoacyltransferases free of activating enzymes were prepared from the cell sap after treatment with Sephadex G-25. The activating enzymes were removed by precipitation at pH 5 and a mixture of transferases I and II was obtained from the supernatant fraction as described by Gasior & Moldave (1965).

Incubation medium. The cell-free protein-synthesizing system for incorporation of free amino acids contained 50 μ -moles of tris-HCl buffer, pH 7.6, 80 μ -moles of NH_4Cl , 4 μ -moles of GSH, 1 μ -mole of GTP, 0.05 μ -mole of a mixture of 19 amino acids (without leucine), 0.02 μ -mole of L-[U- ^{14}C]leucine (0.5 μC), 25 μg . of stripped t-RNA, 500 μg . of mixed activating-enzyme and transferase protein and 500 μg . of polysome protein in a final volume of 1 ml. Incubations were carried out for 15 min. at 37°, and radioactivity incorporated into protein was measured in the residue from treatment with hot trichloroacetic acid (Baliga, Pronczuk & Munro, 1968). Radioactivity was measured with a Nuclear-Chicago gas-flow end-window counter.

The incubation mixture for transfer of aminoacyl-t-RNA to polysomes contained 50 μ -moles of tris-HCl buffer, pH 7.6, 0.2 μ -mole of GTP, 80 μ -moles of NH_4Cl , 5 μ -moles of MgCl_2 , 4 μ -moles of GSH, 100 μg . of transferase protein, 500 μg . of polysome protein and 20000 counts/min. of ^{14}C -labelled aminoacyl-t-RNA in a final volume of 1 ml. After incubation for 5 min. at 37°, uptake of ^{14}C into protein was measured as described above.

Hydrolysis of GTP by the protein-synthesizing system was determined by measuring radioactive inorganic phosphorus released from [γ - ^{32}P]GTP as described by Conway & Lipmann (1964). The reaction mixture was similar to that used for the transfer of ^{14}C -labelled amino acids to peptides, except that only unlabelled aminoacyl-t-RNA was present and the GTP used was labelled with ^{32}P in the terminal phosphate (11000 counts/min./ml. of reaction mixture). The reaction mixture was incubated at 37° for 30 min., and the reaction was terminated by adding an equal amount of 0.2 M-silicotungstic acid in 0.02 N- H_2SO_4 and 1 ml. of 1 M-

potassium phosphate buffer, pH 6.8, as carrier. The extent of GTP hydrolysis was measured by extraction of the released inorganic phosphate as the phosphomolybdate complex into 2-methylpropan-1-ol-benzene; the extract was counted for radioactivity with the Nuclear-Chicago gas-flow counter.

Polysome profiles. The reaction mixture for polysome profiles was first diluted with 0.01 M-tris-HCl buffer, pH 7.6, and then layered over a sucrose gradient (10–40%, w/v). The gradients were centrifuged for 70 min. in the SW 50 rotor of a Spinco L2 ultracentrifuge at 38000 rev./min. and the extinction profile at 260 m μ was recorded automatically with a Gilford model 2000 spectrophotometer. Radioactivity on the gradient was measured on 12-drop fractions; the protein was precipitated with carrier albumin, and the hot-trichloroacetic acid-insoluble precipitate was collected on Millipore filters for counting (Baliga *et al.* 1968).

RESULTS AND DISCUSSION

We have previously reported that in a system *in vitro* consisting of liver polysomes, amino acid-activating and transferring enzymes and GTP, which incorporates free amino acids into protein, the polysomes undergo extensive breakdown to monomer ribosomes and oligosomes as incubation progresses (Pronczuk, Baliga, Triant & Munro, 1968). This polysome disintegration occurs even when peptide-bond formation is restricted by amino acid starvation (Baliga *et al.* 1968). While studying an incorporation system consisting of polysomes, aminoacyltransferases and GTP, which transfers ^{14}C -labelled amino acids from t-RNA to peptide chains, we observed that the polysomes were much more stable during incubation in this medium. Fig. 1 shows that the polysome profile (Fig. 1b) after

Fig. 1. Sucrose gradients showing polysome profiles and amino acid incorporation after the addition of 5 mM-ATP to a cell-free system for incorporating amino acids from ^{14}C -labelled aminoacyl-t-RNA into protein. The incubation mixture contained 5 mM- Mg^{2+} , rat liver polysomes, a mixed transferase preparation and GTP, as described in the Experimental section. The polysomes were resolved on a 10–40% (w/v) sucrose gradient and recorded by E_{260} . (a) The polysome profile (—) at the start of incubation. (b) The profile (—) at the end of incubation without ATP and the amount of radioactivity incorporated into peptide chains along the gradient (----). (c) The profile (—) and uptake of radioactivity (----) after incubation with 5 mM-ATP added to the medium.

Fig. 2. Sucrose gradients showing the effect of increasing concentrations of ATP on liver polysome profiles. The upper four diagrams (a-d) show the effect of increasing concentrations of ATP on polysomes, with a fixed concentration (5 mM) of Mg^{2+} , during incubation for 15 min. in a cell-free system for incorporation of free amino acids into peptides. (a) 1 mM-ATP; (b) 2 mM-ATP; (c) 5 mM-ATP; (d) 15 mM-ATP. The incubation mixture contained liver polysomes, amino acid-activating and -transferring enzymes, t-RNA, amino acids, [^{14}C]leucine, GTP, 5 mM- Mg^{2+} and the various concentrations of ATP. Uptake of ^{14}C into peptide chains is shown in each diagram as counts/min./ml. of incubation mixture. The lower four diagrams (e-h) show the effect of increasing concentrations of ATP on liver polysomes during incubation for 15 min. in a medium containing 5 mM- Mg^{2+} , 80 mM- NH_4Cl and 50 mM-tris-HCl buffer, pH 7.6. (e) 1 mM-ATP; (f) 2 mM-ATP; (g) 5 mM-ATP; (h) 15 mM-ATP.

incubation for 5 min. in this system was similar to the profile (Fig. 1a) at the start of incubation. Fractions collected from various points on the polysome gradient after incubation were found to have considerable ^{14}C radioactivity, mainly confined to the heavier polysome region. This system for transferring ^{14}C -labelled amino acids from t-RNA to peptides differs from the system required for incorporation of free amino acids into peptides in not requiring activating enzymes or ATP. Fig. 1(c) shows that when ATP (5 mM) was added complete disaggregation of the polysomes during incubation and accumulation of monosomes occurred. ATP also inhibited ^{14}C incorporation into peptides at all points on the polysome gradient.

This finding led us to examine the effect of ATP concentration on polysome stability in the complete system for incorporation of free amino acids. This system included activating enzymes, ATP and free amino acids, as well as the components of the above-mentioned system. Figs. 2(a)-2(d) show that, with increasing concentrations of ATP (1, 2, 5 and 15 mM) in the complete system, the polysome profile became progressively degraded at the end of 15 min. of incu-

bation. The effect of ATP concentration on the uptake of [^{14}C]leucine by the system was also examined; total radioactivity incorporated into protein/ml. of reaction mixture is shown on each Figure. Maximum uptake of [^{14}C]leucine occurred at 2 mM-ATP, although the polysome pattern was slightly degraded compared with that at 1 mM-ATP. This is to be expected, since the higher concentration of ATP may allow some ribosomes to complete translation and accumulate in the monomer ribosome pool. With 15 mM-ATP essentially no radioactivity was incorporated into protein and no peaks could be seen on the polysome profile; it is possible that the large amount of ATP at the top of the gradient may have obscured a monomer ribosome peak. In experiments not shown in the Figures, dialysis at the end of incubation failed to restore the polysome profile.

In view of these findings, we tested the effect of ATP on polysome structure during incubation in a buffered medium only. Figs. 2(e)-2(h) show that incubation of polysomes alone in the medium (5 mM-magnesium chloride-80 mM-ammonium chloride-50 mM-tris-hydrochloric acid buffer, pH 7.6) with

Fig. 3. Sucrose gradients showing the effects of increasing concentrations of Mg^{2+} on liver polysomes. The polysome profiles were obtained after incubation in a cell-free system for incorporating free amino acids into peptide chains, as described in the legend to Fig. 2. The concentration of ATP was kept constant at 5 mM, and Mg^{2+} was increased. (a) 1 mM- Mg^{2+} ; (b) 2 mM- Mg^{2+} ; (c) 5 mM- Mg^{2+} ; (d) 15 mM- Mg^{2+} . Uptake of [^{14}C]leucine into peptide chains during incubation is shown on each diagram as counts/min./ml. of incubation mixture.

ATP for 15 min. resulted in profiles showing increasing disaggregation as the concentration of ATP was raised from 1 to 15 mM. Consequently, this action of ATP is not necessarily related to its function in protein synthesis, since no amino acids, activating enzymes or transferring enzymes were present.

Korner (1961) demonstrated that incorporation of amino acids in cell-free systems is affected by the balance between ATP and Mg^{2+} concentrations, and that the inhibitory action of high concentrations of ATP on protein synthesis can be partially reversed by increasing the Mg^{2+} concentration. Accordingly, we examined the effect of changes in Mg^{2+} concentration on incorporation of amino acids and polysome profiles with a fixed 5 mM concentration of ATP in the complete cell-free system for incorporation of free amino acids into protein. Fig. 3 shows the effect of increments of Mg^{2+} from 1 to 15 mM. With increasing concentrations of Mg^{2+} , the polysome pattern was progressively better preserved. Incorporation of amino acids into protein was maximal at 5 mM- Mg^{2+} , but the profile was best retained at 15 mM- Mg^{2+} (Fig. 3). In one experiment (not shown) the system was incubated with 5 mM- Mg^{2+} and 15 mM-ATP, and the concentration of Mg^{2+} was then raised to 15 mM at the end of incubation. This resulted in clumping of the dissociated ribosomes, but failed to restore the polysome profile.

It is clear from these findings that the concentrations of ATP and Mg^{2+} in the medium are critical, not only for amino acid incorporation (Korner, 1961), but also for polysome stability, although the conditions for maximum polysome preservation are not necessarily those for high incorporation. Under our conditions of incubation, 2 mM-ATP and 5 mM- Mg^{2+} appeared to provide optimum conditions for the incorporation of free amino acids (Fig. 2).

Fig. 4. Sucrose gradients showing the effect of increasing concentrations of GTP on polysome profiles during incubation of rat liver polysomes for 15 min. in a buffer containing 5 mM- Mg^{2+} . Concentrations of 1 mM (—), 5 mM (---) and 15 mM (.....) nucleoside triphosphate were used.

Since the effect of ATP on polysome stability is a chemical one, the effects of other nucleoside triphosphates were examined. Figs. 4, 5 and 6 show the effect on rat liver polysomes of incubation with 1 mM, 5 mM and 15 mM concentrations of GTP, UTP and CTP respectively in the presence of 5 mM- Mg^{2+} and 80 mM-ammonium chloride in the buffer. In each case, progressive degradation was observed when the nucleoside triphosphate concentration was increased. In the liver of the well-fed rat

Fig. 5. Sucrose gradients showing the effect of increasing concentrations of UTP on polysome profiles during incubation of rat liver polysomes for 15 min. in a buffer containing 5 mM-Mg²⁺. Concentrations of 1 mM (—), 5 mM (---) and 15 mM (.....) nucleoside triphosphate were used.

Fig. 6. Sucrose density gradients showing the effect of increasing concentrations of CTP on polysome profiles during incubation of rat liver polysomes for 15 min. in a buffer containing 5 mM-Mg²⁺. Concentrations of 1 mM (—), 5 mM (---) and 15 mM (.....) nucleoside triphosphate were used.

Table 1. *Effect of addition of ATP, CTP or UTP on GTP hydrolysis in a cell-free protein-synthesizing system*

The incubation mixture contained rat liver polysomes, aminoacyl-t-RNA, transferases and [γ -³²P]GTP, as described in the Experimental section. Other nucleoside triphosphates were added at either 0.2 mM or 5 mM concentration. Incubation was continued for 30 min., and the ³²P released as inorganic phosphate was measured.

Nucleoside triphosphate added	Release of ³² P from GTP (counts/min./ml. of reaction mixture)	
	0.2 mM-Nucleoside triphosphate	5 mM-Nucleoside triphosphate
None	1050	1110
ATP	830	210
UTP	880	460
CTP	870	390
System minus enzymes	220	280

ATP concentration does not exceed 2 mM (Clark, Goodlad, Chisholm & Munro, 1960) and the other nucleoside triphosphates are present in lower concentrations (Chambon & Mandel, 1959). The concentrations of nucleoside triphosphates in the living cell are therefore consistent with the optimum conditions for polysome stability.

Since incorporation of ¹⁴C-labelled amino acids into protein was found to be sensitive to the concentration of ATP in the incubation medium, the effect of nucleoside triphosphate concentration on the ribosome-dependent release of inorganic phosphate from GTP (Conway & Lipmann, 1964) was also examined. Table 1 shows that addition of 0.2 mM-ATP, -CTP or -UTP to the incubation medium had a slight depressing effect on the capacity of the polysome system to hydrolyse GTP in the presence of aminoacyl-t-RNA and transferases. However, addition of 5 mM concentrations of these nucleoside triphosphates decreased GTP hydrolysis to the value obtained without transferases in the medium. This experiment provides further evidence that the disruption of polysomes caused by high concentrations of nucleoside triphosphates abolishes protein synthesis, since hydrolysis of GTP is a concomitant of peptide-bond formation (Conway & Lipmann, 1964). This is in agreement with the finding of Nishizuka & Lipmann (1966) that hydrolysis of GTP requires intact polysomes.

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