Experimental Conditions Affecting Ribonucleic Acid Polymerase in Isolated Rat Liver Nuclei

EFFECT OF NUCLEOSIDE TRIPHOSPHATE CONCENTRATION, TEMPERATURE, AMMONIUM SULPHATE AND HEPARIN

By F. NOVELLO AND F. STIRPE

Istituto di Patologia generale dell'Università di Bologna, 40126 Bologna, Italy

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1. The conditions affecting the activity of RNA polymerase in isolated rat liver nuclei were studied with Mg^{2+} or Mn^{2+} as activating ions. 2. The enzyme assayed with Mg^{2+} and at low ionic strength is saturated by a lower concentration of nucleotide substrates than if assayed with Mn^{2+} at low ionic strength or with either ion at high ionic strength. 3. At low and at high ionic strength the incorporation of AMP is affected in a similar way by variations in the temperature of incubation. Pre-incubation at 37° impairs the AMP incorporation. 4. Heparin stimulates the RNA polymerase activity in the presence of Mn^{2+} . 5. Both ammonium sulphate and heparin 'restart' the reaction if added after 15 min., the effect being more marked with ammonium sulphate than with heparin, and also more marked in the presence of Mn^{2+} than of Mg^{2+} . 6. α -Amanitin abolishes the effect of ammonium sulphate and of heparin.

The activity of RNA polymerase (nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6) of isolated nuclei or of 'aggregate' enzyme preparations is stimulated if the ionic strength of the reaction mixture is raised by means of high concentrations of a salt, usually ammonium sulphate (Goldberg, 1961; Widnell & Tata, 1966). A stimulatory effect is observed also on addition of heparin (Bresnick, 1966) or of other polyanions such as polyvinyl sulphate and polyethylene sulphonate (Bannai & Terayama, 1967; Chambon, Ramuz, Mandel & Doly, 1968). On the other hand, high ionic strength and heparin are inhibitory for RNA polymerase purified from bacteria or solubilized from mammalian sources (Ballard & Williams-Ashman, 1964; Ramuz, Doly, Mandel & Chambon, 1965; Walter, Zillig, Palm & Fuchs, 1967; Doerfler, Zillig, Fuchs & Albers, 1962). In the experiments reported below the effects of ammonium sulphate and of heparin were examined and compared under various experimental conditions, and with respect to the effect of α -amanitin, which inhibits the Mn^{2+} /ammonium sulphate-activated RNA polymerase (Stirpe & Fiume, 1967).

EXPERIMENTAL

Chemicals. ATP, CTP, UTP and GTP (all as sodium salts) and DNA (calf thymus) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; [$8\text{-}^{14}\text{C}$]ATP (30 mc/

m-mole) was purchased from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.; [$6\text{-}^{14}\text{C}$]orotic acid (44.5 mc/m-mole) was from The Radiochemical Centre, Amersham, Bucks.; heparin (sodium salt) and chondroitin sulphate (sodium salt) were from Mann Research Laboratories Inc., New York, N.Y., U.S.A. All other chemicals were of analytical grade. α -Amanitin was a kind gift from Professor T. Wieland to Dr L. Fiume of this Institute; amylosulphate and amylopectin sulphate were gifts from Professor C. Cessi, Cagliari, Italy.

Isolation of nuclei. This was performed as described by Widnell & Tata (1964a) from pooled livers from two to four normal rats of the Wistar strain.

Assay of RNA polymerase. The activity of RNA polymerase was determined in the presence of Mg^{2+} or of Mn^{2+} with or without the addition of $(\text{NH}_4)_2\text{SO}_4$, basically as described by Widnell & Tata (1966), with the modifications described for each experiment (see the Results section). After the incubation the reaction was stopped with 5 ml. of cold 0.5M-HClO_4 . In some experiments after centrifugation the acid-insoluble material was washed twice with 3 ml. of cold 0.2M-HClO_4 and once with 3 ml. of ethanol-diethyl ether (3:1, v/v). The precipitate was dried and dissolved in 0.5 ml. of 98% (w/v) formic acid. Samples (0.4 ml.) were mixed in counting vials with 5 ml. of ethylene glycol monomethyl ether and 10 ml. of scintillation fluid [0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole in toluene]. The samples were counted in a Nuclear-Chicago Mark I scintillation counter with an efficiency of about 60%. In other experiments after precipitation with HClO_4 the reaction mixture was filtered through Millipore HA filters (25 mm. diam., 0.45 μm .) and the material on the filters washed twice with 3 ml. of cold 0.2M-HClO_4 . The filters were transferred to counting vials,

10 ml. of scintillation fluid was added and the samples were counted as described above, with a counting efficiency of about 80%.

Release of radioactivity from nuclear RNA. Rats were injected intraperitoneally with [6- ^{14}C]orotic acid (10 μC /100 g. body wt.) and the animals were killed after 20 min. Liver nuclei were isolated and incubated in the same media used for the assay of RNA polymerase, except that ATP was entirely unlabelled. Other modifications are described in Table 2. At various times 5 ml. of 0.5 M-HClO₄ was added; after centrifugation the radioactivity was measured in samples of the supernatant.

Determination of DNA. This was performed on samples of nuclear suspensions by the method of Burton (1956), with calf thymus DNA as a standard.

RESULTS

Assay of RNA polymerase at low and at high ionic strength. The RNA polymerase activity assayed in the Mn^{2+} /ammonium sulphate-stimulated system described by Widnell & Tata (1966) was about 50% higher if the concentrations of nucleotide substrates were threefold higher than those used by these investigators (1908 versus 1260 pmoles of AMP incorporated/45 min./mg. of DNA, average of eight experiments). While this paper was in preparation, similar results were reported by Marchok & Wolff (1968). The incorporation proceeded for at least 20 min., approaching linearity only at the highest concentration of nucleotides and provided that the 15 min. preincubation was omitted. No enhancement, and sometimes a decrease, of RNA polymerase activity was observed on raising the concentration of nucleotides in the Mg^{2+} -activated system at low ionic strength described by Widnell & Tata (1966).

To ascertain whether the requirement for a higher concentration of substrates was due to the high ionic strength or to the different ion (Mn^{2+} or Mg^{2+}) the experiments were repeated with these two ions at low and at high ionic strength. In the presence of ammonium sulphate the incorporation was enhanced by increasing the concentration of nucleotides with either Mn^{2+} or Mg^{2+} (Fig. 1a). In most experiments (see e.g. Fig. 5a) maximal stimulation was observed at 6–8% saturation of ammonium sulphate. At low ionic strength (Fig. 1b) the Mg^{2+} -activated system appeared to be saturated by nucleotides at the concentration used by Widnell & Tata (1966), whereas in the presence of Mn^{2+} the reaction rate continued to increase linearly with increased concentrations of nucleotides, to exceed the rate reached in the presence of Mg^{2+} (Fig. 1b).

In the absence of ammonium sulphate the [^{14}C]AMP incorporation in our system was initially faster at 37° than at 25° (Fig. 2a) both with Mg^{2+} and

Fig. 1. Effect of nucleotide triphosphate concentration on RNA polymerase activity. The reaction mixtures contained, in a final volume of 0.5 ml.: 50 μmoles of tris-HCl buffer, pH 7.5; 2.5 μmoles of MgCl_2 or 2 μmoles of MnCl_2 ; concentration 1 of nucleotides corresponded to that used by Widnell & Tata (1966) (0.3 μmole each of CTP, GTP and UTP, 0.015 μmole of non-radioactive ATP and 0.005 μmole of [^{14}C]ATP); other concentrations were multiples or fractions of concentration 1; 0.1 ml. of nuclear suspension; $(\text{NH}_4)_2\text{SO}_4$, when present, was at 10% saturation (final concn.). The reaction was carried out at 37° for 10 min. in the presence (a) or in the absence (b) of $(\text{NH}_4)_2\text{SO}_4$, with MgCl_2 (●) or MnCl_2 (▲).

Mn^{2+} , but the reaction proceeded for a longer time at 25° in either system. The better linearity observed at 25° than at 37° without ammonium sulphate was interpreted by MacGregor & Mahler (1967) as being due to protection of the RNA formed, since 75–80% of the incorporation at 37° occurred at 23°. On the other hand, in the presence of ammonium sulphate the ratio between the incorporation at 37° and that at 25° at 10 min. was similar to the ratio observed at low ionic strength (Fig. 2b). Since destruction of

Fig. 2. Effect of temperature on RNA polymerase in the presence and in the absence of $(NH_4)_2SO_4$. The reaction was assayed: (a) at low ionic strength in the presence of Mg^{2+} as described by Widnell & Tata (1966) at 25° (○) and at 37° (●) and in the presence of Mn^{2+} (with a different preparation of nuclei) as described in Fig. 1 (nucleotide concentration 3) at 25° (Δ) and at 37° (▲); (b) in the presence of $(NH_4)_2SO_4$ at 7% saturation with the system described in Fig. 1 (nucleotide concentration 3) with Mg^{2+} at 25° (○) and at 37° (●) or with Mn^{2+} at 25° (Δ) and at 37° (▲).

RNA is decreased by ammonium sulphate (Steiner & King, 1966; Homoki, Lukács & Sekeris, 1967; see Table 2) the possibility was considered that factors

Table 1. Effect of preincubation on the Mg^{2+} -activated RNA polymerase

Experimental conditions were as described by Widnell & Tata (1966). The reaction mixture contained, in a final volume of 0.5 ml.: 50 μ moles of tris-HCl buffer, pH 8.5, 2.5 μ moles of $MgCl_2$, 10 μ moles of cysteine, 3 μ moles of NaF, 0.3 μ mole each of GTP, CTP and UTP, 0.015 μ mole of non-radioactive ATP, 0.005 μ mole of $[8-^{14}C]ATP$ and 0.1 ml. of nuclear suspension. In the preincubation experiment nuclei were preincubated at 37° in the same medium used for assay. Substances omitted were added at the end of preincubation and the reaction was stopped after 10 min.

Preincubation	RNA polymerase activity (pmoles of AMP incorporated/10 min./mg. of DNA)	
	Expt. 1	Expt. 2
None	456	420
15 min.		
— all nucleotides, — Mg^{2+}	—	147
— all nucleotides	158	150
— ATP (labelled and unlabelled)	135	113
— $[^{14}C]ATP$	63	40

Fig. 3. Stimulation by heparin of Mn^{2+} -activated RNA polymerase reaction at different substrate concentrations. Experimental conditions were as described for Fig. 1(b), with Mn^{2+} . Δ, Concentration of nucleotides was as in Widnell & Tata (1966); ▲, concentration of nucleotides was threefold higher.

other than degradation of RNA could affect the time-course of the reaction at 37°. When nucleotides, with or without Mg^{2+} , were added to nuclei preincubated for 15 min. at 37° the incorporation of AMP was about one-third of that observed in non-preincubated samples, and fell to one-tenth if nuclei were preincubated with the four nucleotides

Fig. 4. Effect of heparin and $(\text{NH}_4)_2\text{SO}_4$, added at various times, on RNA polymerase. Reaction mixtures were as described for Fig. 1 with nucleotide concentration 3 for the Mn^{2+} -activated reaction (a), and as described by Widnell & Tata (1966) for the Mg^{2+} -activated reaction (b). $(\text{NH}_4)_2\text{SO}_4$ was 7% saturated; heparin was $100 \mu\text{g./ml.}$ Addition: ○, none; ●, heparin; □, $(\text{NH}_4)_2\text{SO}_4$.

and only $[^{14}\text{C}]\text{ATP}$ was added after 15 min. (Table 1). These results suggest that during preincubation modifications occur in the system, leading to an impairment of the incorporation, which was maximal if all nucleotides were present, i.e. if RNA synthesis occurred during preincubation.

Effect of heparin. When heparin was added to the incorporation system without ammonium sulphate in the presence of Mn^{2+} at low concentration of nucleotides, the stimulation observed was maximal (fourfold) with $100 \mu\text{g.}$ of heparin/ml. (Fig. 3), no further stimulation being obtained with higher concentrations of heparin. A somewhat less marked stimulation (up to twofold) was seen when the concentration of nucleotides was higher; under these conditions, however, a slight inhibitory effect was exerted by low concentrations of heparin, and the stimulation was not observed with more than $300 \mu\text{g.}$ of heparin/ml.

Fig. 5. Effect of heparin on the stimulation of RNA polymerase by $(\text{NH}_4)_2\text{SO}_4$. Experimental conditions were as described for Fig. 1, with nucleotides at concentration 3 (a) in the presence of Mn^{2+} and (b) in the presence of Mg^{2+} (obtained with a different preparation of nuclei). ●, No heparin added; ○, heparin, $100 \mu\text{g./ml.}$

Other polyanions (amylosulphate, chondroitin sulphate, amylopectin sulphate) had no stimulatory effect on Mn^{2+} -activated RNA polymerase when added at concentrations up to $200 \mu\text{g./ml.}$

The effect of heparin at 25° on the time-course of the RNA polymerase reaction in the presence of

Table 2. *Effect of ammonium sulphate and of heparin on the release of label from liver nuclei of rats injected with [6-¹⁴C]orotic acid*

Experimental conditions were as described in the Experimental section. The incubation mixtures were the same as those used for assay of the activities of the Mg^{2+} - and the Mn^{2+} -activated reactions (see Table 1 and Fig. 1 respectively). Nucleotide triphosphates, all non-radioactive, were at concentrations 3 and 1 (see Fig. 1) for the Mn^{2+} - and the Mg^{2+} -activated systems respectively. The incubation mixture contained 160 μ g. of nuclear DNA. The counting efficiency was about 60%.

Time of incubation (min.)	Addition	...	Radioactivity in acid-soluble supernatant (c.p.m.)		
			None (control)	(NH ₄) ₂ SO ₄ (7% saturated)	Heparin (100 μg./ml.)
Mn ²⁺ -activated system					
0			241	—	—
10			1815	247	1058
25			2736	330	1402
45			3396	398	1760
Mg ²⁺ -activated system					
0			273	—	—
10			1100	426	825
25			2103	330	1168

Mn^{2+} was negligible in the first 25 min.; after this time the reaction slowed down without heparin, but continued to proceed in the presence of heparin. At 37° the effect of heparin was more obvious, owing to the fact that the incorporation of [¹⁴C]-AMP was practically stopped after 10 min. (Fig. 4a). Addition of heparin after 15 min. of incubation had no effect at 25° but 'restarted' the reaction at 37°. The effect of heparin was compared with that of ammonium sulphate at 37° (Fig. 4a): both substances stimulated the incorporation of [¹⁴C]AMP when they were added to the mixtures with Mn^{2+} before the reaction was started, the stimulation by ammonium sulphate being greater than that by heparin. Both substances 'restarted' the reaction when added after 15 min. of incubation, and again the stimulation was more marked after addition of ammonium sulphate than of heparin. In the Mg^{2+} -activated system as described by Widnell & Tata (1966) the addition of heparin either at the beginning or after 15 min. had only a slight effect on the incorporation rate (Fig. 4b), whereas the addition of ammonium sulphate after 15 min. 'restarted' the reaction.

The similarities between the effects of heparin and of ammonium sulphate on the Mn^{2+} -activated reaction led us to investigate further the possible interrelationships between these substances, by studying the effect of increasing concentrations of ammonium sulphate in the presence of heparin (100 μ g./ml.). The effect of ammonium sulphate was 'anticipated' in the presence of heparin, i.e. maximal stimulation was reached with lower concentrations of the salt (Fig. 5a). At optimum concentrations of

ammonium sulphate and above no effect of heparin could be demonstrated. A similar effect was observed when Mg^{2+} was substituted for Mn^{2+} at pH 7.5 and without cysteine and fluoride (Fig. 5b).

To ascertain whether ammonium sulphate and heparin acted by inhibiting ribonuclease the effect of these substances on the release of radioactivity from nuclei labelled *in vivo* with [¹⁴C]orotic acid was tested. In the presence of ammonium sulphate there was almost no release of radioactivity both in the Mn^{2+} - and in the Mg^{2+} -activated system; heparin had a less marked effect (Table 2).

Effect of α -amanitin. It has been reported that α -amanitin inhibits *in vitro* the RNA polymerase activity in the presence of ammonium sulphate of nuclei isolated from mouse liver (Stirpe & Fiume, 1967). When the effect of α -amanitin was studied on nuclei from rat liver in the presence of various concentrations of ammonium sulphate or of heparin, it was observed that the stimulation by these substances was abolished by the toxin (Fig. 6). It was confirmed that α -amanitin had little effect on the polymerase activity in the absence of ammonium sulphate or of heparin.

DISCUSSION

The results reported above allow a comparison of the effects of ammonium sulphate and of heparin. The action of heparin differs from that of ammonium sulphate since ammonium sulphate exerts a stronger stimulation on Mn^{2+} -activated RNA polymerase and also stimulates the Mg^{2+} -activated

Fig. 6. Effect of α -amanitin on the stimulation of RNA polymerase by $(\text{NH}_4)_2\text{SO}_4$ or by heparin. RNA polymerase was assayed as described for Fig. 5(a), with increasing concentrations of $(\text{NH}_4)_2\text{SO}_4$ (a) or of heparin (b) without (○) or with (●) α -amanitin (0.05 $\mu\text{g./ml.}$).

reaction on which heparin has a negligible effect. Ammonium sulphate and heparin presumably do not act by preserving nucleotides from destruction by nucleotidases; if the polymerase reaction at low ionic strength stopped because of lack of substrates, it should not 'restart' on the addition of these substances. A comparison of the effects of ammonium sulphate and heparin on the release of labelling and on the RNA polymerase activity in the Mn^{2+} -containing mixtures suggests an inter-relationship between the inhibition of ribonuclease and the stimulation of RNA polymerase. However, the inhibition of ribonuclease can account only in

part for the action of ammonium sulphate and of heparin on RNA polymerase since: (i) in the Mg^{2+} -activated system heparin does not affect RNA polymerase, although it diminishes RNA degradation (Table 2); (ii) ammonium sulphate inhibits ribonuclease to about the same extent in both the Mg^{2+} - and the Mn^{2+} -containing system, although it gives a greater stimulation of RNA polymerase in the presence of Mn^{2+} (see Fig. 1); (iii) α -amanitin abolishes the effects of ammonium sulphate and of heparin on RNA polymerase without restoring the release of labelled material from nuclei incubated with ammonium sulphate (Stirpe & Fiume, 1967).

The effect of heparin on nuclear RNA polymerase is opposite to the action exerted by the same substance on the bacterial enzyme (Walter *et al.* 1967). This could be due to differences between the two enzymes; however, the effect of heparin on the nuclear polymerase is greatly favoured by the presence of Mn^{2+} , and its action on the bacterial enzyme has been studied in the presence of Mg^{2+} . Further, purified preparations of the bacterial polymerase have been used, whereas nuclei are a complex system; since heparin caused swelling of nuclei (Davies & Spencer, 1962; Korohoda, Forrester, Moreman & Ambrose, 1968) and displaces histones from DNA (Kent, Hichens & Ward, 1958), it is possible that heparin could affect RNA synthesis indirectly, by acting on some nuclear component(s) other than the enzyme. In part, at least, heparin acts by allowing the RNA polymerase reaction to proceed for longer than 10–15 min. and in this respect it is similar to ammonium sulphate.

An effect of ammonium sulphate is to modify the requirement for nucleotides in both the Mg^{2+} - and the Mn^{2+} -activated system. Our results also show that the Mn^{2+} -activated system has a greater requirement for nucleotides even in the absence of ammonium sulphate (Fig. 1).

A comparison of the activating effects of Mg^{2+} and of Mn^{2+} with the concentrations of nucleotides used by various investigators is reported in Table 3. Our observation that Mn^{2+} becomes a better activator for RNA polymerase at higher concentrations of nucleotides may perhaps explain some controversial reports.

α -Amanitin 'abolishes' the stimulation of RNA polymerase by ammonium sulphate or by heparin. This should not be interpreted as a 'prevention' of the effect of these substances, since the inhibition by α -amanitin is detectable even if the toxin is added after starting the RNA polymerase reaction (L. Fiume & F. Stirpe, unpublished work). If ammonium sulphate acts directly on RNA polymerase, as was postulated by Jacob, Sajdel & Munro (1968), the possibility should be considered that α -amanitin makes the enzyme insensitive to high ionic strength.

Table 3. Comparison of the effects of Mg^{2+} and Mn^{2+} on RNA polymerase assayed with various concentrations of nucleotides at low ionic strength

References: (a) Pogo, Allfrey & Mirsky (1966); (b) Bresnick (1966); (c) Breuer & Florini (1966); (d) Widnell & Tata (1964b, 1966); (e) Barnabei, Romano, Di Bitonto, Tomasi & Sereni (1966); (f) MacGregor & Mahler (1967); (g) Weiss (1960); (h) Marchok & Wolff (1968); (i) Chambon *et al.* (1968); (j) present paper (Fig. 1).

Reference	Source of enzyme	Concn. of nucleotides	Stimulation
(a)	Rat liver nuclei	0.1 mM-ATP, 0.1 mM-CTP, 0.1 mM-GTP, 0.012 mM-UTP	$Mg^{2+} > Mn^{2+}$
(b)	Rat liver nuclei (aggregate)	1 mM-ATP, 1 mM-CTP, 1 mM-GTP, 0.016 mM-UTP	$Mg^{2+} > Mn^{2+}$
(c)	Rat muscle nuclei (aggregate)	1.33 mM-ATP, 5.31 mM-CTP, 5.31 mM-GTP, 0.0134 mM-UTP	$Mg^{2+} > Mn^{2+}$
(d)	Rat liver nuclei	0.6 mM-CTP, 0.6 mM-GTP, 0.6 mM-UTP, 0.04 mM-ATP	$Mg^{2+} > Mn^{2+}$
(e)	Rat liver nuclei	0.6 mM-CTP, 0.6 mM-GTP, 0.6 mM-UTP, 0.05 mM-ATP	$Mg^{2+} > Mn^{2+}$
(f)	Rat liver nuclei	0.05 mM-ATP, 0.05 mM-CTP, 0.05 mM-GTP, 0.05 mM-UTP	$Mg^{2+} > Mn^{2+}$
(g)	Rat liver nuclei (aggregate)	0.91 mM-ATP, 0.91 mM-UTP, 0.91 mM-GTP, 0.054 mM-CTP	$Mg^{2+} > Mn^{2+}$
(h)	Nuclei from developing chick muscle	0.4 mM-ATP, 0.4 mM-CTP, 0.4 mM-GTP, 0.4 mM-UTP	$Mn^{2+} \geq Mg^{2+}$
(i)	Rat liver nuclei (aggregate)	4 mM-ATP, 4 mM-CTP, 4 mM-GTP, 4 mM-UTP	$Mn^{2+} > Mg^{2+}$
(j)	Rat liver nuclei	0.6 mM-CTP, 0.6 mM-GTP, 0.6 mM-UTP, 0.04 mM-ATP	$Mg^{2+} > Mn^{2+}$
		1.2 mM-CTP, 1.2 mM-GTP, 1.2 mM-UTP, 0.08 mM-ATP	$Mg^{2+} > Mn^{2+}$
		1.8 mM-CTP, 1.8 mM-GTP, 1.8 mM-UTP, 0.12 mM-ATP	$Mn^{2+} \geq Mg^{2+}$

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