The Effects of Copper and other Ions on the Ribonucleic Acid Polymerase Activity of Isolated Rat Liver Nuclei

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(Received 5 July 1968)

1. The effects of various ions on the Mg^{2+} - and Mn^{2+} /ammonium sulphate-activated RNA polymerase activities of isolated liver nuclei were studied. 2. The Mg^{2+} -activated RNA polymerase reaction was inhibited by more than 60% by Cd^{2+} , SeO_3^{2-} , Be^{2+} , Cu^{2+} , Co^{2+} , Ca^{2+} and La^{3+} , all at 1 mM concentrations. 3. The Mn^{2+} /ammonium sulphate-activated RNA polymerase reaction was strongly inhibited by Hg^{2+} , Cd^{2+} , Cu^{2+} and Ag^+ . The effect of Hg^{2+} , Cd^{2+} and Ag^+ was relieved by cysteine or mercaptoethanol. 4. Inhibition by Cu^{2+} was not affected by addition of DNA, and was relieved only partially by EDTA or histidine. 5. No changes of RNA polymerase activities were observed in nuclei isolated from the liver of rats treated with copper albuminate.

The activity of RNA polymerase (nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6) from various sources is affected by several ions and salts. The enzymic reaction is dependent on the presence of metal ions, the most effective ones being Mg^{2+} and/or Mn^{2+} (Furth, Hurwitz & Anders, 1962; Ballard & Williams-Ashman, 1964; Widnell & Tata, 1966), which in some cases can be replaced by Co^{2+} (Ballard & Williams-Ashman, 1964) but not by other metal ions (Furth *et al.* 1962; Ballard & Williams-Ashman, 1966). In addition, the RNA polymerase activity is stimulated and modified by increased ionic strength, and for this purpose ammonium sulphate is the most commonly used salt (Widnell & Tata, 1966).

The interrelation of metal ions with nucleic acids is well known, and has been much investigated in recent years (see Bryan & Frieden, 1967). However, data on the effects of ions on RNA polymerase activity are surprisingly few: it has been reported that Ca^{2+} is inhibitory in the presence of Mg^{2+} (Weiss, 1960) and that *p*-chloromercurobenzoate inhibits the enzyme activity (Furth, Rosenberg & Ho, 1967; Ro & Busch, 1967).

The present paper is concerned with the effects of several metal ions on the activity of RNA polymerase *in vitro*, the enzyme being assayed at both low and high ionic strength. The inhibitory effects of some ions tested were relieved by cysteine. Among ions whose effect was not counteracted by cysteine, Cu^{2+} was inhibitory at very low concentrations, and this was studied in greater detail.

EXPERIMENTAL

Animals. Male Wistar rats weighing 200–250 g. were used. All animals were fed *ad libitum* with the standard diet used in this Institute. In some experiments rats were poisoned with daily injections of copper albuminate as described by Verity, Gambell, Reith & Brown (1967).

Chemicals. [$8\text{-}^{14}C$]ATP (27 $\mu C/\mu\text{mole}$) was purchased from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. Other nucleotides, DNA (calf thymus) and bovine serum albumin were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Copper albuminate was prepared from $CuSO_4$ and bovine serum albumin as described by Verity *et al.* (1967). All other chemicals were of the purest available grade. Solutions were prepared with glass-redistilled water.

Preparation of nuclei and determination of RNA polymerase activity. Nuclei were prepared as described by Widnell & Tata (1964), and RNA polymerase activity was determined at low and high ionic strength essentially as described by Widnell & Tata (1966). After the incubation, the reaction was stopped with 5 ml. of 0.5 N-HClO₄, 200 μg . of RNA dissolved in 0.1 ml. of water was added, and the precipitated material was centrifuged, washed twice with 5 ml. each time of 0.2 N-HClO₄, once with ethanol-diethyl ether (3:1, v/v), dried at 60° and redissolved in 0.5 ml. of formic acid. Samples (0.4 ml.) were put in counting vials, with 5 ml. of methylCellosolve and 10 ml. of scintillation fluid [0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% diphenyloxazole in toluene]. The radioactivity was determined in a Nuclear-Chicago Mark I scintillation counter with an efficiency of about 60%. The results were corrected for the efficiency and for the dilution of the [^{14}C]ATP, and expressed as $\mu\mu\text{moles}$ of AMP incorporated/mg. of DNA.

Other determinations. The DNA content of nuclear suspensions was determined by the method of Burton

Table 1. *Effect of metal ions on the Mg²⁺-activated RNA polymerase activity*

Experimental conditions were as described by Widnell & Tata (1966). The reaction mixture contained (in 0.5 ml.): 50 μ moles of tris-HCl buffer, pH 8.5, 2.5 μ moles of MgCl₂, 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 [¹⁴C]ATP, the appropriate salt (final concn. 1 mM, added before the nuclei) and 0.1 ml. of nuclear suspension (200–400 μ g. of DNA). Activities are expressed as μ moles of AMP incorporated/10 min./mg. of DNA.

Addition	RNA polymerase activity	Inhibition (%)	Addition	RNA polymerase activity	Inhibition (%)
None	396	—	None	276	—
HgCl ₂	376	5	Cr(CH ₃ ·CO ₂) ₃	293	—
CdCl ₂ *	136	66	CaCl ₂	102	63
Na ₂ SeO ₃ *	156	61	SrCl ₂	196	29
BeSO ₄	110	72			
CuSO ₄	97	75	None	416	—
ZnCl ₂	386	2	TiCl ₄	214	49
CoCl ₂	137	65	NiCl ₂	364	12
None	337	—	None	308	—
LiCl	400	—	La(NO ₃) ₃	124	60
AlCl ₃	269	20			
			None†	467	—
			AgNO ₃ †	421	10

* Turbidity and coloured material in the reaction mixture.

† In this experiment tris-acetate, 2-mercaptoethanol and MgSO₄ were used instead of tris-HCl, cysteine and MgCl₂. Some turbidity was noticed on the addition of 2-mercaptoethanol in the presence of AgNO₃.

Table 2. *Effect of metal ions on the Mn²⁺/ammonium sulphate-activated RNA polymerase activity*

Experimental conditions were as described by Widnell & Tata (1966). The reaction mixture contained (in 0.5 ml.): 50 μ moles of tris-HCl buffer, pH 7.5, 2 μ moles of MnCl₂, 0.05 ml. of a solution of (NH₄)₂SO₄ saturated at room temperature and adjusted to pH 7.5 with aq. NH₃, 10 μ moles of cysteine (when added), 0.3 μ mole each of GTP, CTP and UTP, 0.015 μ mole of non radioactive ATP, the appropriate salt (final concn. 1 mM added before the nuclei) and 0.1 ml. of nuclear suspension (200–400) μ g. of DNA). After preincubation for 15 min. at 37°, 0.005 μ mole of [¹⁴C]ATP was added, and the reaction was stopped after a further 45 min. Activities are expressed as μ moles of AMP incorporated/45 min./mg. of DNA.

Addition	RNA polymerase activity		Inhibition (%)		Addition	RNA polymerase activity		Inhibition (%)	
	— Cyst-eine	+ Cyst-eine	— Cyst-eine	+ Cyst-eine		— Cyst-eine	+ Cyst-eine	— Cyst-eine	+ Cyst-eine
None	1376	1391	—	—	None	1013	1104	—	—
HgCl ₂	0	1369	100	2	Cr(CH ₃ ·CO ₂) ₃	1164	1120	—	—
CdCl ₂ *	0	359	100	75	CdCl ₂ *	44	199	96	82
Na ₂ SeO ₃ *	990	1045	28	25	CaCl ₂	986	981	8	12
BeSO ₄	1178	986	14	29	SrCl ₂	1154	991	—	10
CuSO ₄	52	189	97	87					
ZnCl ₂	881	1422	36	0	None	775	825	—	—
CoCl ₂ *	1214	351	12	75	TiCl ₄	880	665	—	19
					NiCl ₂ *	803	645	—	21
None	1041	—	—	—					
Na ₂ SeO ₃	699	—	33	—	None	1070	—	—	—
CoCl ₂	700	—	33	—	LiCl	1042	—	5	—
					AlCl ₃	1042	—	5	—
None	715	742	—	—					
La(NO ₃) ₃	486	528	31	28					

* Turbidity or coloured material in the reaction mixture.

(1956). The copper content of the livers and of the preparations of copper albuminate was determined by the method of Eden & Green (1940).

RESULTS

Effects of metal ions in vitro. A preliminary screening was performed, in which various ions were tested at a relatively high concentration (1mM). Cd²⁺, SeO₃²⁻, Be²⁺, Cu²⁺, Co²⁺, Ca²⁺ and La³⁺ all caused more than 60% inhibition of RNA polymerase activity assayed at low ionic strength in the presence of Mg²⁺ (Table 1). With the Mn²⁺/ammonium sulphate-activated RNA polymerase reaction, assayed as described by Widnell & Tata (1966), a strong inhibitory effect was observed with Hg²⁺, Cd²⁺ and Cu²⁺ (Table 2). Among the differences between the two assay systems there is the presence of cysteine, which is added only to the Mg²⁺-activated system at the concentration 20mM. The addition of the same concentration of cysteine relieved the inhibition exerted by Hg²⁺ on the Mn²⁺/ammonium sulphate-activated RNA polymerase. On the other hand, a marked inhibitory effect was observed on the addition of Co²⁺ in the presence of cysteine. During these experiments it was noticed that the incorporation of [¹⁴C]AMP in the Mn²⁺/ammonium sulphate-activated system was greater if the concentration of nucleotide

substrates was increased. Essentially similar effects of ions were obtained (Table 3) when the experiments were repeated at a saturating concentration of nucleotides (three times those in the original method). In these experiments, the effect of Ag⁺ was tested: this ion had a strong inhibitory effect, which was prevented by mercaptoethanol.

Experiments with Cu²⁺. From the results reported above (Tables 1 and 2) it appeared that Cu²⁺ inhibited RNA polymerase activity under all conditions of assay, and that its effect was not counteracted by cysteine. Therefore the effect of Cu²⁺ was investigated further. The effect of various concentrations of Cu²⁺ is shown in Fig. 1. The inhibition was stronger on the Mn²⁺/ammonium sulphate-activated than on the Mg²⁺-activated reaction, 50% inhibition occurring at 1μM- and 10μM-Cu²⁺ respectively. The inhibition exerted by 10μM-Cu²⁺ was not relieved by increased concentrations of nucleotide substrates, nor by addition of DNA (Table 4). EDTA had only a partial counteracting effect, which was more marked in the Mn²⁺/ammonium sulphate-activated system at the highest concentration of nucleotides, and under these conditions histidine also had a partial protective effect.

In view of the hepatotoxic effects of copper (Underwood, 1962) the RNA polymerase activity was determined in liver nuclei from rats subjected

Table 3. *Effect of metal ions on the Mn²⁺/ammonium sulphate-activated RNA polymerase activity at higher substrate concentration*

Experimental conditions were as in Table 2, except that the concentration of nucleotide substrate was three times as high. Other details were as in Table 1.

Addition	Expt. 1		Expt. 2			
	RNA polymerase activity	Inhibition (%)	RNA polymerase activity		Inhibition (%)	
			- Cysteine	+ Cysteine	- Cysteine	+ Cysteine
None	1672	—	2699	2466	—	—
HgCl ₂	42	98	50	2198	97	11
CdCl ₂ *	356	79	1011	2159	62	13
Na ₂ SeO ₃ *	556	67	1661	1731	38	30
BeSO ₄	1260	25	1638	1679	40	32
ZnSO ₄	1009	35	1847	2433	31	1
CoCl ₂ *	1461	13	2288	2188	15	12
Cr(CH ₃ ·CO ₂) ₃	1496	—	—	—	—	—
CaCl ₂	1440	14	2020	2718	25	0
SrCl ₂	1566	7	1898	1991	29	19
LiCl	1585	5	—	—	—	—
AlCl ₃	1126	23	2241	2129	17	14
TlCl	1631	3	2289	1913	15	22
NiCl ₂ *	—	—	2044	2356	24	2
La(NO ₃) ₃	884	47	1917	2042	29	18
None†	—	—	1492	1414	—	—
AgNO ₃ †	—	—	2	1265	100	10

* Turbidity or coloured material in the reaction mixture.
† See Table 1.

to sub-acute poisoning with copper albuminate, but no differences were observed between the control and copper-treated rats, in spite of the increased copper content in the liver of the latter animals (Table 5).

DISCUSSION

From a comparison of the results reported in Table 1 with those of Tables 2 and 3 a difference

Fig. 1. Inhibition of RNA polymerase activity by Cu²⁺: ○, Mg²⁺-activated reaction; ●, Mn²⁺/(NH₄)₂SO₄-activated reaction. The values obtained without CuSO₄ were 163 μmoles of AMP incorporated/10 min./mg. of DNA for the Mg²⁺-activated reaction and 2372 μmoles AMP incorporated/45 min./mg. of DNA for the Mn²⁺/(NH₄)₂SO₄-activated reaction. The curves were obtained in two separate experiments. Experimental conditions were as in Table 1 for the Mg²⁺-activated reaction, and as in Table 3 for the Mn²⁺/(NH₄)₂SO₄-activated reaction.

appears in that some ions inhibit the RNA polymerase reaction activated by Mg²⁺ and assayed at low ionic strength but have less or even no effect on the Mn²⁺/ammonium sulphate-activated system. Ag⁺ and Cd²⁺ are exceptions, but their effects are relieved by the addition of cysteine to the system. This indicates that an essential thiol group is probably involved in the inhibition by these metal ions, and this is in agreement with the observed effect of *p*-chloromercuribenzoate (Furth *et al.* 1967; Ro & Busch, 1967). With Cd²⁺ the inhibition is relieved by cysteine only at the highest concentrations of nucleotides, thus indicating that the inhibition may in part be attributed to the interaction of nucleotides with the metal ion. The inhibition by Ca²⁺ in the Mg²⁺-activated system (Weiss, 1960; MacGregor & Mahler, 1967) was confirmed. It should be noticed that Sr²⁺ was much less inhibitory than Ca²⁺.

The most consistent effect was obtained with Cu²⁺, which inhibited RNA polymerase under all conditions of assay. Cu²⁺ also inhibits ribonuclease, but only as purified enzyme, and this inhibition is relieved by boiled liver supernatant (interpreted as due to binding of Cu²⁺, presumably by protein; Wojnar & Roth, 1964), whereas its effect on RNA polymerase is evident at very low concentrations in the system of whole nuclei used in our experiments. It should be noticed also that Zn²⁺, which is inhibitory for ribonuclease, does not affect RNA polymerase, provided that cysteine is present. The effect of Cu²⁺ was not relieved by cysteine, in contrast with Hg²⁺ and other thiol-binding ions. It is known that Cu²⁺ binds to nucleotides as well as to DNA (see review in Eichhorn, Clark & Becker, 1966). The binding of Cu²⁺ to DNA results in 'destabilization' of DNA, as demonstrated by the lower transition temperatures when thermal denaturation of DNA is performed in the presence of

Table 4. *Effects of various additions on the inhibition of RNA polymerase activity by Cu²⁺*

Experimental conditions were as in Table 1 for the Mg²⁺-activated reaction, and as in Table 2 for the Mn²⁺/(NH₄)₂SO₄-activated reaction. 1 indicates the concentration of nucleoside triphosphates that was used by Widnell & Tata (1966), 2 and 3 indicate concentrations that were two- and three-fold higher respectively.

Additions	RNA polymerase activity					
	Mg ²⁺ -activated			Mn ²⁺ /(NH ₄) ₂ SO ₄ -activated		
	1	2	3	1	2	3
None	364	216	194	1111	1346	1518
10 μM-CuSO ₄	147	129	88	242	213	272
10 μM-CuSO ₄ + 200 mg. of DNA	184	—	—	213	—	—
10 μM-CuSO ₄ + 1 mM-EDTA	283	—	—	226	—	—
None	482	—	—	—	—	1824
10 μM-CuSO ₄	214	—	—	—	—	313
10 μM-CuSO ₄ + 1 mM-EDTA	268	—	—	—	—	678
10 μM-CuSO ₄ + 1 mM-histidine	185	—	—	—	—	625

Table 5. *RNA polymerase activities of liver nuclei from rats treated with copper albuminate*

Rats were killed 24 hr. after the last injection. The experimental procedure was as described by Widnell & Tata (1966) except for the concentration of nucleoside phosphates in the $\text{Mn}^{2+}/(\text{NH}_4)_2\text{SO}_4$ -activated system, which was three times as high. Results are mean values $\pm$ s.e.m. of four experiments for each group. Other details are as in the preceding Tables.

No. of injections	Injection	Copper content in the liver ($\mu\text{g./g. wet wt.}$)	RNA polymerase activity	
			Mg^{2+} -activated	$\text{Mn}^{2+}/(\text{NH}_4)_2\text{SO}_4$ -activated
3	Albumin	13.1 ± 2.6	554 ± 44	2431 ± 188
	Copper albuminate	18.9 ± 2.9	614 ± 145	2794 ± 297
5	Albumin	7.3 ± 2.4	451 ± 96	2204 ± 194
	Copper albuminate	28.6 ± 7.0	421 ± 25	2455 ± 208
9	Albumin	5.5 ± 0.2	325 ± 47	1579 ± 249
	Copper albuminate	50.5 ± 7.8	349 ± 24	1450 ± 19

0.1 mM- Cu^{2+} (Eichhorn, 1962). The possibility that the inhibition of RNA polymerase could be due to some interaction of the metal ion with nucleotides or with template DNA is excluded by the fact that no difference was observed when the concentration of nucleotides was increased or when DNA was added to the reaction mixture. The RNA-synthesizing system is formed by (a) the polymerase, (b) the nucleotide substrates and (c) the template DNA. If Cu^{2+} does not inhibit by interfering with nucleotides or DNA, one must conclude that it acts on the enzyme, for which it should have a very high affinity, since the inhibition is abolished only in part by EDTA or histidine. However, it must be considered: (i) that in our experiments the activity of RNA polymerase was studied with whole nuclei: thus some nuclear component(s) could interfere with the reaction (for instance histones), and this interference could be affected by Cu^{2+} ; (ii) that the synthesis of RNA is a complex process, consisting of several steps (initiation, condensation of nucleotides, detachment of newly formed RNA) each of which could be affected by Cu^{2+} . A clear interpretation of these results will be possible only when a better knowledge of mechanism of RNA polymerase reaction has been achieved.

We are indebted to Professor E. Bonetti for advice and helpful criticism, and to Sig. E. Lorenzoni for performing the copper analyses and for skilled technical assistance.

The work was supported by a grant from Consiglio Nazionale delle Ricerche, Rome.

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