Influence of Nucleotides, Cations and Nucleoside Triphosphatase Inhibitors on the Release of Ribonucleic Acid from Isolated Rat Liver Nuclei

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The reasons underlying reported discrepancies in the effects of ATP, ADP, adenosine 5'-[β -methylene]triphosphate, AMP + PP_i, *p*-chloromercuribenzoate and F⁻ on RNA efflux from isolated rat liver nuclei and on nuclear-envelope nucleoside triphosphatase activity were investigated. The stimulatory effect of ADP was attributed to myokinase activity associated with the nuclei; this activity was eluted on repeated washing with nuclear incubation medium. In the absence of Ca²⁺ and Mn²⁺, ATP, adenosine 5'-[β -methylene]triphosphate and AMP + PP_i were found to promote release of both DNA and RNA. In the presence of 0.5 mM-Ca²⁺ and 0.3 mM-Mn²⁺, only ATP promoted RNA efflux to a significant extent. In the absence of spermidine, Ca²⁺ and Mn²⁺, nuclei released large quantities of DNA and RNA into the medium; this effect was promoted by *p*-chloromercuribenzoate. In the presence of the three cations, however, *p*-chloromercuribenzoate inhibited RNA efflux. F⁻ caused a slight leakage of DNA from nuclei. The results are discussed in terms of models for the effects of ATP and analogues on RNA efflux and nuclear stability.

The stimulation of RNA release from isolated nuclei by ATP and related compounds has been investigated in several laboratories and the effect has been interpreted in at least four different ways.

(1) The ATP chelates bivalent metal ions in the medium, promoting release of heterogeneous nuclear RNA–protein complexes from the chromatin (Ishikawa *et al.*, 1974) and hence from the nuclei (Chatterjee & Weissbach, 1973; Sauermann, 1974).

(2) The ATP, possibly again via a chelating action, causes expansion of the nuclei and increases the tendency for nuclear contents to leak through the envelope (Stuart *et al.*, 1975, 1977).

(3) The ATP interacts with a structure (possibly a component of the pore complex) in the nuclear envelope, inducing a conformation change which results in the release of bound mature mRNA and ribosomal subunits from the nucleus (Raskas & Bhaduri, 1973; Raskas & Rho, 1973).

(4) ATP hydrolysis occurs, supplying the energy required for RNA transport across the nuclear envelope (Yu *et al.*, 1972; Schumm & Webb, 1972, 1974; Racevskis & Webb, 1974).

Investigations in my laboratory (Agutter *et al.*, 1976, 1979b) and by others (Clawson & Smuckler,

Abbreviations used: NTPase, nucleoside triphosphatase (EC 3.6.1.15); p[CH₂]ppA, adenosine 5'-[β -methylene]triphosphate.

1978a,b; Clawson *et al.*, 1978) supported the fourth of these interpretations. More specifically, Agutter *et al.* (1976, 1979b) have provided evidence that ATP hydrolysis occurs at an NTPase located in the nuclear-pore complex (Yasuzumi & Tsubo, 1966; Yasuzumi *et al.*, 1967; Chardonnet & Dales, 1972; Franke, 1974), and that this event is necessary for RNA transport. However, the findings of Agutter *et al.* (1979b) and Clawson *et al.* (1978) are not wholly consistent with one another. The main differences are that: (a) Clawson *et al.* (1978) reported that ADP stimulated RNA release to a significant extent, but Agutter *et al.* (1979b) were unable to repeat this observation; (b) Clawson *et al.* (1978) found that *p*-chloromercuribenzoate markedly stimulated RNA release in the presence of ATP, whereas Agutter *et al.* (1979b) found that it inhibited. In addition, Clawson *et al.* (1978) found that F⁻ inhibited the NTPase, but did not affect RNA efflux.

A thorough investigation of the ATP effect by Ishikawa *et al.* (1978) led them, in contrast with those workers discussed above, to accept the third interpretation, i.e. that ATP induces a conformation change in a nuclear-envelope structure, promoting RNA release. Although Ishikawa *et al.* (1978) could not exclude the possibility that ATP hydrolysis occurred, they concluded that its role in RNA transport was secondary. Their experimental ob-

servations differed from those of Agutter *et al.* (1979b) in three important respects; (a) once again, ADP was found to stimulate RNA release; (b) p[CH₂]ppA was an effective substitute for ATP, in agreement with the work of Raskas & Rho (1973), but contradicting the findings of Yu *et al.* (1972) and Agutter *et al.* (1979b); (c) AMP + PP_i were also found to be effective substitutes for ATP.

The aim of the present paper is to investigate the reasons for these conflicts in the current literature on the stimulation of RNA release by ATP. At the outset of the investigation, it was assumed that the differences in experimental findings were most likely to result from differences in the incubation media used, particularly in their cation content. Spermidine (Yu *et al.*, 1972; Sato *et al.*, 1977a,b) and bivalent metal ions (Ishikawa *et al.*, 1969; Leake *et al.*, 1972; Giese *et al.*, 1979) are known to affect RNA release dramatically, and it has been reported that Cu²⁺ and Cu²⁺ complexes inhibit RNA transport (Baru *et al.*, 1978).

Materials and Methods

ATP, ADP, AMP, *p*-chloromercuribenzoate, hexokinase (ATP-D-hexose 6-phosphotransferase, EC 2.7.1.1) and glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP⁺ 1-oxidoreductase, EC 1.1.1.49) were obtained from Sigma Chemical Co., London S.W.6, U.K. p[CH₂]ppA was obtained from Boehringer, Mannheim, Germany. [5-³H]Uridine was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents used were of AnalaR grade from BDH Chemicals, Poole, Dorset, U.K.

Male albino rats (100–150 g body wt.) were used throughout. In some experiments, they were injected intraperitoneally with 50 µCi of [5-³H]uridine 1 h before they were killed.

Isolation of nuclei and nuclear envelopes

Nuclei were isolated by the method of Blobel & Potter (1966) and nuclear envelopes by the method of Harris & Milne (1974).

Assay of NTPase activity

NTPase activity was assayed as previously described (Agutter *et al.*, 1979a) by using 2.85 mM-ATP + 2.85 mM-MgCl₂. When inhibitors (F⁻ and *p*-chloromercuribenzoate) were used, these were pre-incubated for 10 min at 0°C with the envelopes (Agutter *et al.*, 1979a).

Assay of myokinase (ATP-AMP phosphotransferase, EC 2.7.4.3) activity

Nuclei (50–100 µg of protein) were incubated in 3.5 ml of 50 mM-Tris/HCl/2 mM-ADP/2 mM-MgCl₂/5 mM-D-glucose/1 mM-NADP⁺, pH 8.0 at 37°C,

containing 2 µg of hexokinase and 2 µg of glucose 6-phosphate dehydrogenase/ml, and the rate of increase of A₃₄₀ was measured. Since under these assay conditions (cf. Colowick, 1955) the rate was proportional to the concentration of nuclear protein and virtually independent of the concentrations of the other reagents, this rate was taken to be a measure of myokinase activity in the nuclei.

Measurement of RNA efflux rate

RNA efflux rate was routinely measured by using a 10 min incubation at 37°C in one of the following media.

(a) 0.25 M-D-glucose, 10 mM-Tris/HCl buffer, 25 mM-KCl, 5.0 mM-spermidine, 5.0 mM-2-mercaptoethanol, 2.0 mM-MgCl₂, 0.5 mM-CaCl₂, 0.3 mM-MnCl₂ and 300 µg of yeast RNA/ml, pH 7.6 (Agutter & McCaldin, 1979; Agutter *et al.*, 1979b). In some experiments, the CaCl₂ and MnCl₂ were omitted (Ishikawa *et al.*, 1978), or their concentrations were varied or the MgCl₂ concentration was increased to 5 mM.

(b) 0.88 M-ribonuclease-free sucrose, 50 mM-Tris/HCl buffer, 25 mM-KCl, 5 mM-MgCl₂, pH 7.6 (Clawson *et al.*, 1978).

Radioactivity counting was carried out as described previously (Agutter & McCaldin, 1979).

Chemical determinations

Protein was determined by the modification by Maddy & Spooner (1970) of the method of Lowry *et al.* (1951), with bovine serum albumin as a standard, and DNA by the method of Burton (1956), with 2'-deoxyadenosine as a standard.

Results and Discussion

Effect of ADP on RNA release

The stimulation of RNA release by ADP in six nuclear preparations correlated significantly with myokinase activity, which was found to vary markedly between preparations (Table 1). In contrast, the extent of ATP-stimulated RNA release was fairly reproducible. Myokinase activity was decreased to about 20–25% of its initial value by repeated washing in the nuclear incubation medium of Agutter & McCaldin (1979) and Agutter *et al.* (1979b). This may account for the failure of Agutter *et al.* (1979b) to find evidence for ADP hydrolysis by isolated nuclear envelopes or ADP-stimulated RNA release from isolated nuclei. In view of the correlation found in Table 1, formation of ATP from ADP followed by hydrolysis of the ATP by the NTPase presumably accounts for (a) the release of P_i from ADP and (b) the stimulation of RNA release from the nuclei by ADP, which were observed by Clawson *et al.* (1978) and Ishikawa *et al.* (1978).

Table 1. *Effect of ADP and ATP on RNA release in six preparations of nuclei*

Myokinase activity is given as nmol of NADPH/min per mg of protein. RNA efflux during a 10 min incubation in the medium used by Agutter *et al.* (1979b) was corrected by controls in which the nucleotide was replaced by 2.5 mM-EDTA. Each value is the mean of triplicate determinations. Experiments were performed before (a) and after (b) the nuclei were washed three times with incubation medium at 0°C. The correlation between myokinase activity and ADP-stimulated RNA release is highly significant ($r = +0.998, P < 0.001$).

Preparation No.	RNA release (% of total nuclear radioactivity)					
	Myokinase		2.5 mM-ADP		2.5 mM-ATP	
	(a)	(b)	(a)	(b)	(a)	(b)
1	220	50	1.9	0.22	3.8	3.3
2	27	24	0.1	Zero	3.1	2.6
3	210	71	2.1	0.46	3.6	3.0
4	300	48	2.9	0.30	4.1	3.2
5	82	53	0.6	0.35	2.8	2.6
6	390	92	3.7	0.82	4.6	3.4
Mean $\pm$ s.d.	210 $\pm$ 130	57 $\pm$ 25	1.9 $\pm$ 1.4	0.39 $\pm$ 0.28	3.7 $\pm$ 0.7	3.0 $\pm$ 0.3

The physiological importance of ADP in supplying energy for nucleocytoplasmic RNA transport *in vivo* must remain in doubt at present.

Effects of ATP analogues on RNA release

In a medium containing 2.0 mM-Mg²⁺ but lacking Ca²⁺, p[CH₂]ppA stimulated some release of RNA from isolated nuclei; under the incubation conditions used, this amounts to about half the RNA released in the presence of ATP (Fig. 1). This is consistent with the findings of Raskas & Rho (1973) and Ishikawa *et al.* (1978). When the Ca²⁺ concentration is increased to 0.5 mM, however, p[CH₂]ppA no longer promotes more RNA release than is found in controls (Fig. 1). This in turn is consistent with the findings of Yu *et al.* (1972) and Agutter *et al.* (1979b). ATP and analogues promote the leakage of DNA from nuclei, but the extent of this leakage is markedly lowered by increasing the Ca²⁺ and Mn²⁺ concentrations in the medium to 0.5 mM and 0.3 mM respectively (Fig. 2). It is noteworthy that release of neither RNA nor DNA into the medium was significantly altered by the inclusion of EDTA (Figs. 1 and 2). This supports the view (Agutter *et al.*, 1976, 1979b; Ishikawa *et al.*, 1978; Schumm & Webb, 1972, 1974) that the chelation model proposed by Chatterjee & Weissbach (1973) to explain the effect of ATP on RNA release is untenable. Increase of the Mg²⁺ concentration to 5 mM (Ishikawa *et al.*, 1978) did not significantly alter the results shown in Figs. 1 and 2.

The data summarized in Fig. 1(a) can be accommodated in a modification (Fig. 3b) of the scheme for RNA release proposed by Ishikawa *et al.* (1978). Both Fig. 3(a), which is taken from Ishikawa *et al.* (1978), and Fig. 3(b) are speculative. Investigation of their validity cannot be undertaken until purified active preparations of the nuclear-envelope preparations concerned become available. In its simplest form, this modification predicts that,

Fig. 1. *Efflux of RNA from isolated nuclei*
(a) Medium used by Ishikawa *et al.* (1978) supplemented with 0.3 mM-Mn²⁺ and 300 µg of yeast RNA/ml. (b) Medium used in Fig. 1(a) without 0.3 mM-Mn²⁺; 2.5 mM-ATP (●), -p[CH₂]ppA (■), -AMP + PP_i (□) or -EDTA (△) was added to the media; ○, controls. Values are means of three triplicate determinations. The half-error-bars represent 1 s.e.m.

Fig. 2. Efflux of DNA from isolated nuclei
The media used correspond to those described in the legends to Figs. 1(a) and 1(b), and the symbols used are as in Fig. 1.

although in the absence of Ca^{2+} (Fig. 3a) the RNA-release step (II) is rate-limiting and the ATP- or ATP-analogue-release step (III) may be either hydrolytic (IIIa) or non-hydrolytic (IIIb), in the presence of Ca^{2+} (Fig. 3b) step IIIa becomes rate-limiting and step IIIb cannot occur. It is believed in this laboratory that this modification (Fig. 3b) approximates more closely to the situation *in vivo* than does the scheme given in Fig. 3(a), since nuclei *in vivo* contain Ca^{2+} (Mirsky & Osawa, 1961; cf. Yu *et al.*, 1972). It is noteworthy that, if step I in Fig. 3(b) is more rapid than step IV (i.e. if RNA binding to structure S stimulates dephosphorylation), this scheme accounts for the stimulation of NTPase activity by RNA (Agutter *et al.*, 1977). That a phosphorylated polypeptide in the nuclear envelope represents an intermediate in NTPase activity is apparent from the results of Agutter *et al.* (1979a) and Lam & Kasper (1979).

Fig. 3. Schemes for mechanism of RNA efflux from nuclei

(a) According to Ishikawa *et al.* (1978), the RNA binds to a conformationally modified structure (S^*) in the nuclear envelope (step I) and is displaced into the cytoplasm by ATP or an ATP analogue (step II). In step III, the ATP is removed hydrolytically (IIIa) or non-hydrolytically (IIIb). Step II is rate-limiting. (b) Modification of the scheme summarized in Fig. 3(a) to account for the evidence from Fig. 1 and from Agutter *et al.* (1979b). Step IIIb of Fig. 3(a) does not occur; step IIIa is rate-limiting, and involves phosphorylation of S and re-association with ATP may occur either with associated binding and release of RNA (steps I and II), as in Fig. 3(a), or independently of RNA (steps IV and V).

The effect of $\text{AMP} + \text{PP}_i$ on RNA release (Fig. 1) and on elution of DNA (Fig. 2) from nuclei is very dependent on Ca^{2+} and Mn^{2+} concentrations in the medium. The effect is therefore likely to be attributable partly to a non-specific destabilizing effect on the nuclei in the absence of these bivalent cations, and partly to the substitution of $\text{AMP} + \text{PP}_i$ for ATP at step IIIb in low (Fig. 3a), but not at higher (Fig. 3b), Ca^{2+} concentrations (Ishikawa *et al.*, 1978). The fact that ATP stimulates release of mature mRNA (Agutter & McCaldin, 1979; Palayoor *et al.*, 1979) without destabilizing the nuclei (i.e. promoting release of DNA) under the conditions used by Agutter *et al.* (1979b) is clear from Figs. 1 and 2. This finding is inconsistent with the view that ATP stimulates RNA release from nuclei by promoting leakage of nuclear contents (Stuart *et al.*, 1975, 1977).

It must be emphasized that, although the results shown in Figs. 1 and 2 are inconsistent with the

explanations advanced by Chatterjee & Weissbach (1973), Sauermann (1974) and Stuart *et al.* (1975, 1977) for the stimulation of RNA release by ATP, they do not refute the model proposed by Raskas & Rho (1973) and Ishikawa *et al.* (1978). The alternative schemes in Fig. 3 differ in that (i) they postulate different rate-limiting steps, and (ii) in one case ATP hydrolysis is obligatory, whereas in the other it is optional. These inconsistencies are unlikely to be resolved until we understand the events taking place at the pore complex during RNA transport in greater detail.

Effects of cations and NTPase inhibitors on nuclear stability and RNA release

The omission of spermidine from a nuclear incubation medium results in extensive non-specific leakage of nuclear contents (Yu *et al.*, 1972; Schumm & Webb, 1972, 1974; Sato *et al.*, 1977a,b; Ishikawa *et al.*, 1978), and this largely accounts for the differences in extent of DNA and RNA release into the media used by Agutter *et al.* (1979b) [medium (a)] and Clawson *et al.* (1978) [medium (b)] (Table 2). F^- , which inhibits the NTPase (Table 2; cf. also Clawson *et al.*, 1978), significantly inhibits RNA release only in the medium used by Agutter *et al.* (1979b); it also appears to destabilize the nuclei, since it promotes slight efflux of DNA, possibly because it sequesters Ca^{2+} in the medium (cf. Fig. 2).

The RNA released into the medium used by

Agutter *et al.* (1979b) and into similar media (Schumm & Webb, 1972, 1974; Ishikawa *et al.*, 1978) is predominantly 8–18S material, and approx. 50% of it contains poly(A) tracts (Agutter & McCaldin, 1979). Some of this RNA is intact messenger, since it can be translated *in vitro*, for example to serum albumin (Palayoor *et al.*, 1979). Under the conditions of prelabelling and incubation described in the present paper, ribosomal subunits cannot be detected in the medium (Racvskis & Webb, 1974; Agutter & McCaldin, 1979). The RNA released under the conditions used by Clawson *et al.* (1978) contains higher-molecular-weight material, and about 10–20% of it is polyadenylated (P. S. Agutter, unpublished work).

The inhibitory effect of Cu^{2+} on RNA release (Baru *et al.*, 1978) is simulated by Co^{2+} (Table 2), but not by Mg^{2+} , Ca^{2+} or Mn^{2+} unless very high concentrations of these ions are used (Ishikawa *et al.*, 1969). The results shown in Table 2 suggest that the effects of Cu^{2+} and Co^{2+} are attributable partly to increased stabilization of the nuclei (they decrease the rate of efflux of DNA) and partly to inhibition of the NTPase. The NTPase inhibition is probably mediated through reaction of the ion with protein thiol groups (cf. Agutter *et al.*, 1979a,b), since the effects of Cu^{2+} and Co^{2+} were not additive with those of *p*-chloromercuribenzoate and could be reversed by a 5-fold molar excess of dithiothreitol.

The mechanism whereby cations enhance the

Table 2. *Effects of inhibitors on nuclear stability, RNA release and NTPase activity*

ATP-stimulated DNA and RNA release are expressed as percentages of total nuclear contents (release in presence of 2.5 mM-ATP—release in presence of 2.5 mM-EDTA). NTPase activity (μ moles of P_i /h per mg of protein) was determined by using isolated nuclear envelopes. All values are means $\pm$ 1 S.E.M. of three triplicate determinations. Key: ND, not determined; * = significant inhibition, † = significant stimulation ($P < 0.05$ by analysis of variance).

Inhibitor	Concn. (mM)	ATP-stimulated release					
		Medium (a)		Medium (b)		NTPase	
		DNA	RNA	DNA	RNA		
None	—	0.8 $\pm$ 0.3	3.1 $\pm$ 0.7	18.3 $\pm$ 4.9	26.2 $\pm$ 6.1	14.6 $\pm$ 2.1	
F^-	0.1	0.7 $\pm$ 0.4	3.3 $\pm$ 0.6	17.7 $\pm$ 4.1	28.6 $\pm$ 5.7	10.1 $\pm$ 1.9	
	0.2	1.1 $\pm$ 0.6	2.4 $\pm$ 0.4	22.1 $\pm$ 5.6	24.3 $\pm$ 5.5	5.8 $\pm$ 1.2*	
	0.5	1.7 $\pm$ 0.5†	1.6 $\pm$ 0.4*	16.6 $\pm$ 4.2	30.2 $\pm$ 7.9	3.5 $\pm$ 0.6*	
<i>p</i> -Chloromercuribenzoate	0.2	0.7 $\pm$ 0.4	2.6 $\pm$ 0.7	21.6 $\pm$ 4.8	33.6 $\pm$ 7.4	10.4 $\pm$ 1.2	
	0.5	1.1 $\pm$ 0.4	2.3 $\pm$ 0.6	29.8 $\pm$ 5.7†	45.7 $\pm$ 9.2†	8.8 $\pm$ 1.2*	
	1.0	0.6 $\pm$ 0.2	1.6 $\pm$ 0.6*	31.5 $\pm$ 6.2†	43.4 $\pm$ 9.2†	7.5 $\pm$ 0.9*	
Cu^{2+}	0.2	0.5 $\pm$ 0.2	2.6 $\pm$ 0.4	ND	ND	12.0 $\pm$ 1.7	
	0.5	0.5 $\pm$ 0.2	1.4 $\pm$ 0.5*	14.3 $\pm$ 3.1	13.1 $\pm$ 3.9*	11.0 $\pm$ 1.6	
	1.0	0.2 $\pm$ 0.1*	Zero*	ND	ND	8.9 $\pm$ 1.3*	
	2.0	0.3 $\pm$ 0.2*	Zero*	6.1 $\pm$ 1.4*	4.2 $\pm$ 0.8*	8.3 $\pm$ 1.2*	
Co^{2+}	0.2	0.7 $\pm$ 0.3	2.9 $\pm$ 0.5	ND	ND	13.9 $\pm$ 2.2	
	0.5	0.5 $\pm$ 0.3	2.1 $\pm$ 0.6	15.1 $\pm$ 3.3	15.4 $\pm$ 2.9*	12.6 $\pm$ 1.9	
	1.0	<0.2*	0.7 $\pm$ 0.3*	ND	ND	10.2 $\pm$ 1.5	
	2.0	Zero*	Zero*	9.4 $\pm$ 2.3*	7.7 $\pm$ 1.2*	7.4 $\pm$ 1.1*	

stability of nuclei is not wholly clear at present. Preliminary results from our laboratory indicate that DNA leakage is extensive only when the nuclei appear swollen in phase-contrast microscopy; it is minimal when the nuclear volume is low. It is well established that nuclei swell in media containing low concentrations of bivalent cations and shrink in media containing higher concentrations (Leake *et al.*, 1972), and spermidine appears to have a similar effect on nuclear volume. From the extensive work of Wunderlich's group on *Tetrahymena* macro-nuclei (Wunderlich & Herlan, 1977; Giese *et al.*, 1979; Herlan *et al.*, 1979), the effects of the cations appear to be mediated through the nuclear matrix (cf. Berezney & Coffey, 1974). This mechanism, if it is valid for liver nuclei, could explain the effect of *p*-chloromercuribenzoate (Table 2), which in swollen unstable nuclei may interact with matrix thiol groups (Shelton & Cochran, 1978) and cause further swelling, but may have no such effect on shrunken nuclei in which the matrix thiol groups are masked. The study of RNA efflux in *Tetrahymena* nuclei has so far only concerned the marked difference in the extent of ATP-independent leakage between expanded and contracted nuclei (Herlan *et al.*, 1979). It will be interesting to see if the conditions necessary for specific RNA transport from stable contracted nuclei resemble those for isolated mammalian liver nuclei.

In the present paper, it has been shown that changes in the incubation media used can apparently alter the dependence of RNA efflux on nucleotides by altering nuclear stability. Pre-treatment of the nuclei, including the extent of washing before incubation, can also alter RNA release by affecting the myokinase activity in the preparations. After repeated pre-washing and incubation of the nuclei in a medium in which they remain stable (i.e. in which no significant DNA efflux occurs), only ATP of the adenine nucleotides investigated promotes RNA efflux to a significant extent.

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