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Ribonucleic Acid Stimulation of Mammalian Liver Nuclear-Envelope Nucleoside Triphosphatase

A POSSIBLE ENZYMIC MARKER FOR THE NUCLEAR ENVELOPE

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1. The specific activity of rat and pig liver nuclear-envelope nucleoside triphosphatase (EC 3.6.1.3) decreases when the system is depleted of RNA. The activity can be restored by adding high concentrations of yeast RNA to the assay medium. 2. Exogenous RNA also increases the activity of the enzyme in control envelopes (not RNA-depleted). The effect appears to be largely specific for poly(A) and poly(G); it is not stimulated by rRNA or tRNA preparations, ribonuclease-hydrolysed RNA, AMP, or double- or single-stranded DNA. 3. Inhibitors of the enzyme, in concentrations at which half-maximal inhibition of the enzyme is achieved, do not affect the percentage stimulation of the enzyme by yeast RNA. 4. The stimulation is abolished by the inclusion of 150mM-KCl or -NaCl in the assay medium, but not by increasing the assay pH to 8.5. 5. The results are discussed in the light of the possible role of the nucleoside triphosphatase *in vivo* in nucleo–cytoplasmic ribonucleoprotein translocation. 6. It is proposed that poly(G)-stimulated Mg^{2+}-activated adenosine triphosphatase activity should be adopted as an enzymic marker for the nuclear envelope.

Several authors have described a nucleoside triphosphatase (Mg^{2+}-dependent ATPase,‡ EC 3.6.1.3) in mammalian liver nuclear envelopes [see Franke (1974) for a thorough review], and there is evidence that this activity is localized exclusively in or near the nuclear pore complexes (Yasuzumi & Tsubo, 1966; Franke, 1974). The enzyme has been characterized with respect to its substrate specificity and other properties (Agutter *et al.*, 1976*b*), its kinetic behaviour (Agutter *et al.*, 1976*c*) and its sensitivity to various inhibitors (Agutter *et al.*, 1976*d*). Recent evidence has suggested that its functions *in vivo* include the provision of energy for nucleo–cytoplasmic translocation of ribonucleoproteins (Agutter *et al.*, 1976*a*). This hypothesis is supported by the well-established observation that ATP is required for the elution of ribonucleoproteins from isolated nuclei (Ishikawa *et al.*, 1969, 1970; Raskas, 1971; Schumm *et al.*, 1973; McNamara *et al.*, 1975), and by the argument that the translocation process must involve a conformation change in the pore complex (Paine, 1975; Paine *et al.*, 1975). The possibility of such a function has led us to investigate the effect of exogenously added polyribonucleotides on the enzyme activity itself, and the present paper summarizes the results of this investigation.

Since the enzyme has proved intractable to all attempts at solubilization (Agutter *et al.*, 1976*b*), the work was carried out on whole isolated nuclear envelopes. These can be obtained from rat liver by the method of Harris & Milne (1974), and from pig liver by a modification of this procedure (J. F. Milne, J. R. Harris & P. S. Agutter, unpublished work). Pig liver envelopes can be obtained in bulk, and the results presented below were obtained with such material. In all cases, rat liver material gave essentially similar results.

Experimental

ATP (disodium salt, grade II), GTP (disodium salt, grade III), CTP (trisodium salt), UTP (disodium salt), NADH, glucose 6-phosphate, DNAase-free pancreatic RNAase, DNAase, poly(A), poly(G), poly(C) and poly(U) were obtained from Sigma Chemical Co., London S.W.6, U.K. $MgCl_2$ (1.0M) yeast RNA and DNA were obtained from British Drug Houses, Poole, Dorset, U.K. Mixed bovine transfer ribonucleic acids were obtained from Boehringer, Mannheim, Germany. All other reagents used were of AnalaR grade.

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‡ Abbreviations: ATPase, adenosine triphosphatase; DNAase, deoxyribonuclease; RNAase, ribonuclease.

Before use in the experiments described below, RNA and DNA solutions (1mg/ml) were dialysed exhaustively, in the presence of 5μg of proteinase K (Boehringer)/ml, against 5mM-triethanolamine/HCl+1mM-$MgCl_2$, pH 7.7, at 4°C (Wiegers & Hilz, 1972). At least 90% of the proteinase K activity was removed from the dialysed nucleic acid by passage through a column (10cm×1cm) of Sephadex G-25 and discarding the retarded material. The proteolytic activity was determined with denatured haemoglobin as substrate as described by Dounce & Umaña (1962). The homopolyribonucleotides used were phosphorylated at the 5'-termini; the poly(A), poly(U) and poly(C) had average molecular weights of about 100000, and the range of mol.wts. of poly(G) was approx. 15000–50000.

Nuclear envelopes

These were isolated from rat and pig liver essentially by the method of Harris & Milne (1974). In some experiments, the purified envelopes were incubated for 10min at room temperature (22°C) with 10μg of RNAase/ml and centrifuged for a further 90min at 100000g ($r_{av.}$ = 11.6cm) on a 0–50% (w/v) sucrose gradient in 10mM-Tris/HCl, pH7.4, in the SW 27 rotor of a Beckman L2 65B ultracentrifuge. The gradient behaviour of the envelopes (banding at a density of 1.22±0.01g/ml) was not altered by this treatment, nor was the RNAase activity significantly greater than that of control envelopes. When RNA was added to the assay mixture the amount of 260nm-absorbing material precipitable with 5% (w/v) trichloroacetic acid decreased by 20±5% during the 15min incubation in both control and RNAase-pretreated envelopes.

Enzyme assays

Nucleoside triphosphatase. This was assayed as previously described (Agutter *et al.*, 1976*b*), with 1mM-$MgATP^{2-}$, -$MgGTP^{2-}$ or -$MgUTP^{2-}$ as substrate. In brief, the assay depends on determination of the P_i released from the substrate during the incubation (Wahler & Wollenberger, 1958). The initial catalytic rate of the enzyme at the substrate concentration used is nearly maximal (Agutter *et al.*, 1976*c*); in most experiments, 15min incubations were used. ATP^{4-} and $MgATP^{2-}$ solutions were prepared and stored as described by Agutter *et al.* (1976*b*). For experiments involving inhibitors, the envelopes were preincubated for 5min at 35°C with the inhibitor at the stated concentration; in cases where 95% (v/v) ethanol was used as solvent, solvent controls were run. The inhibitor concentrations used were close to the concentrations required to give a half-maximal inhibitory effect (Agutter *et al.*, 1976*d*). These were as follows: phenyl arsenoxide 1.0mM, dithiothreitol 5.0mM, oligomycin 10μg/ml, quercetin 5μg/ml, *p*-phenylmercuribenzenesulphonate 0.6mM, adenosine 1.0mM, and AMP 1.0mM. Dithiothreitol was added to envelopes after 5min preincubation with phenyl arsenoxide at 4°C, under which conditions it relieves the inhibition by the arsenical completely (Agutter *et al.*, 1976*d*).

NADH–ferricyanide reductase (*EC* 1.6.99.3). This was assayed by the method of Zamudio *et al.* (1969).

Glucose 6-phosphatase (*EC* 3.1.3.9). This was assayed by incubating the envelopes with 5mM-glucose 6-phosphate in 50mM-sodium succinate, pH6.5, at 35°C for 30min, and determining the P_i released by the method of Wahler & Wollenberger (1958).

All enzyme activities in the present paper are given in units of μmol of substrate/h per mg of protein, mean values ± 1 S.E.M. being quoted throughout.

Assays

Protein was assayed by the modification by Maddy & Spooner (1970) of the method of Lowry *et al.* (1951), DNA by the modification (Giles & Myers, 1965) of the method of Burton (1956), RNA by the method of Drury (1948), and phospholipid by determining the P_i content (Wahler & Wollenberger, 1958) of a chloroform/methanol (2:1, v/v) extract of the nuclear envelopes (Folch *et al.*, 1957) after digestion at 100°C for 90min with 70% (v/v) $HClO_4$. Overall contents of protein, DNA, RNA and phospholipid in the nuclear-envelope preparations were expressed to the nearest 1% total weight, the contribution of carbohydrate being ignored.

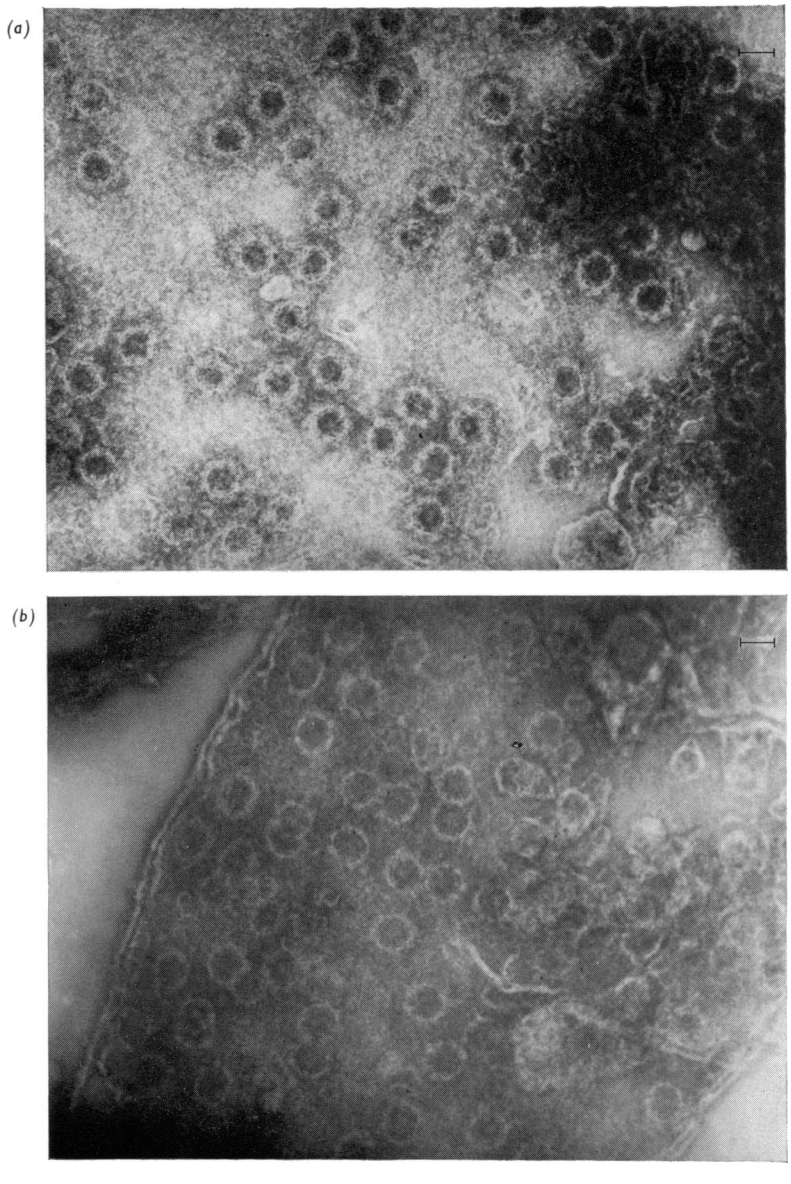
Nucleic acids

Double- and single-stranded DNA. These were separated by the method of Miyazawa & Thomas (1965) from DNA obtained from British Drug Houses.

rRNA. This was prepared by the formamide/sucrose-gradient method of Lewis *et al.* (1974) from a crude microsomal pellet obtained from pig liver homogenate.

Partial hydrolysis of the RNA preparations. This was done by incubation (at 500μg/ml concentration) with 0.1M-NaOH at 4°C for various times. The hydrolysis was terminated by neutralization with HCl. When material prepared in this way was added to the enzyme-assay media, 0.1M-NaOH neutralized with HCl was added to the controls.

Translocated ribonucleoprotein. This, which can be obtained from nuclei *in vitro* (Ishikawa *et al.*, 1969; Schumm *et al.*, 1973; Agutter *et al.*, 1976*a*), was prepared by the following method. Pig liver nuclei (10mg of protein) were incubated in 2.5ml of 25mM-triethanolamine/HCl/2mM-$MgCl_2$/1mM-ATP, pH7.7, at 35°C for 5min, and, after removal of the nuclei by centrifugation at 2000g for 5min at 0°C, EDTA (pH7.7) was added to a concentration of 1mM. The final medium thus contained approx.



EXPLANATION OF PLATE 1

Control and RNAase-pretreated rat liver nuclear envelopes

Electron micrographs of negatively stained rat liver nuclear envelopes. Scale bar = 0.1 μm. (*a*) Control envelopes; (*b*) envelopes pretreated with RNAase (50 μg/ml) for 60 min at 22°C. RNAase treatment removes amorphous material from the annuli of the pore complexes. Similar results were obtained with pig liver material.

1mM-Mg^{2+} and 1mM-ATP. Excess of Mg^{2+} is necessary during the incubation to stabilize the nuclei. The 'translocated ribonucleoprotein control' was prepared in the same way, except that ATP was omitted from the medium. After adjustment of the $MgATP^{2-}$ concentration to 1mM, samples of the supernatant were taken for nucleoside triphosphatase determination.

Gel electrophoresis

This was done on RNA fractions with the formamide/polyacrylamide-gel system described by Staynov *et al.* (1972), by using a Quickfit Instrumentation Polyacrylamide-Gel Electrophoresis Module. The gels were overlayered with water and allowed to polymerize at 30°C for 16h. The samples were loaded in formamide-containing 15% (v/v) glycerol (R. W. Old, personal communication), and the gels stained overnight in Pyronine Y and destained in 10% (v/v) acetic acid.

Electron microscopy

This was carried out on the nuclear envelopes with a Philips EM 301 electron microscope, the samples being negatively stained with 2.0% (w/v) ammonium molybdate, pH7.2, and photographed on Ilford SP 332 film.

Results

Table 1 compares the compositions and enzymic properties of untreated and RNAase-pretreated envelopes. The decreases in RNA content and nucleoside triphosphatase activity after RNAase pretreatment are significant ($P<0.01$ by analysis of variance by Student's t test). Prolongation of the RNAase digestion to 30min had no further significant effect on the RNA content of the material, nor was there a further decrease in nucleoside triphosphatase activity. Electron microscopy of the envelopes (Plate 1) showed that ultrastructural integrity was preserved after RNAase pretreatment: the only observable change was a slight enhancement of the clarity with which the annuli of the pore complexes could be seen, suggesting that some amorphous material had been removed from this region. This is consistent with the claim by Franke & Scheer (1970) that the pore complexes contain RNA. Isolation of the envelopes in the presence of 1.5M-KCl (Franke *et al.*, 1970) had a similar effect to RNAase pretreatment on the RNA content of the material and on the enzymic activity.

After RNAase pretreatment, the rate of phosphate release from $MgATP^{2-}$ was, in pig liver envelopes, significantly non-linear after 20min, in contrast with the control envelopes (Agutter *et al.*, 1976*b*), but linearity of the rate up to 1h could be restored, and the overall specific activity increased, by performing the assays in the presence of 50μg of total yeast RNA/ml (Fig. 1). The specific-activity increase depended on the amount of exogenous RNA added, but the effect appeared to be saturable (Table 2). Rapid stirring of the assay mixture was also found largely to eliminate the non-linearity. This RNA effect was not simulated by double- or single-stranded DNA, in the presence of which the activity of control and RNAase-pretreated envelopes was not significantly altered (Table 3). Effects of these various conditions on phosphate release from $MgATP^{2-}$ were found to be closely paralleled when

Table 1. *Effect of different conditions of isolation on composition and enzymology of pig liver nuclear envelopes*

Composition and enzymic activities of pig liver nuclear envelopes under various conditions of isolation and pretreatment. A: control envelopes. B: envelopes digested for 10min at 20°C with 10μg of RNAase/ml. C: envelopes incubated for 10min at 20°C with 10mM-Tris/HCl, pH7.4. D: envelopes incubated for 10min at 20°C with 10μg of DNAase I/ml. E: as B, but 30min incubation. F: as C, but 30min incubation. G: envelopes isolated by the method of Franke *et al.* (1970). H: as B, with 100μg of yeast RNA/ml included in the buffer. The activity of the nucleoside triphosphatase, but not of the other enzymes, correlates closely with the envelope RNA content.

	Envelope composition (%, by wt.)				Enzyme activities (μmol/h per mg of protein)		
Conditions	Protein	Lipid	DNA	RNA	Nucleoside triphosphatase	NADH–ferricyanide reductase	Glucose 6-phosphatase
A	66	21	4	9	10.1±1.4	8.1±1.3	13.9±2.4
B	75	19	4	2	7.2±1.8	6.2±1.4	9.1±2.2
C	69	19	4	8	10.3±1.7	6.7±1.8	8.9±2.9
D	66	23	3	8	9.8±1.6	6.5±2.0	9.4±2.0
E	78	17	3	2	6.9±1.3	7.2±1.2	2.0±0.5
F	71	17	4	8	9.6±1.8	6.0±1.6	1.5±0.5
G	75	20	2	3	6.9±1.0	2.7±1.1	0.1±0.2
H	67	19	4	10	10.0±1.5	6.8±1.7	8.4±2.4

$MgGTP^{2-}$ and $MgUTP^{2-}$ were used as substrates, supporting the conclusion (Agutter *et al.*, 1976*b*) that these substrates are all hydrolysed by the same broad-specificity enzyme in the nuclear envelope.

Fig. 2 shows the effect of preincubation of control and RNAase-pretreated envelopes at a range of temperatures on the subsequent activity of the enzyme at 35°C. The percentage thermal inactivation at each temperature was calculated by assuming 100% activity in envelopes which were assayed without preincubation. Clearly RNA has no effect on the thermal-stability of the enzyme. Preincubation of the envelopes for 5min at temperatures exceeding 57°C (not shown in Fig. 2) resulted in a sharp rise in the rate of $MgATP^{2-}$ hydrolysis subsequently measured at 35°C: thus, after preincubation at 60°C, the specific activity rose to 29.2 ± 4.8 μmol/h per mg of protein.

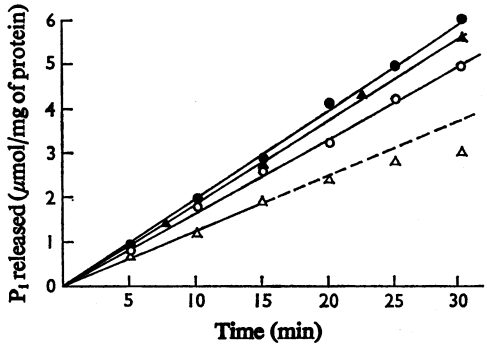


Fig. 1. *Time course of phosphate release from $MgATP^{2-}$*
Rate of hydrolysis catalysed by pig liver nuclear-envelope nucleoside triphosphatase. ○, Control envelopes; ●, control envelopes with total yeast RNA (50μg/ml) present in the assay medium; △, RNAase-pretreated envelopes; ▲, RNAase-pretreated envelopes with total yeast RNA (50μg/ml) present in the assay medium.

This presumably indicates the presence of a cryptic enzyme, not necessarily identical with the nucleoside triphosphatase present without such preincubation. Indeed, the rise in activity was not reflected by any significant increase in the rate of hydrolysis of $MgUTP^{2-}$, nor was the additional $MgATP^{2-}$-hydrolytic activity susceptible to stimulation by yeast RNA. These observations support the conclusion that the cryptic enzyme is indeed different from the nucleoside triphosphatase, and on this basis it was considered irrelevant to the subject of the present paper. The possibility that it represented mitochondrial F_1-ATPase activity resulting from contamination of the envelopes by submitochondrial fragments is excluded by the observation that it was susceptible neither to stimulation by dinitrophenol nor to inhibition by oligomycin.

The deviation of the enzyme from Michaelis–Menten kinetics (Agutter *et al.*, 1976*c*) was not affected by RNAase pretreatment or by addition of

Table 2. *Stimulation of pig liver nuclear-envelope nucleoside triphosphatase by total yeast RNA*
Effect of various concentrations of yeast RNA in assay medium on hydrolysis rate of $MgATP^{2-}$ (μmol/h per mg of protein) in (*a*) control and (*b*) RNAase-pretreated nuclear envelopes.

RNA in assay medium (μg)	Rate of hydrolysis	
	a	*b*
0	10.1±1.4	7.2±1.8
5	9.5±1.6	8.7±1.8
10	10.5±1.9	9.0±1.6
20	11.3±1.6	10.1±1.9
40	12.3±1.5	11.6±1.9
60	11.7±1.4	11.8±1.6
100	12.6±1.1	11.4±1.4

Table 3. *Effect of nucleic acids on rate of nucleoside triphosphatase hydrolysis*
Effects of nucleic acids on nucleoside triphosphatase activity in control and RNAase-pretreated pig liver nuclear envelopes. All nucleic acid preparations used were present at a final concentration of 50μg/ml in the assay media. Concentrations of nucleoside triphosphate and $MgCl_2$ were 1.0mM in all cases.

Envelopes	Assay conditions	Nucleoside triphosphatase (μmol of P_i/h per mg of protein) from:		
		ATP	GTP	UTP
Control	Control	10.1±1.4	10.6±1.9	4.8±0.7
	+double-stranded DNA	10.3±1.9	9.9±1.7	5.1±0.7
	+single-stranded DNA	9.7±1.9	10.2±1.7	4.4±0.8
	+yeast RNA	12.6±1.2	12.7±1.4	6.3±0.6
RNAase-pretreated	Control	7.2±1.8	6.8±2.0	3.6±0.9
	+double-stranded DNA	6.9±1.8	6.9±2.0	3.5±0.9
	+single-stranded DNA	7.6±1.8	7.3±1.7	3.1±1.0
	+yeast RNA	11.7±1.2	12.1±1.4	5.7±0.7

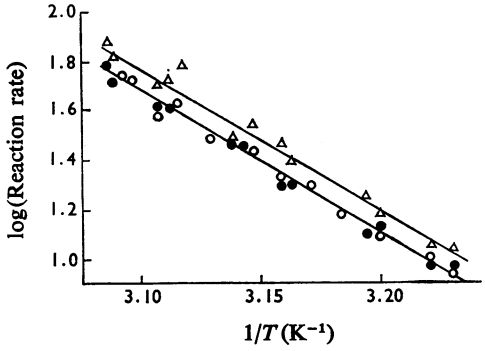


Fig. 2. *Thermal inactivation of nucleoside triphosphatase* Arrhenius plot of thermal inactivation for pig liver nuclear-envelope enzyme. ○, Control envelopes; △, RNAase-pretreated envelopes; ●, control envelopes with total yeast RNA (50μg/ml) present in assay medium.

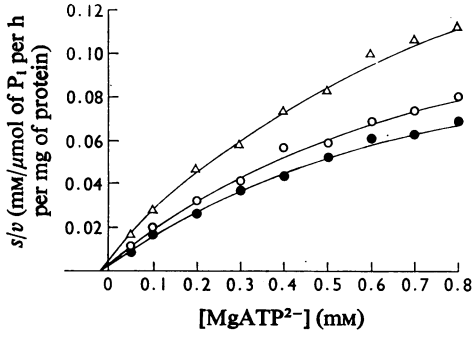


Fig. 3. *Kinetics of pig liver nuclear-envelope nucleoside triphosphatase* Eadie plot showing effect of RNA on kinetics of enzyme, with $MgATP^{2-}$ as substrate. ○, Control envelopes; ●, control envelopes with total yeast RNA (50μg/ml) present in assay medium; △, RNAase-pretreated envelopes. In all cases, the concentration of the inhibitory species Mg^{2+} (Agutter *et al.*, 1976*b*) was maintained at 0.2mM throughout the substrate-concentration range. Each point represents the mean of six experimental determinations.

yeast RNA (50μg/ml) to the assay medium. The stimulation of the enzyme by RNA was apparent at all substrate concentrations investigated (Fig. 3). Table 4 shows that inclusion of 150mM-KCl in the assay medium, under which conditions linear Eadie plots are obtained (Agutter *et al.*, 1976*c*), removed the effect of RNA on the activity. This treatment lowers the RNA content of the envelope by approx. 50%. Increase of the pH to 8.5, which has a similar

Table 4. *Sensitivity of RNA stimulation of nucleoside triphosphatase to conditions of assay*

Nucleoside triphosphatase activities (μmol/h per mg of protein) in (*a*) control envelopes, (*b*) RNAase-pretreated envelopes, (*c*) RNAase-pretreated envelopes with yeast RNA (50μg/ml) added in the assay medium, with $MgATP^{2-}$ as substrate. Assay conditions were as follows: A, control (assay pH7.7); B, activity at pH8.5; C, activity at pH7.7 in the presence of 150mM-KCl; D, activity under control conditions after pretreatment of the enzyme at 4°C for 10min with 1mM-fluorodinitrobenzene.

	Nucleoside triphosphatase		
Conditions	*a*	*b*	*c*
A	10.1±1.4	7.2±1.8	11.7±1.2
B	7.6±1.7	6.7±1.9	9.0±1.6
C	7.7±1.7	7.9±1.8	7.9±1.6
D	9.2±1.4	6.4±1.9	10.5±1.3

effect on the Eadie plot (Agutter *et al.*, 1976*c*), did not completely destroy the RNA effect.

Table 5 summarizes the effects of RNAase pretreatment of the envelopes and inclusion of RNA in the assay medium on the sensitivity of the enzyme to various inhibitors. In the presence of RNA or after RNAase pretreatment of the material, the percentage inhibition obtained is the same in all cases as it is in the control envelopes. Ethanol at the concentration (6%, v/v) used has no significant effect on the activity; AMP does not behave as an inhibitor in 1mM concentration (Agutter *et al.*, 1976*d*).

Table 6 compares the effects on the enzyme in control and RNAase-pretreated envelopes of rRNA, tRNA and the homopolyribonucleotides. Although rRNA and tRNA have no significant effect, the homopolyribonucleotides stimulate the activity, and poly(A) and poly(G) are particularly effective. Considerable stimulation is also obtained by using the translocated ribonucleoprotein from pig liver nuclei. RNA prepared from this ribonucleoprotein by chloroform/phenol extraction and ethanol precipitation had an effect on the nucleoside triphosphatase not significantly different from that of the ribonucleoprotein itself. RNAase treatment of this material, however, destroys the effect. Treatment of the yeast RNA with 10μg of RNAase/ml for 30min at 35°C, followed by passage through a column (5cm×1cm) of Sephadex G-10 to remove the enzyme together with larger RNA fragments, also resulted in loss of the stimulation. Alkaline hydrolysis of poly(A) at 4°C ultimately decreases its stimulating effect (Fig. 4). It may therefore be concluded that the effect of this polymer is a function of its molecular weight. Fig. 5 illustrates the electrophoretic mobility of the poly(A) after different times of hydrolysis; it appears that poly(A) segments of

Table 5. *Effect of inhibitors of nucleoside triphosphatase on RNA stimulation of activity*
Effects of various inhibitors of nucleoside triphosphatase on activity in (*a*) control envelopes, (*b*) RNAase-pretreated envelopes and (*c*) RNAase-pretreated envelopes with RNA (50 μg/ml) yeast present in the assay medium. Activities are given in μmol/h per mg of protein, with $MgATP^{2-}$ as substrate throughout (see the Experimental section).

Inhibitor	Concentration	Nucleoside triphosphatase *a*	*b*	*c*
None (control)	—	10.1±1.4	7.2±1.8	11.7±1.2
Phenyl arsenoxide	1.0mM	5.9±1.1	4.3±0.7	6.9±1.1
Phenyl arsenoxide+dithiothreitol	1.0mM+5.0mM	9.8±1.0	7.6±0.6	11.0±1.5
Oligomycin	10μg/ml	6.2±1.2	5.5±0.9	7.2±1.3
Quercetin	5μg/ml	5.2±0.8	3.7±0.6	5.6±1.1
p-Phenylmercuribenzenesulphonate	0.6mM	4.0±0.5	2.6±0.4	4.9±0.8
Adenosine	1.0mM	10.3±1.6	7.7±1.9	11.3±1.5
AMP	1.0mM	9.8±1.6	7.4±1.8	12.1±1.5
Ethanol	6% (v/v)	10.0±1.2	7.2±1.5	11.9±1.3

Table 6. *Comparison of stimulating effects of different RNA types*
Activities of nucleoside triphosphatase (μmol/h per mg of protein) with $MgATP^{2-}$ as substrate in (*a*) control and (*b*) RNAase-pretreated envelopes.

RNA preparation used	Nucleoside triphosphatase *a*	*b*
(Control)	10.1±1.4	7.2±1.8
Yeast RNA	12.6±1.3	11.7±1.2
RNAase-digested yeast RNA	9.6±1.9	6.9±1.0
rRNA (28S+18S)	9.9±1.7	7.9±1.0
tRNA	9.7±1.1	7.5±1.3
Poly(A)	11.7±1.4	11.2±1.2
Poly(G)	13.2±1.4	12.4±1.1
Poly(C)	10.5±1.2	8.1±1.7
Poly(U)	10.2±1.4	8.5±1.5
Transported ribonucleoprotein	11.3±1.3	11.1±1.3
Transported RNA control	10.3±1.6	7.7±1.7

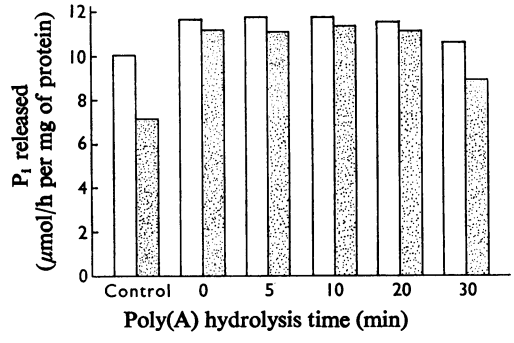


Fig. 4. *Effect of alkaline hydrolysis of poly(A) on enzyme stimulation*
Poly(A) was hydrolysed for various times under the conditions described in the text and added to the assay media to a final concentration of 50μg/ml. The control values are those obtained without addition of poly(A). □, Control; ▨, RNAase-pretreated envelopes.

length exceeding 10–15 residues will give significant stimulation of the enzyme.

Discussion

The loss of linearity of phosphate release with time consequent on depletion of the RNA content of the envelopes suggests that the stimulation of the nucleoside triphosphatase by RNA is at least partially attributable to protection against thermal denaturation, as described for the chloroplast F_2-ATPase by Livne & Racker (1969). This possibility is, however, refuted by the Arrhenius plot of denaturation (Fig. 2), which shows that the activation energy of the thermal-denaturation process is not affected by the presence or absence of RNA. It is concluded therefore that the effect is a genuine stimulation of the enzyme. The non-linearity of phosphate release after RNAase pretreatment (Fig. 1) may be attributable, since rapid stirring largely eliminates it, to either substrate depletion or product accumulation in the micro-environment of the active site; however, it is not clear how RNA prevents such diffusion-limitation effects. Very similar results (not shown) have been reproducibly obtained by using rat liver nuclear envelopes.

The possibility that RNA is activating another enzyme catalysing ATP hydrolysis, inactive under the conditions under which the nucleoside triphosphatase is normally assayed, is essentially refuted (a) by the similar specificity for substrate shown by the enzyme in the presence and absence of RNA (Table 3) and (b) by the results obtained by using inhibitors of the nucleoside triphosphatase (Table 4). RNA and RNAase pretreatment do not affect the percentage

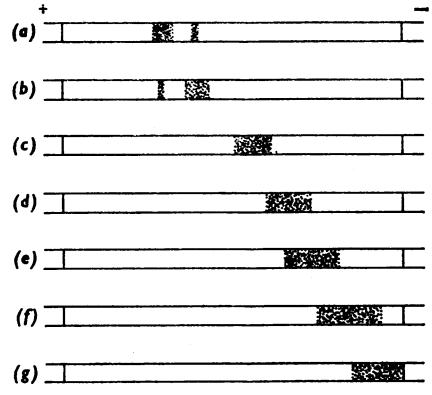


Fig. 5. *Gel electrophoresis of partially hydrolysed poly(A)* Staining pattern on disc gels given by (*a*) 28S rRNA, (*b*) 18S rRNA, and poly(A) hydrolysed for (*c*) 0, (*d*) 5, (*e*) 10, (*f*) 20 and (*g*) 30min. Assuming that the electrophoretic system used results in a linear relationship between log (molecular weight) and electrophoretic mobility (Staynov *et al.*, 1972), and accepting molecular-weight values for 28S and 18S rRNA of 1.69×10^6 and 0.67×10^6 respectively (Loening, 1968), the molecular-weight values for the hydrolysed poly(A) samples are: 0min, 0.7×10^5–1.6×10^5; 5min, 1.6×10^4–6.3×10^4; 10min, 0.8×10^4–4.0×10^4; 20min, 1.8×10^3–12.6×10^3; 30min, less than 4.0×10^3.

inhibition caused by any of these compounds. Table 1 shows that RNA exerts no stimulating effect on the other enzymes investigated. Glucose 6-phosphatase is unstable in the nuclear envelope, and exposure to room temperature quickly inactivates it. This observation may account for the wide range of values of nuclear-envelope glucose 6-phosphatase activity reported in the literature: long preparation procedures and vigorous conditions, such as sonication, probably result in extensive loss of activity. NADH–ferricyanide reductase activity (EC 1.6.99.3) for which once again widely varying values have been reported, also shows some inactivation on incubation at 22°C. It may be noted that this enzyme is less than 5% rotenone-sensitive; this observation, along with the findings that succinate dehydrogenase (EC 1.3.99.1) and cytochrome oxidase (EC 1.9.3.1) activities are not detectable in nuclear-envelope preparations, and that the nucleoside triphosphatase is not dinitrophenol-stimulated, argues against the possibility that the partial inhibition of the nucleoside triphosphatase by oligomycin that we have repeatedly observed (Agutter *et al.*, 1976*d*) is attributable to mitochondrial contamination. The inconsistency between our observation on the effect of oligomycin and that of Jarasch *et al.* (1973),

Table 7. *Correlation between envelope RNA content and nucleoside triphosphatase activity*

Comparison of (*a*) mean rates of hydrolysis of $MgATP^{2-}$ (μmol/h per mg of protein) and (*b*) percentage RNA contents in a range of nuclear-envelope preparations described in the literature. The symbols 'h' and 'l' represent heavy and light bands respectively in the density gradients used in the preparations.

Source	Reference	*a*	*b*
Rat liver (h)	Kashnig & Kasper (1969)	2.3	3.1
(l)		1.7	1.6
Rat liver	Franke *et al.* (1970)	5.6	2.5
Rat liver	Agutter (1972)	16.1	5.0
Rat liver	J. R. Milne, J. F. Harris & P. S. Agutter (unpublished work)	16.4	7.0
Rat liver (h)	Zbarsky *et al.* (1969)	14.0	2.1
(l)		23.1	3.3
Pig liver	Franke *et al.* (1970)	6.9	3.5
Pig liver	J. R. Milne, J. F. Harris & P. S. Agutter (unpublished work)	10.1	9.0
Bovine liver	Berezney *et al.* (1970)	7.0	8.5

who found no such inhibition, is not easy to explain. However, after subjection of our nuclear-envelope preparations to 1.5M-KCl [the conditions used by Jarasch *et al.* (1973) in envelope isolation; cf. Franke *et al.* (1970)], we have observed that oligomycin-sensitivity is no longer apparent. It is therefore possible that this sensitivity is lost in high-ionic-strength media.

The fact that 150mM-KCl completely suppresses the stimulation by RNA of the nucleoside triphosphatase and decreases the control activity to about the value obtained with RNA-depleted envelopes is consistent with previous observations on the effect of increasing ionic strength (Agutter *et al.*, 1976*b*,*c*). NaCl has an effect indistinguishable from that of KCl, and once again very similar results were obtained with rat and pig liver material. On removal of the salt and inclusion of RNA in the assay medium, stimulation is again seen. Envelopes isolated from pig liver nuclei after suspension in 1.5M-KCl (Franke *et al.*, 1970) have a nucleoside triphosphatase activity (7.2 μmol/h per mg of protein) similar to that seen when envelopes isolated by the method of Harris & Milne (1974) were assayed in the presence of 150mM-KCl. This value is in close agreement with that given by Franke *et al.* (1970) for pig liver. At the same time, the RNA content of the material is decreased by high-ionic-strength treatment. The correlation between envelope RNA content and nucleoside triphosphatase activity in a range of preparations of nuclear envelope is fair (Table 7). The activity reported by Zbarsky *et al.* (1969) is in this respect anomalous: however, it must be noted that

envelopes prepared in this way show dinitrophenol stimulation of ATP hydrolysis (Delektorskaya & Perevoshchikova, 1969), in contrast with our own findings and reports from other laboratories (Jarasch *et al.*, 1973; Franke, 1974). In view of the claim by Zbarsky's group (Buldyayeva *et al.*, 1972) that oxidative phosphorylation occurs in the nuclear envelope, it may be suggested that the anomalously high rate of ATP hydrolysis is the result of mitochondrial contamination. There is certainly good evidence that the occurrence of mitochondrial cytochromes in nuclear-envelope preparations is an artifact (Jarasch & Franke, 1974). The general correlation between nucleoside triphosphatase activity and envelope RNA content leads us to propose that the salt-induced inactivation of the enzyme on storage, previously not explained (Agutter *et al.*, 1976*b*), is in fact attributable to removal of RNA from the system by salt.

Given the observation that the translocated ribonucleoprotein stimulates the nucleoside triphosphatase to an extent comparable with that observed when poly(A) is used, and accepting (a) the fact that many mRNA sequences carry a poly(A) segment at their 3'-ends (Darnell *et al.*, 1971; Edmonds *et al.*, 1971), (b) the probability that the enzyme is involved in the translocation of messenger ribonucleoprotein (Agutter *et al.*, 1976*a*), it is tempting to suggest that the specific stimulating effect of poly(G) and poly(A) is relevant to the mechanism of this process. Such an argument is rendered less tenable by three observations. First, the poly(A) segment is by no means a universal feature of mRNA molecules (Milcavek *et al.*, 1974; Nemer *et al.*, 1975). Secondly, the percentage stimulation of the enzyme observed in these experiments is, though significant, not great. Thirdly, modification of the apparently allosteric behaviour of the enzyme (Agutter *et al.*, 1976*c*) by RNA is not apparent; irrespective of the presence of RNA, the concave-downward curvature of the Eadie plot (Fig. 3) is maintained. Against these reservations, the following considerations may be set. First, it is clear from Figs. 4 and 5 that only a very short poly(A) sequence (of the order of 10–15 nucleotides) is needed for activation, and in any case the effect is not absolutely specific for poly(A). Secondly, the assay conditions (pure RNA added to a bulk suspension of envelopes) do not simulate conditions *in vivo* at all closely, and may not permit a maximally effective interaction between the enzyme and RNA to occur; thus local concentrations of RNA may be very high *in vivo*, whereas under the assay conditions the added RNA may interact with and stimulate only a small percentage of the total population of enzyme sites; moreover, RNAase pretreatment does not completely deplete the system of RNA to begin with. Thirdly, failure to alter the Eadie-plot curvature cannot be regarded as a serious reservation at this stage, because the details of the mechanism responsible for the curvature are not completely clear (Agutter *et al.*, 1976*c*,*d*). Thus it remains possible that the stimulation by RNA is physiologically significant in the sense suggested above. The hypothesis is strengthened by the fact that we have not been able to demonstrate similar stimulation by RNA of ATP hydrolysis in other subcellular fractions, nor are we aware of any reports of such stimulation in the literature. This remark pertains, of course, only to cases where the $\beta\gamma$-anhydride bond of the triphosphate is hydrolysed, as is the case for the nucleoside triphosphatase (Agutter *et al.*, 1976*b*). Stimulation of the ATP/polynucleotidyl exotransferase by poly(A), for example, would be expected (Maale *et al.*, 1975), but here the $\alpha\beta$-bond is lysed.

It has often been pointed out (e.g. Franke, 1974; Harris & Agutter, 1976) that there is no established enzymic marker for the nuclear envelope. In view of the failure to demonstrate stimulation by RNA of $\beta\gamma$-anhydride hydrolysis of ATP in other cell fractions, and of the particular efficacy of poly(G) in stimulating it in the nuclear envelope, it seems reasonable to propose poly(G)-stimulated Mg^{2+}-activated ATPase activity as such a marker. However, the above results suggest that this marker cannot be used when preparations have been made in high-ionic-strength media, or when there are high concentrations of endogenous RNA or ribonucleoprotein to interact with and stimulate the enzyme. Moreover, if cells or cell fractions have been subjected to conditions sufficiently vigorous to disrupt the nuclear envelope, it should be remembered that the enzyme is probably associated only with the pore complexes (Yasuzumi & Tsubo, 1966; Franke, 1974) and will not therefore indicate the presence of inner or outer nuclear-membrane vesicles. It must also be demonstrated that the products of stimulated hydrolysis are ADP and P_i, rather than PP_i. It may be noted that the rate of hydrolysis of ADP by nuclear envelopes is less than 2% that of ATP, and that of PP_i less than 10%. Notwithstanding these reservations, poly(G) stimulation of Mg^{2+}-activated ATPase activity at pH7.7 may prove useful for indication of contamination by nuclear envelope in other subcellular fractions under appropriate conditions of preparation and assay.

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