Properties of Mammalian Nuclear-Envelope Nucleoside Triphosphatase

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The nucleoside triphosphatase activities of the nuclear envelopes from rat liver, pig liver and simian-virus-40-transformed mouse-embryo 3T3 cells were shown to exhibit similar properties. All three preparations hydrolyse ATP, 2'-dATP, 3'-dATP, GTP, CTP and UTP in the presence of Mg^{2+} , Ca^{2+} , Mn^{2+} and Co^{2+} with a pH optimum of 8.0, are sensitive to inhibition by mercurials, arsenicals, quercetin, proflavin and adenosine 5'-[γ -thio]triphosphate and are partially inactivated by exposure to high ionic strength. The kinetic behaviour is similar for all substrates irrespective of the source of material. The typical Eadie-Hofstee plot, which is concave upwards at pH 8.0 when the ionic strength is 20 mM, becomes linear when the pH is increased to 8.5 or the ionic strength to 160 mM. The overall evidence, particularly the labelling of only one polypeptide by [γ - ^{32}P]ATP, suggests that under the conditions of preparation and assay used only one class of nucleoside triphosphatase active sites is detectable in nuclear envelopes. The importance of these results for an understanding of the role of the enzyme *in vivo* is discussed.

Mg^{2+} -activated ATPase activity (EC 3.6.1.4) has been reported in isolated nuclei (Schneider, 1946; Rees & Rowland, 1961) and in isolated nuclear envelope from rat liver (Zbarski *et al.*, 1969; Delektorskaya & Perevoshchikova, 1969; Kashnig & Kasper, 1969; Franke *et al.*, 1970; Harris & Agutter, 1976), pig liver (Franke *et al.*, 1970; Harris & Agutter, 1976), bovine liver (Berezney *et al.*, 1970), neoplastic tissues (Delektorskaya & Perevoshchikova, 1969), calf thymus (Jarasch *et al.*, 1973) and hen erythrocytes (Zentgraf *et al.*, 1971). Other NTPase activities have been reported in liver nuclear-envelope preparations (Kartenbeck *et al.*, 1973). In addition, cytochemical staining studies have indicated an association of the ATPase activity with the nuclear-pore complexes (Klein & Afzelius, 1966; Yasuzumi & Tsubo, 1966; Yasuzumi *et al.*, 1967, 1968; Franke, 1974). Whether or not this indication is valid, the observation cannot be explained as a non-enzymic

redistribution of the lead phosphate deposited, since similar studies on glucose 6-phosphatase (EC 3.1.3.9) show this enzyme to be more or less homogeneously distributed through the perinuclear cisterna (Kartenbeck *et al.*, 1973; Franke, 1974).

The ATPase is not stimulated by Na^+ or K^+ ions and is not inhibited by ouabain (Delektorskaya & Perevoshchikova, 1969; Franke *et al.*, 1970; Zentgraf *et al.*, 1971), and it therefore appears not to be involved in the transport of univalent cations across the nuclear envelope. This conclusion is corroborated in the present paper. There have been some reports of oxidative phosphorylation in the nuclear envelope (Rees & Rowland, 1961; Buldayeva *et al.*, 1972), but these are controversial (Berezney & Crane, 1972; Agutter *et al.*, 1977). Given the very low content of mitochondrial cytochromes in the envelope (Berezney *et al.*, 1970; Berezney & Crane, 1972) and the likelihood that such of these as occur are artifacts of preparation (Jarasch & Franke, 1974, 1977), it does not seem probable that the mitochondrial type of ATP-generating system could be present in sufficient concentration to account for all the observed NTPase activity. Several reports have indicated that the addition of ATP to isolated nuclei stimulates the release of ribonucleoproteins, predominantly mRNA, into the supernatant (Schneider, 1959; Ishikawa *et al.*, 1969, 1970*a,b,c*; Raskas, 1971; Brunner & Raskas, 1972; Schumm & Webb, 1972, 1974; Schumm *et al.*, 1973), and we have already presented evidence indicating that the hydrolysis of nucleoside triphosphate in the nuclear envelope is

Abbreviations used: ATPase, adenosine triphosphatase; NTPase, nucleoside triphosphatase; SV40-3T3 cells, simian-virus-40-transformed mouse-embryo 3T3 cells.

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necessary for this translocation process to occur (Agutter *et al.*, 1976, 1977).

In the present paper we provide evidence suggesting that the ATPase activity and other NTPase activities mentioned above are attributable to a single type of enzyme in the nuclear envelope; this is referred to below simply as NTPase. The properties of this enzyme, including its kinetic behaviour, substrate and bivalent metal ion specificity, sensitivity to a range of inhibitors, and dependence on pH, ionic strength and temperature, are described. The consistency in these properties between the preparations obtained from rat liver, pig liver and cultured SV40-3T3 fibroblasts is demonstrated. This detailed knowledge of the properties of the enzyme is necessary for critical examination of the hypothesis that it is essential for nucleocytoplasmic translocation of RNA. The investigation was carried out with whole nuclear envelopes; all attempts to solubilize and purify the enzyme were unsuccessful. Butanol extraction (Maddy, 1966; Rega *et al.*, 1967), treatment with deoxycholate or non-ionic detergents (Dunlany & Touster, 1970; Tzagoloff & Penefsky, 1971; Konopka *et al.*, 1972; Umbreit & Strominger, 1973) or with urea or guanidinium chloride rapidly inactivated the enzyme. Phospholipases A and C did not affect the activity but did not solubilize it. A previous report has emphasized the instability of the enzyme and described its inactivation by media of high ionic strength and by sonication (Porteous *et al.*, 1978).

Materials and Methods

ATP (disodium salt, grade II), 2'-dATP (disodium salt), 3'-dATP (disodium salt), GTP (disodium salt, grade II), CTP (trisodium salt, grade II), UTP (disodium salt, grade III), ADP (disodium salt), 2'-deoxyadenosine, AMP (disodium salt), actinomycin D, colchicine, atracyloside, gramicidin, oligomycin, ouabain, proflavin, quercetin, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate and iodoacetamide were obtained from Sigma Chemical Co., London S.W.6, U.K. Adenine and adenosine were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Cyclic AMP (sodium salt), phenyl arsenoxide and 1.0M stock solutions of MgCl₂ and CaCl₂ were obtained from BDH Chemicals, Poole, Dorset, U.K. Adenosine 5'-[β -methylene]triphosphate and adenosine 5'-[γ -thio]triphosphate were obtained from Boehringer, Mannheim, Germany. [γ -³²P]ATP was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. *p*-[(4,6-Diamino-1,3,5-triazin-2-yl)amino]phenyl arsenoxide (Melarsen) and *p*-amino-phenyl arsenoxide (Atoxyl) were kindly given to the authors by Dr. I. A. Flynn. All other reagents used were of AnalaR grade from BDH Chemicals.

Bivalent cation-nucleoside triphosphate solutions

These were prepared in the following manner. Stock nucleoside triphosphate solutions were adjusted to pH 8.0–8.5 with NaOH and stored at –20°C; under these conditions they showed no detectable increase in P_i concentration in 3 months. Solutions of bivalent metal ion salts (chloride or sulphate) were added to these, and the final concentrations of free bivalent cation, free nucleoside triphosphate and bivalent cation-nucleoside triphosphate complex were computed by assuming the dissociation constants given by Phillips (1966).

Nuclear envelope

This was isolated from rat and pig liver by the method of Harris & Milne (1974). In the isolation of pig liver envelope, a second incubation of the material with deoxyribonuclease (10 min at 20°C; first incubation 20 min at 20°C) was used, and ten washes in bicarbonate buffer were performed before final purification of the material on a sucrose gradient. SV40-3T3-cell nuclear envelope was prepared as follows. SV40-3T3 cells were grown for 3–4 days in roller culture in Dulbecco's modification of Eagle's medium (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) containing newborn-calf serum (10%, v/v). The cells were washed in 10mM-Tris/HCl/2mM-MgCl₂, pH 7.4, and homogenized in this medium by 15–20 passages through a syringe needle of 1.1 mm internal diameter. Then 60% (w/v) sucrose in 10mM-Tris/HCl/2mM-MgCl₂, pH 7.4, was added slowly until the final sucrose concentration was 55 ± 1% (w/v) by refractometry, when the suspension was layered over 7.0 ml of 58% (w/v) sucrose in 10mM-Tris/HCl/2mM-MgCl₂, pH 7.4, in 37.5 ml-capacity centrifuge tubes, and centrifuged at 130000g for 60 min at 4°C in the SW 27 rotor of a Beckman L2-65B ultracentrifuge. The pellet of nuclei was resuspended in 10 ml of homogenization buffer and re-pelleted by centrifugation at 1000g for 10 min at 4°C, then resuspended in 1mM-NaHCO₃/0.1mM-MgCl₂, pH 7.6–8.0, and incubated for 10 min at 0°C. The pellet obtained on centrifugation at 1000g for 10 min was resuspended in the same NaHCO₃/MgCl₂ buffer to a concentration of approx. 2 mg of protein/ml, and deoxyribonuclease I (BDH Chemicals) was added to a concentration of 10 µg/ml. After incubation at 20°C for 20 min, the material was then subjected to the procedure described by Harris & Milne (1974) to obtain purified nuclear envelopes.

NTPase activity

Nuclear envelope (normally 0.1 mg of protein/ml) was incubated for 10–30 min in 25mM-triethanolamine/HCl buffer (pH 8.0 for most experiments) at 35°C in the presence of 1mM-bivalent cation-nucleoside triphosphate complex. For experiments

with the SV40–3T3-cell material, a 2.5 mM substrate concentration was normally used. Release of P_i was determined by the absorbance at 310 nm of an ethyl acetate extract of phosphomolybdic acid (Wahler & Wollenberger, 1958). For experiments in which the substrate concentration was varied, the concentration of free bivalent metal ion was kept constant, since these species appeared to be more potent inhibitors of the enzyme than were free nucleoside triphosphates (see below). For experiments in which the temperature was varied, the pH of the buffer was re-adjusted at each temperature.

Isotachopheresis

This was performed with the LKB Tachophor at pH 3.75, with β -alanine as the leading electrolyte and hexanoic acid as the terminating electrolyte. The temperature was maintained at $15 \pm 0.01^\circ\text{C}$ and the current at $100 \pm 1 \mu\text{A}$, the potential increasing from 2 to 10 kV during the 20 min separation. These conditions, which were used to demonstrate that ADP rather than AMP was produced by hydrolysis of ATP by the NTPase, were derived from the work of Beckers & Everaerts (1972) and Dunn & Kemp (1974).

Labelling of the enzyme with ^{32}P

This was done by incubating the envelopes with [γ - ^{32}P]ATP as described by Kyte (1972) and Lane *et al.* (1973).

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis

Envelope protein (approx. 0.1 mg) was incubated at 100°C for 5 min in 0.4 ml of 20 mM-Tris/glycine buffer, pH 8.1, containing 2% (w/v) sodium dodecyl sulphate, 10 mM-2-mercaptoethanol and 15% (v/v) glycerol. Portions (20 μl) of the cooled solution were layered on 10% (w/v) acrylamide/0.25% NN' -dimethylbis(acrylamide) gels, and electrophoresis was performed as described by Shapiro *et al.* (1967) with a Quickfit disc gel electrophoresis apparatus. Protein staining was done with Coomassie Brilliant Blue as described by Fairbanks *et al.* (1971). After being dried, labelled gels were radioautographed for 28 days with Ilford Industrial G X-ray film.

Inhibitors

These were added to the nuclear-envelope suspension in aqueous or ethanolic solution and preincubated with the envelope for 5 min at 35°C before the addition of substrate. Solvent controls showed that ethanol in the concentration used [up to 7% (v/v) in the final assay mixture] had no detectable effect on the enzyme activity.

Modification of thiol groups

This was effected by incubation of nuclear-en-

velope suspensions for various times before assay with the modifier at 0°C at pH 8.0. It was assumed that at any inhibitor concentration maximal inhibition had been reached when further incubation gave no further increase of inhibition. On this basis the minimal inhibitor concentration required for maximal final inhibition, and the concentration required for half-maximal final inhibition, were determined. The reversibility of thiol-group modification was determined by treatment of phenyl arsenoxide-inhibited envelopes with dithiothreitol in 5-fold excess.

Enzyme assays

Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of King (1963), 5'-nucleotidase (EC 3.1.3.5) by the method of Belfield & Goldberg (1969) and NADH-ferricyanide reductase (EC 1.6.99.3) by the method of Zamudio *et al.* (1969).

Chemical assays

Protein was determined by a modification (Maddy & Spooner, 1970) of the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and RNA by the method of Ashwell (1957).

Significance testing

In experiments involving inhibitors of the NTPase, the significance of the inhibition was determined by analysis of variance as described by Freund (1967). Significance was accepted at the 5% level.

Results and Discussion

General

With ATP or UTP as substrate in the presence of either Mg^{2+} or Ca^{2+} ions, release of P_i was linear with time for up to 30 min at 35°C . The pH optimum of the enzyme was 8.0 irrespective of the substrate used; activity was halved by lowering the pH to 7.5 or increasing it to 8.3. Arrhenius plots were linear between 1 and 40°C ; above 40°C thermal denaturation became significant over a 30 min incubation. With MgATP^{2-} , MgGTP^{2-} and CaATP^{2-} as substrate the Arrhenius plots were parallel for nuclear envelopes obtained from the same tissue. ADP rather than AMP was detected by isotachopheresis after the incubation, indicating that hydrolysis of the β - γ bond had occurred.

Dixon plots (Fig. 1) show that free Mg^{2+} and free ATP (predominantly ATPH^{3-} at the pH of the experiments; Phillips, 1966) inhibit the enzyme competitively, and maximum activity is obtained with a ratio of Mg^{2+} to ATP of approximately 1:1. In common with other Mg^{2+} -activated ATPases, therefore, the substrate is presumably MgATP^{2-} or other bivalent cation-nucleoside triphosphate complex (see below under 'Substrate specificity'). In these

experiments it was assumed that the dissociation constant for MgATP^{2-} was 0.05 mM (Phillips, 1966).

Composition of the nuclear envelopes

The specific activity of succinate dehydrogenase in the envelopes from all sources was less than 2% of

Fig. 1. Dixon plots showing inhibition of rat liver nuclear-envelope NTPase activity by Mg^{2+} and ATP^{3-} . (a) Competitive inhibition of enzyme by Mg^{2+} ; $\circ$, 1 mM- MgATP^{2-} ; $\blacksquare$, 2 mM- MgATP^{2-} ; $\bullet$, 5 mM- MgATP^{2-} . (b) Competitive inhibition by free ATP: $\circ$, 1 mM- MgATP^{2-} ; $\blacksquare$, 2 mM- MgATP^{2-} . Each value shown is the mean for four experimental determinations. K_i for Mg^{2+} = 0.4 mM; K_i for ATP^{3-} = 0.9 mM.

that in whole homogenates, and that of 5'-nucleotidase less than 5%. NADH-ferricyanide reductase activity values were 252, 128 and 195 nmol of NADH/min per mg of envelope protein for rat liver, pig liver and SV40-3T3-cell preparations respectively, but in all cases less than 5% of the activity was rotenone-sensitive. These results are consistent with those obtained by Milne *et al.* (1978), and are taken to indicate that the preparations were substantially free of mitochondrial and plasma-membrane contamination. Sialic acid was not detectable. The RNA content of the envelopes was reproducible (6.1 ± 0.9 , 4.4 ± 0.7 and $6.8 \pm 1.1\%$ by wt. in rat liver, pig liver and SV40-3T3-cell nuclear envelopes respectively), so marked differences of NTPase activity would not be expected to occur as a result of differences in RNA content of different preparations (Agutter *et al.*, 1977).

Orientation of the enzyme in the nuclear envelope

Trypsin (0.01%) inactivated the enzyme within 2 min at 20°C. After incubation of intact rat liver nuclei with trypsin insolubilized on agarose beads (a gift to the authors from Dr. W. E. Hornby) in the nuclear suspension medium described by Schumm *et al.* (1973) for 3 min, ultrastructurally recognizable nuclear envelopes could still be isolated by the procedure of Harris & Milne (1974), but the NTPase activity was less than 5% of that normally found. When the trypsin was inactivated by preincubation of the beads with 1 M-NaOH followed by repeated washing in the nuclear suspension medium, the NTPase activity was within 10% of the normal value. This was taken to indicate that the active site of the enzyme is on the cytoplasmic surface of the envelope.

Substrate specificity

The substrate specificities of NTPases from different sources are summarized in Tables 1 and 2. The breadth of substrate specificity observed is in quali-

Table 1. Substrate specificity of nuclear-envelope NTPases

NTPase activities of rat liver and pig liver nuclear envelopes were measured with 1.25 mM- MgCl_2 plus 1.25 mM-ATP, 2'-dATP, 3'-dATP, GTP, CTP or UTP as substrate (columns a), or with 2.5 mM- MgCl_2 plus 1.25 mM-ATP plus 1.25 mM other nucleoside triphosphate (columns b). Values are means $\pm$ s.e.m. for five determinations. For SV40-3T3-cell nuclear envelopes all concentrations were 2.2-fold higher.

Nucleoside triphosphate	NTPase activity ($\mu\text{mol of P}_i \text{ released/h per mg of protein}$)					
	Rat liver nuclear envelopes		Pig liver nuclear envelopes		SV40-3T3-cell nuclear envelopes	
	(a)	(b)	(a)	(b)	(a)	(b)
ATP	16.4 ± 1.2	17.1 ± 1.1	10.1 ± 2.1	10.7 ± 0.8	7.4 ± 1.1	7.9 ± 1.2
2'-dATP	16.4 ± 1.0	18.0 ± 1.3	10.7 ± 1.0	10.2 ± 1.1	6.9 ± 1.0	7.7 ± 1.4
3'-dATP	15.8 ± 1.9	17.2 ± 2.0	9.5 ± 1.7	10.6 ± 0.9	7.9 ± 1.3	8.4 ± 0.9
GTP	18.0 ± 1.0	20.2 ± 1.5	11.0 ± 1.3	10.6 ± 1.1	8.0 ± 1.0	8.6 ± 1.3
CTP	9.8 ± 0.9	14.6 ± 1.2	4.4 ± 0.8	9.8 ± 1.0	2.8 ± 0.6	6.8 ± 1.2
UTP	10.5 ± 1.1	14.9 ± 1.2	4.8 ± 0.8	10.0 ± 0.7	3.5 ± 0.8	7.1 ± 1.2

Table 2. *Effects of different bivalent cations on NTPase activity*

Activities of NTPases were measured with 1 mM-bivalent metal ion-ATP complexes (2.5 mM with SV40-3T3-cell material) as substrate, and in the bottom row with 1 mM-MgCl₂ + 1 mM-CaCl₂ + 2 mM-ATP as substrate. Values are means $\pm$ S.E.M. for four determinations. N.D., Not determined.

Bivalent metal ion salt	NTPase activity (μ mol of P _i released/h per mg of protein)		
	Rat liver nuclear envelopes	Pig liver nuclear envelopes	SV-40-3T3-cell nuclear envelopes
MgCl ₂	16.4 $\pm$ 1.2	10.1 $\pm$ 2.1	7.4 $\pm$ 1.1
MgSO ₄	16.7 $\pm$ 1.1	10.1 $\pm$ 1.0	7.2 $\pm$ 0.8
CaCl ₂	13.4 $\pm$ 1.0	7.6 $\pm$ 0.8	6.0 $\pm$ 0.7
MnSO ₄	11.5 $\pm$ 1.1	7.4 $\pm$ 0.7	4.4 $\pm$ 0.6
CoCl ₂	9.2 $\pm$ 1.0	6.3 $\pm$ 0.5	3.8 $\pm$ 0.9
ZnSO ₄	1.3 $\pm$ 0.5	2.1 $\pm$ 0.4	Trace
NiSO ₄	Trace	Trace	N.D.
BeCl ₂	Zero	Zero	Zero
MgCl ₂ + CaCl ₂	15.5 $\pm$ 1.8	10.4 $\pm$ 1.0	N.D.

tative, though not quantitative, agreement with that observed by Kartenbeck *et al.* (1973). The effects of the different ions and nucleoside triphosphates are not additive. These results may suggest that only one type of active site is involved in the hydrolysis of all the bivalent cation-nucleoside triphosphate complexes examined in these preparations.

Labelling of the enzyme by [γ -³²P]ATP

Fig. 2(a) shows a sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the nuclear envelope, and Fig. 2(c) shows a radioautograph of such a gel after incubation of the intact envelope with 100 μ Ci of [γ -³²P]ATP for 5 min at 20°C at pH 8.0 (ATP concentration 0.4 mM), followed by addition of unlabelled ATP to a final concentration 10 mM. There appears to be a sodium dodecyl sulphate-resistant association of label with a single polypeptide, corresponding to the major 62000-dalton band visible on Coomassie Blue staining. The persistence of the labelling of this band in the presence of excess of unlabelled ATP suggests that it represents phosphorylation of the polypeptide rather than ATP binding to it. Moreover, labelling of the band is prevented by inclusion of 20 μ g of quercetin/ml or 1 mM-adenosine 5'-[γ -thio]triphosphate in the incubation buffer; these reagents completely inhibit the NTPase (see below). The appearance of the radioautograph was not significantly altered if the film was exposed for 3 months. The small trace of label in the lower part of the gel probably represents some phosphorylation of the histones that generally appear in nuclear-envelope preparations. It is noteworthy

that the major band labelled apparently corresponds to one of the three polypeptides observed by Aaronson & Blobel (1975); indeed, the label is not extracted from the envelopes with 2.0% Triton X-100 or with 1 M-KCl, the media used in the isolation of the pore-lamina. This observation is consistent with the claim that the NTPase is located in the pore complex (Yasuzumi & Tsubo, 1966).

Polypeptides other than the 62000-dalton band may have been labelled by the ³²P and the label lost in subsequent manipulations; moreover, it is not certain that this band is the NTPase itself, since the possibility of phosphate exchange between adjacent proteins in the envelope cannot be excluded. After incubation of labelled envelopes with unlabelled ADP, we have been unable to detect labelled ATP in the assay medium by using the t.l.c. procedure described by Renderath & Renderath (1964). This lack of phosphate exchange may cast some doubt on the validity of the labelling studies. However, in support of the identification of the labelled band as the

Fig. 2. *Labelling of nuclear-envelope proteins by [γ -³²P]-ATP*

Pig liver nuclear-envelope proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after labelling with radioactive ATP, as described in the text. (a) Gel stained with Coomassie Blue. (b) Molecular-weight standards: I, bovine serum albumin (mol.wt. 68000); II, ovalbumin (mol.wt. 45000); III, chymotrypsin (mol.wt. 23000); IV, lysozyme (mol.wt. 14400). (c) Gel radioautographed with Industrial G X-ray film. The position of the labelled band corresponds to a mol.wt. of 62000 $\pm$ 3000.

NTPase, it is noteworthy that Allen *et al.* (1977) have observed that a band of similar molecular weight becomes labelled by ATP in preparations of the pore-complex-rich nuclear matrix. In addition, envelopes prepared by the method of Bornens (1977), which includes heparin in the isolation medium, show extremely low NTPase activities ($0.3\text{--}0.9\text{ }\mu\text{mol of P}_i/\text{h per mg of protein}$ in rat liver preparations with MgATP^{2-} as substrate), and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of this material shows virtual absence of the 62000-dalton band.

Effect of ionic strength

When nuclear envelopes were assayed in media of increased ionic strength, the activity of the NTPase decreased (Fig. 3). This effect seems comparable with the effects of media of high ionic strength on microsomal ATPases (Schwartz & Laseter, 1964; Bray, 1973; Izutsu *et al.*, 1974). Sucrose at 250 mM concentration had no effect on activity. When the envelopes were recovered after a 15 min assay in 200 mM-KCl and re-assayed without KCl, the activity was not significantly less than that in controls. This suggests

Fig. 3. Effect of increasing ionic strength on rat liver nuclear-envelope NTPase activity

Activities were measured in the presence of NaCl (○) and KCl (●) with 1 mM-MgATP²⁻ as substrate and in the presence of KCl (▲) with 2 mM-MgATP²⁻ as substrate. The pH of the buffer was re-adjusted to pH 8.0 at each salt concentration. The values shown represent the means of four experimental determinations.

that activity was not lost because of solubilization of envelope components at high ionic strength. By assuming dissociation constants of 0.1 M for NaATP^{3-} and KATP^{3-} (Phillips, 1966), the inclusion of 200 mM-NaCl or -KCl in the assay medium would decrease the concentration of MgATP^{2-} to 0.38 mM and increase that of Mg^{2+} to 0.87 mM. Kinetic studies and the known inhibitory effect of Mg^{2+} (Figs. 3 and 4) would account for an activity lower than control values by 40–45%. That these changes could account for the observed effects of increased ionic strength on the enzyme is further suggested by the finding that increase of the MgATP^{2-} concentration in the assay medium to 2 mM significantly protects against the effect of KCl. Perhaps the similar effects noted in microsomal ATPase preparations (see above) could be explained in the same way.

That univalent ion-ATP complexes do not themselves act as substrates for the NTPase was demonstrated by omitting MgCl_2 from the assay medium and including various concentrations of NaCl or KCl. The activity was in all cases decreased to less than 5% of that observed in the presence of optimal Mg^{2+} concentration. Simultaneous inclusion of 60 mM-NaCl and 10 mM-KCl with MgCl_2 in the medium did not result in an increase in the observed activity. This indicates that the amount of $\text{Na}^+ + \text{K}^+$ -stimulated Mg^{2+} -dependent ATPase activity in the preparations was negligible, a finding consistent with the reports by Delektorskaya & Perevoshchikova (1969), Franke *et al.* (1970) and Zentgraf *et al.* (1971).

Kinetic studies

Fig. 4 shows the dependence of initial reaction rate on substrate (MgATP^{2-}) concentration at pH 8.0 for the three nuclear-envelope preparations used. Deviation from linearity of the Eadie-Hofstee plots is most marked at low substrate concentration, where the reaction rate is significantly higher than would have been predicted from simple Michaelis-Menten kinetics. Inhibition by free Mg^{2+} or free ATP^{3-} cannot account for this effect: the concentration of free Mg^{2+} was maintained at 0.25 mM throughout for all the results given in Fig. 4, so the ATP^{3-} must have risen from 2.5 μM concentration at 10 μM - MgATP^{2-} to 0.25 mM at 1.0 mM- MgATP^{2-} . If ATP^{3-} inhibition alone were being observed, the curve would be concave-downwards, not concave-upwards. A curve of similar shape was obtained if the free Mg^{2+} concentration was maintained at 0.75 mM. Similar arguments apply, and similar results are obtained, if the free ATP^{3-} concentration is kept constant at either 0.25 mM or 2 mM.

Similarly curved Eadie-Hofstee plots are obtained with CaATP^{2-} and MgGTP^{2-} as substrates (Fig. 4). The interpretation of these results is complicated by the heterogeneity of the whole nuclear-envelope system, in which the NTPase has at present to be

assayed (Levitzki & Koshland, 1969; Koshland, 1970). The possibility that two or more enzymes differing in K_m (Michaelis constant) and V_{max} values are acting is, however, difficult to support. Apart from the evidence described above, which is indicative of one class of NTPase sites in the system, it would argue, given the results in Fig. 4, that both enzymes show similarly broad substrate and bivalent cation specificities, and that the ratio of K_m to V_{max} is more or less constant for all bivalent cation-nucleoside triphosphate complexes used. Further difficulties for this interpretation arise from the results shown in Fig. 5, which shows that the Eadie-Hofstee plots become linear when the pH is raised to 8.5 (Fig. 5a) and when the ionic strength is increased by inclusion

of 150mM-KCl in the assay medium (Fig. 5b; the Mg^{2+} concentration in this case was 0.75 mM throughout). If one of two enzymes detectable at pH 8.0 were

Fig. 4. Eadie-Hofstee plot for NTPase activity at pH 8.0 (a) Rat liver nuclear envelopes; (b) SV40-3T3-cell nuclear envelopes. Activities were measured with $MgATP^{2-}$ ($\circ$), $CaATP^{2-}$ ($\bullet$) and $MgGTP^{2-}$ ($\square$) as substrate. Values are the means for between five and 13 experimental determinations. The curvature is significant ($P < 0.001$ by regression analysis and χ -squared) in each case.

Fig. 5. Linearization of Eadie-Hofstee plot by increase of pH or ionic strength

(a) Rat liver nuclear envelopes at pH 8.5. Activities were measured with $MgATP^{2-}$ ($\square$) and $MgGTP^{2-}$ ($\blacksquare$) as substrate. (b) Rat liver nuclear envelopes at pH 8.0. Activities were measured in the presence of 150mM-KCl with $MgATP^{2-}$ as substrate. Each value is the mean for four experimental determinations. Similar results were obtained with other substrates and other nuclear-envelope preparations.

totally inactive at pH 8.5, it is unlikely that the pH optimum of the two enzymes could be identical; but the NTPase activity shows only a single pH optimum (8.0).

Feldherr (1974) presented evidence to show that, under physiological conditions, the cytoplasmic face of the nuclear-pore complex is positively charged. The curvature of the Eadie-Hofstee plot at pH 8.0 could therefore be interpreted as resulting from an electrostatic interaction between the enzyme (assuming it to be located in this positively charged region) and the negatively charged substrate, producing a higher-than-bulk concentration of substrate in the micro-environment of the active site. The effect of increasing the pH to 8.5 is consistent with this interpretation. Electrostatic partitioning of this kind has been described in other systems by Engasser & Horvath (1975) and Katchalski *et al.* (1971). However, under the assay conditions used at pH 8.0, the bulk Cl^- concentration is 14 mM. This would be expected to saturate the double layer and overcome electrostatic partitioning of this kind.

This reasoning does not, however, exclude the possibility of preferential adsorption of the substrate. To examine this, 2.0 ml of 40 mM-triethanolamine/HCl, pH 8.0, containing 0.5 mg of pig liver nuclear-envelope protein/ml, was incubated with 1 mM-1-fluoro-2,4-dinitrobenzene for 1 h at 0°C. The absorbance of a 1% (w/v) sodium dodecyl sulphate solution of the dialysed envelopes at 450 nm was not increased by prolonging the incubation time to 4 h or by increasing the fluorodinitrobenzene concentration to 2 mM. This was taken to indicate that all accessible amino groups of the envelope were dinitrophenylated under the conditions used; moreover, Feldherr (1974) has shown that such treatment removes the positive charge of the pore complex. Though preferential adsorption does not necessarily require that the surface be positively charged, the high density of dinitrophenol residues that must have accumulated round the pore complex during this treatment would have radically altered the properties of the surface and made it highly likely that the extent of preferential substrate adsorption would have been changed. The dinitrophenylation effected a slight (10%) decrease in the enzyme activity, but the curvature of the Eadie-Hofstee plot was not significantly affected. This result presumably refutes the electrostatic-partitioning hypothesis. Repulsion between substrate and negatively charged regions of the envelope cannot account for the results either, since such repulsion would be not less significant at pH 8.5 than at 8.0, but this pH increase gives rise to linear Eadie-Hofstee plots (Fig. 5).

A Hill plot that uses velocity fractions (Fig. 6) indicates a minimum of 0.5 substrate-binding site per enzyme subunit at low substrate concentration and of 6.0 substrate-binding sites at concentrations

over 0.8 mM. There is a clear discontinuity between the two parts of the plot. Binding curves have not been obtained, since the number and heterogeneity of non-catalytic ATP-binding sites on the envelope are unknown, and the results would therefore be difficult to interpret.

The possibility that the deviation from Michaelis-Menten kinetics is attributable to substrate-induced conformation changes in the NTPase is to some extent supported by the observation that adenosine 5'-[γ -thio]triphosphate, an inhibitor of the enzyme (see below), renders the Eadie-Hofstee plot linear. It is possible that adenosine 5'-[γ -thio]triphosphate behaves as a substrate-like ligand with respect to its effect on the conformation. It is noteworthy that adenosine 5'-[β -methylene]triphosphate has no such effect. With an impure enzyme preparation it is not possible to distinguish between the different possible types of ligand-induced conformation change (Koshland, 1970). The existence of two or more polymorphic forms of the enzyme, non-interconvertible at pH 8.0 (Levitzki & Koshland, 1969), would be difficult to reconcile with the existing evidence unless increase of pH and ionic strength (Fig. 5) or inclusion of adenosine 5'-[γ -thio]triphosphate in the medium preferentially inactivated all but one form, or converted all the enzyme into a single form. Moreover, the ultrastructural homogeneity of the pore complexes might suggest that an enzyme associated with them would always have the same polymorphic form.

Finally, the non-linearity of the Eadie-Hofstee plot is unlikely to be due to diffusion limitation, a phenomenon that can result in deviation from Michaelis-Menten kinetics in artificially immobilized enzymes. The thickness of the nuclear envelope (about 500 nm)

Fig. 6. Hill plot (velocity fractions) for rat liver nuclear-envelope NTPase activity

The data were obtained from the experiments used for Fig. 4, with $MgATP^{2-}$ as substrate. The slopes, calculated by the method of least squares, are 0.5 (for substrate concentrations below 0.8 mM) and 6.0 (for higher substrate concentrations).

is insignificant compared with the thickness of membranes used for artificial immobilization (about 1 mm; Hamilton *et al.*, 1974). In any case it would be difficult to reconcile this interpretation with the linearization of the plot in media of increased pH or ionic strength or media containing adenosine 5'-[γ -thio]triphosphate.

Inhibitor studies

Table 3 shows the effects of various adenine derivatives on NTPase activity. Adenine, adenosine and 2'-deoxyadenosine have no effect, whereas AMP, ADP and cyclic AMP inhibit slightly, but reproducibly. The trace release of P_i from ADP is probably attributable to a slight contamination of this reagent, detectable on isotachopheresis, by ATP. The release of P_i from AMP may represent trace 5'-nucleotidase activity (EC 3.1.3.5), which may be intrinsic to the nuclear envelope or may represent some trace plasma-membrane contamination. Adenosine 5'-[$\beta\gamma$ -methylene]triphosphate does not inhibit the enzyme. At high concentration, there is a trace release of P_i from adenosine 5'-[$\beta\gamma$ -methylene]triphosphate that may arise from small traces of ATP in the material, though slight hydrolysis of the reagent itself cannot be excluded; from the kinetic studies (Fig. 4) and the fact that adenosine 5'-[$\beta\gamma$ -methylene]triphosphate does not inhibit (Table 3), this trace cannot exceed 0.2% of the total. Adenosine 5'-[γ -

thio]triphosphate inhibits the enzyme, and it also has the effect of making the Eadie-Hofstee plot linear. It must be emphasized that these results could only be obtained with freshly prepared adenosine 5'-[γ -thio]triphosphate solutions: on storage at -20°C the reagent was largely hydrolysed to ATP, to the detriment of its potency as an inhibitor. The results given in Table 3 were obtained with rat liver material, but pig liver and SV40-3T3-cell envelopes showed the same behaviour.

Table 4 shows the effects of a range of inhibitors on the NTPase activities. Ouabain has no effect, in accordance with the claim that there is no detectable sodium-pump activity in the envelope (see above under 'Effect of ionic strength'). Gramicidin and the mitochondrial nucleotide-transport inhibitor atracytuloside (Pfaff *et al.*, 1965) do not inhibit. 2,4-Dinitrophenol does not enhance the activity, a result consistent with those obtained by Jarasch *et al.* (1973) and Franke (1974), but inconsistent with the finding by Delektorskaya & Perevoshchikova (1969). However, the partial inhibition shown by oligomycin was not observed by Jarasch *et al.* (1973). Although the explanation for this discrepancy is not clear, we have found that subjection of our envelope preparations to the isolation conditions used by Jarasch *et al.* (1973), namely 1.5M-KCl, results in loss of the partial sensitivity to oligomycin. Colchicine, which is a potent inhibitor of nucleo-cytoplasmic RNA

Table 3. *Effects of adenine derivatives on rat liver nuclear-envelope NTPase activity*
Activities were measured in the presence and in the absence of 1.0mM-MgATP $^{2-}$ under otherwise the same assay conditions. Values are means $\pm$ s.e.m. for the numbers of experiments given in parentheses.

Reagent	Concn. (mM)	NTPase activity (μmol of P_i released/h per mg of protein)			
		1.0mM-MgATP $^{2-}$ present		MgATP $^{2-}$ absent	
None (control)	—	16.4 $\pm$ 2.1	(25)	—	(25)
Adenine	1.0	16.6 $\pm$ 2.7	(9)	Zero	(9)
	2.0	15.8 $\pm$ 3.2	(6)	Zero	(6)
Adenosine	1.0	15.9 $\pm$ 3.4	(6)	Zero	(6)
	2.0	16.2 $\pm$ 3.8	(6)	Zero	(6)
2'-Deoxyadenosine	1.0	17.5 $\pm$ 4.2	(3)	Zero	(3)
	2.0	16.4 $\pm$ 4.2	(3)	Zero	(3)
AMP	1.0	14.8 $\pm$ 2.8	(6)	0.9 $\pm$ 0.4	(6)
	5.0	12.4 $\pm$ 3.6	*(9)	3.2 $\pm$ 2.0	(9)
Cyclic AMP	1.0	15.2 $\pm$ 3.4	(6)	Zero	(6)
	5.0	13.1 $\pm$ 3.1	*(9)	Trace	(9)
ADP	1.0	15.6 $\pm$ 3.8	(6)	Trace	(6)
	5.0	13.7 $\pm$ 2.0	*(9)	1.2 $\pm$ 0.4	(9)
[$\beta\gamma$ -Methylene]ATP	1.0	16.0 $\pm$ 3.0	(5)	Zero	(5)
	5.0	16.9 $\pm$ 3.8	(5)	Trace	(5)
[γ -Thio]ATP	1.0	9.1 $\pm$ 3.0	*(5)	1.2 $\pm$ 0.5	(5)
	2.0	6.2 $\pm$ 2.0	*(5)	2.2 $\pm$ 0.8	(5)

* Significant inhibition compared with control ($P < 0.05$ by analysis of variance).

Table 4. *Percentage activities of NTPases surviving in the presence of various inhibitors*

Activities were measured in the presence of a range of possible inhibitors. The activities represented by the control values (100%) are 16.4 ± 2.1 , 10.1 ± 1.5 and 7.4 ± 1.4 μmol of P_i released/h per mg of protein for rat liver, pig liver and SV40-3T3-cell material respectively. Values are means $\pm$ S.E.M. for the numbers of experiments given in parentheses. N.D., Not determined.

Inhibitor	Concn.	NTPase activity (% of control value)			Concn. required for half-maximal inhibitory effect ($\mu\text{g}/\text{ml}$)
		Rat liver nuclear envelopes	Pig liver nuclear envelopes	SV40-3T3-cell nuclear envelopes	
None (control)	—	100 ± 13 (25)	100 ± 15 (14)	100 ± 19 (10)	—
Quabain	1.0 mM	97 ± 18 (6)	104 ± 12 (4)	98 ± 10 (4)	—
	2.0 mM	96 ± 11 (6)	95 ± 10 (4)	106 ± 12 (4)	—
Gramicidin	5 $\mu\text{g}/\text{ml}$	107 ± 10 (6)	109 ± 12 (6)	113 ± 16 (3)	—
	10 $\mu\text{g}/\text{ml}$	105 ± 11 (4)	102 ± 9 (6)	111 ± 14 (3)	—
	50 $\mu\text{g}/\text{ml}$	111 ± 13 (6)	109 ± 13 (6)	106 ± 11 (3)	—
Oligomycin	5 $\mu\text{g}/\text{ml}$	85 ± 16 (3)*	85 ± 11 (6)*	79 ± 12 (6)*	4.5 ± 0.5
	10 $\mu\text{g}/\text{ml}$	55 ± 10 (3)*	67 ± 8 (6)*	N.D.	
	50 $\mu\text{g}/\text{ml}$	60 ± 10 (3)*	65 ± 8 (3)*	55 ± 9 (3)*	
Atractyloside	5 $\mu\text{g}/\text{ml}$	N.D.	95 ± 16 (3)	110 ± 13 (3)	—
	50 $\mu\text{g}/\text{ml}$	N.D.	91 ± 16 (3)	94 ± 14 (3)	—
Actinomycin D	1 $\mu\text{g}/\text{ml}$	103 ± 9 (8)	N.D.	96 ± 11 (3)	—
	10 $\mu\text{g}/\text{ml}$	89 ± 16 (3)	N.D.	110 ± 17 (3)	—
Proflavin	200 $\mu\text{g}/\text{ml}$	N.D.	55 ± 7 (4)*	44 ± 7 (4)*	100 ± 20
Quercetin	2 $\mu\text{g}/\text{ml}$	81 ± 7 (6)*	82 ± 11 (6)*	76 ± 13 (6)*	5.5 ± 1.0
	5 $\mu\text{g}/\text{ml}$	58 ± 10 (6)*	52 ± 6 (6)*	66 ± 7 (6)*	
	10 $\mu\text{g}/\text{ml}$	11 ± 5 (6)*	21 ± 4 (6)*	14 ± 2 (6)*	
	20 $\mu\text{g}/\text{ml}$	Zero (6)*	Zero (6)*	Zero (6)*	
Colchicine	0.5 mM	106 ± 11 (4)	102 ± 9 (3)	N.D.	—
	1.0 mM	95 ± 7 (4)	97 ± 8 (3)	N.D.	—
	2.0 mM	78 ± 9 (4)*	70 ± 6 (3)*	N.D.	—
	4.0 mM	52 ± 8 (4)*	59 ± 4 (3)*	N.D.	—

* Significant inhibition compared with control ($P < 0.05$ by analysis of variance).

transport (Schumm & Webb, 1974), inhibits the NTPase significantly only at high concentration.

Table 5 indicates the maximal extent of inhibition attainable with a range of thiol-blocking reagents and the concentrations of these reagents required for half-maximal effect. The results were obtained by using a 5 min incubation of the reagent with the envelopes at 0°C ; prolongation of the treatment to 1 h did not lead to further loss of activity, beyond that observed with control envelopes (Porteous *et al.*, 1978). The rapidity of completion of all thiol-group reaction in the envelope is supported by the observation that on treatment with *p*-hydroxymercuribenzoate the absorbance at 250 nm [determined after dispersion of the envelopes in 1% (w/v) sodium dodecyl sulphate] reached a maximum in 30 s and thereafter showed no further change. The results in Table 5 suggest that the NTPase active site contains no thiol group necessary for substrate binding or catalysis. The partial inactivation by mercurials and arsenicals could have resulted from alkylation of thiol groups close to the active site, the modifying group then interfering sterically or electrostatically

with binding or catalysis, or from alkylation of groups more distant from the active site, producing a conformation change resulting in partial loss of activity. Melarsen, which has a large aromatic substituent, is no more or less active than phenyl arsenoxide as an inhibitor, but this does not refute the steric-interference hypothesis, since presumably the orientation of the modifying group will be important, as well as its size. Atoxyl, which is similar in size to phenyl arsenoxide but is positively charged at the pH of the experiment (pK 8.5), is a less potent inhibitor. This may be due to electrostatic repulsion from the pore complex (Feldherr, 1974). The greater inhibition obtained with the water-soluble mercurial *p*-chloromercuribenzenesulphonate may indicate that it reacts with groups in a hydrophilic environment not accessible to *p*-hydroxymercuribenzoate or the arsenicals examined.

Inclusion of MgATP^{2-} (1 mM) in the envelope suspension did not affect the percentage inhibition achieved with *p*-chloromercuribenzenesulphonate, *p*-hydroxymercuribenzoate or phenyl arsenoxide, or the rate of increase of A_{250} . This is consistent with the

Table 5. *Effects of thiol-blocking reagents on NTPase activity*

Percentage activities surviving after incubation with the minimum concentration of thiol-blocking reagent necessary to effect maximal inhibition were measured. Full details are given in the Materials and Methods section. The 100% values are given in the legend to Table 4. Values are means $\pm$ S.E.M. for six experiments. Except for iodoacetamide, all reagents showed significant inhibition ($P < 0.05$ by analysis of variance).

Reagent	Range of concns. tested (mM)	Rat liver nuclear envelopes		SV40-3T3-cell nuclear envelopes		Concn. required for half-maximal inhibitory effect (mM)
		Minimum concn. required (mM)	NTPase activity (% surviving)	Minimum concn. required (mM)	NTPase activity (% surviving)	
Iodoacetamide	0.5-4.0	—	100 $\pm$ 8	Not determined		—
N-Ethylmaleimide	0.5-12.0	6.5	84 $\pm$ 10	5.0	89 $\pm$ 8	—
Dithionitrobenzoate	0.2-3.0	0.5	79 $\pm$ 10	1.0	82 $\pm$ 7	—
p-Chloromercuri-benzenesulphonate	0.1-2.0	1.0	18 $\pm$ 5	0.8	13 $\pm$ 4	0.3 $\pm$ 0.1
p-Hydroxymercuri-benzoate	0.1-2.0	1.0	48 $\pm$ 4	0.8	59 $\pm$ 6	0.6 $\pm$ 0.1
Phenyl arsenoxide	0.1-2.0	1.0	42 $\pm$ 5	1.0	52 $\pm$ 6	0.5 $\pm$ 0.1
Atoxyl	0.2-2.0	0.5	61 $\pm$ 3	Not determined		0.2 $\pm$ 0.05
Melarsen	0.2-2.0	1.5	46 $\pm$ 8	Not determined		0.7 $\pm$ 0.2

conclusion that the thiol groups labelled are not located in the active site. Dithiothreitol, which itself does not affect the NTPase activity, reverses the inhibition achieved with phenyl arsenoxide when added in 5-fold molar excess, suggesting that the arsenical had reacted with thiol groups in the envelope. Dithioerythritol behaved in an indistinguishable manner under the same conditions.

Uniqueness of the NTPase

Several lines of evidence presented in this paper suggest that, under the assay conditions used, the nuclear-envelope preparations manifested only one class of NTPase active sites: (a) the activities obtained with different nucleoside triphosphates, or with different bivalent cations with ATP as substrate, are not additive; (b) there is a single pH optimum irrespective of the substrate used; (c) the Arrhenius plots obtained with different substrates are parallel; (d) the Eadie-Hofstee-plot curvatures at pH 8.0 are similar for all substrates; (e) a single polypeptide on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis is labelled by [γ - 32 P]ATP.

The conclusion that there is only one active NTPase in the system is consistent with the evidence for its localization in the pore complexes, which appear to form a homogeneous population. The evidence in question includes cytochemical staining studies (Yasuzumi & Tsubo, 1966), the relatively low specific activity of the enzyme in avian erythrocyte nuclear envelopes (Zentgraf *et al.*, 1971) and the probable involvement of the enzyme in nucleocytoplasmic RNA transport (Agutter *et al.*, 1976).

There is evidence for a second ATPase activity in mammalian nuclear envelopes, not apparent under the conditions of preparation and assay used but

revealed after treatment of the envelopes at 60°C for 3 min (Agutter *et al.*, 1977). This second activity is specific for MgATP $^{2-}$, insensitive to proflavin and not stimulated by exogenous RNA; its physiological significance is as yet unclear.

The NTPase discussed in the present paper exhibits strikingly similar properties in the three nuclear-envelope sources examined, and preliminary results suggest that its properties in sheep liver and endometrium are again similar. This point may be important if the hypothesis that it is involved in nucleo-cytoplasmic RNA transport is valid.

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References

- Aaronson, R. P. & Blobel, G. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1007-1011
- Agutter, P. S., McArdle, H. J. & McCaldin, B. (1976) *Nature (London)* **263**, 165-167
- Agutter, P. S., Harris, J. R. & Stevenson, I. (1977) *Biochem. J.* **162**, 671-679
- Allen, S. L., Berezney, R. & Coffey, D. S. (1977) *Biochem. Biophys. Res. Commun.* **75**, 111-116
- Ashwell, G. (1957) *Methods Enzymol.* **3**, 73-105
- Beckers, J. L. & Everaerts, F. M. (1972) *J. Chromatog.* **71**, 380-390

- Belfield, A. & Goldberg, D. M. (1969) *J. Clin. Pathol.* **22**, 144-151
- Berezney, R. & Crane, F. L. (1972) *J. Biol. Chem.* **247**, 5562-5568
- Berezney, R., Funk, L. K. & Crane, F. L. (1970) *Biochim. Biophys. Acta* **223**, 61-70
- Bornens, M. (1977) *Nature (London)* **270**, 80-82
- Bray, G. M. (1973) *Biochim. Biophys. Acta* **298**, 239-245
- Brunner, M. & Raskas, H. J. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3101-3104
- Buldyayeva, T. W., Kuzmina, S. N. & Zbarski, I. B. (1972) *Dokl. Acad. Nauk SSSR* **202**, 467-472
- Delektorskaya, S. N. & Perevoshchikova, K. A. (1969) *Biokhimiya* **34**, 199-203
- Dunlaney, J. T. & Touster, O. (1970) *Biochim. Biophys. Acta* **196**, 29-34
- Dunn, J. P. D. & Kemp, R. B. (1974) *Protides Biol. Fluids Proc. Colloq.* **22**, 727-736
- Engasser, J. M. & Horvath, C. (1975) *Biochem. J.* **145**, 431-435
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606-2612
- Feldherr, C. M. (1974) *Exp. Cell Res.* **85**, 271-277
- Franke, W. W. (1974) *Int. Rev. Cytol. Suppl.* **4**, 71-236
- Franke, W. W., Deumling, B., Ermen, B., Jarasch, E. D. & Kleinig, H. (1970) *J. Cell Biol.* **46**, 379-395
- Freund, J. E. (1967) *Modern Elementary Statistics*, 3rd edn., pp. 302-308, Prentice-Hall, Englewood Cliffs
- Hamilton, B. K., Gardner, C. R. & Colton, C. K. (1974) *AICRE J.* **20**, 503-510
- Harris, J. R. & Agutter, P. S. (1976) in *Biochemical Analysis of Membranes* (Maddy, A. H., ed.), pp. 132-173, Chapman and Hall, London
- Harris, J. R. & Milne, J. F. (1974) *Biochem. Soc. Trans.* **2**, 1251-1253
- Ishikawa, K., Kuroda, C. & Ogata, K. (1969) *Biochim. Biophys. Acta* **179**, 316-331
- Ishikawa, K., Kuroda, C., Ueki, M. & Ogata, K. (1970a) *Biochim. Biophys. Acta* **213**, 495-504
- Ishikawa, K., Kuroda, C. & Ogata, K. (1970b) *Biochim. Biophys. Acta* **213**, 505-512
- Ishikawa, K., Ueki, M., Nagai, K. & Ogata, K. (1970c) *Biochim. Biophys. Acta* **213**, 542-544
- Izutsu, K. T., Siegel, I. A. & Brisson, D. L. (1974) *Biochim. Biophys. Acta* **373**, 361-368
- Jarasch, E. D. & Franke, W. W. (1974) *J. Biol. Chem.* **249**, 7245-7254
- Jarasch, E. D. & Franke, W. W. (1977) *Exp. Cell Res.* **109**, 450-454
- Jarasch, E. D., Reilly, C. E., Comes, P., Kartenbeck, J. & Franke, W. W. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 979-986
- Kartenbeck, J., Jarasch, E. D. & Franke, W. W. (1973) *Exp. Cell Res.* **81**, 175-194
- Kashnig, D. M. & Kasper, C. B. (1969) *J. Biol. Chem.* **244**, 3786-3792
- Katchalski, E., Silman, I. & Goldman, R. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol.* **34**, 445-536
- King, T. E. (1963) *J. Biol. Chem.* **238**, 4037-4042
- Klein, R. L. & Afzelius, B. A. (1966) *Nature (London)* **212**, 609
- Konopka, K., Gross-Bellard, M. & Turski, W. (1972) *Enzyme* **13**, 269-277
- Koshland, D. E. (1970) *Enzymes 3rd Ed.* **1**, 342-397
- Kyte, J. (1972) *J. Biol. Chem.* **247**, 7642-7649
- Lane, L. K., Copenhaver, J. H., Lindenmayer, G. E. & Schwartz, A. (1973) *J. Biol. Chem.* **248**, 7197-7200
- Levitzki, A. & Koshland, D. E. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 1121-1128
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Maddy, A. H. (1966) *Biochim. Biophys. Acta* **117**, 193-200
- Maddy, A. H. & Spooner, R. O. (1970) *Vox Sang.* **18**, 34-41
- Milne, J. F., Agutter, P. S., Harris, J. R. & Stubbs, G. (1978) *Biochem. Soc. Trans.* **6**, 271-273
- Pfaff, E., Klingenberg, M. & Heldt, H. W. (1965) *Biochim. Biophys. Acta* **104**, 312-315
- Phillips, R. (1966) *Chem. Rev.* **66**, 501-527
- Porteous, E. R., Jamieson, S. A. & Agutter, P. S. (1978) *Biochem. Soc. Trans.* **6**, 1174-1177
- Raskas, H. J. (1971) *Nature (London) New Biol.* **233**, 134-136
- Rees, K. R. & Rowland, G. F. (1961) *Biochem. J.* **78**, 89-95
- Rega, A. F., Weed, R. I., Reed, C. F., Berg, G. G. & Rothstein, A. (1967) *Biochim. Biophys. Acta* **147**, 297-312
- Renderath, K. & Renderath, E. (1964) *J. Chromatog.* **16**, 111-117
- Schneider, J. H. (1959) *J. Biol. Chem.* **234**, 2728-2732
- Schneider, W. C. (1946) *J. Biol. Chem.* **165**, 585-593
- Schumm, D. E. & Webb, T. E. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1259-1265
- Schumm, D. E. & Webb, T. E. (1974) *Biochem. J.* **139**, 191-196
- Schumm, D. E., McNamara, D. J. & Webb, T. E. (1973) *Nature (London) New Biol.* **245**, 201-203
- Schwartz, A. & Laseter, A. H. (1964) *Biochem. Pharmacol.* **13**, 337-348
- Shapiro, A. V., Vinuela, E. & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun.* **28**, 815-821
- Tzagoloff, A. & Penefsky, H. S. (1971) *Methods Enzymol.* **22**, 219-230
- Umbreit, J. N. & Strominger, J. L. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2997-3001
- Wahler, B. E. & Wollenberger, A. (1958) *Biochem. Z.* **329**, 508-520
- Yasuzumi, G. & Tsubo, I. (1966) *Exp. Cell Res.* **43**, 284-292
- Yasuzumi, G., Nakai, Y., Tsubo, I., Yasuda, M. & Sugioka, T. (1967) *Exp. Cell Res.* **45**, 261-276
- Yasuzumi, G., Tsubo, I., Okada, K., Terawaki, A. & Enomoto, Y. (1968) *J. Ultrastruct. Res.* **23**, 321-332
- Zamudio, I., Cellino, M. & Canessa-Fisher, M. (1969) in *The Molecular Basis of Membrane Function* (Tosteson, D. C., ed.), pp. 545-571, Academic Press, New York
- Zbarski, I. B., Perevoshchikova, K. A., Delektorskaya, L. N. & Delektorsky, V. V. (1969) *Nature (London)* **221**, 257-259
- Zentgraf, H., Deumling, B., Jarasch, E. D. & Franke, W. W. (1971) *J. Biol. Chem.* **246**, 2986-2995