Tamm, I., Hand, R., & Caliguiri, L. A. (1976) *J. Cell Biol. 69*, 229–240.
Wang, D., & Moore, S. (1978) *J. Biol. Chem. 253*, 7216–7219.
Weinberg, R. A., & Penman, S. (1968) *J. Mol. Biol. 38*, 289–304.
Zurawski, G., Brown, K., Killingly, D., & Yanofsky, C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4271–4275.

Pertinence of Nuclear Envelope Nucleoside Triphosphatase Activity to Ribonucleic Acid Transport†

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ABSTRACT: Nuclear envelopes were isolated from purified rat and calf liver nuclei via different methods. Basic characterization of nuclear envelopes demonstrated phospholipid distributions similar to those found in microsomes, but enzymatic compositions and fatty acid moieties of phosphatidylserine differed from those in microsomes. A substantial Mg^{2+}-dependent NTPase was found in nuclear envelopes from both sources. The activity was linear with protein concentration and showed a sharp pH dependency with maximal activity near pH 7.5. Arrhenius analysis of the activity in rat liver preparations disclosed an activation energy of 13.8 kcal/mol, and Lineweaver–Burk plots showed a K_m of 1.8 mM ATP. Under similar conditions, calf liver preparations showed an activation energy of 13.3 kcal/mol and a K_m of 1.9 mM ATP; with Mg^{2+} added in 5 mM excess (over nucleotide concentration) they yielded linear Eadie plots. The NTPase activity in nuclear envelopes from both sources showed a broad substrate specificity and induced declines in the energy charge of various nucleotide additives that paralleled stimulation of RNA transport in vitro by these additives. Nuclear envelopes from both sources were able to hydrolyze the high-energy phosphate bonds of diphosphate nucleotides. The ability to utilize ADP was not dependent on coupled oxidative phosphorylation or on electron transport; rather, it apparently proceeds via a myokinase-like activity that furnishes ATP. A number of agents that modify RNA transport in vitro similarly modified the NTPase activity. For instance, cAMP increases RNA transport and the NTPase activity, and further investigation showed that cAMP increased the K_m of the NTPase activity only slightly and the V_{max} by 65%. Further studies in vivo following CCl_4 or thioacetamide treatment of rats demonstrated a parallelism between alterations in RNA transport in vivo and nuclear envelope NTPase activity. Histochemical studies demonstrated that the NTPase activity was distributed along the nuclear envelope and was not localized to nuclear pores under the conditions employed. Supporting this result, we found that thioacetamide-induced nuclear swelling produces changes in nuclear envelope surface area which parallel increases in the nuclear envelope NTPase activity produced by this treatment. The reciprocity between the NTPase activity in nuclear envelopes and RNA transport, with regard to substrate behavior and to effects of activators and inhibitors and perturbations induced by in vivo treatments, suggests that this activity participates in RNA transport.

Isolated nuclei have been used in vitro to study nucleocytoplasmic RNA transport to a surrogate cytoplasm. A potential problem with such systems is to what extent the "selection" of RNA transcripts for transport from isolated nuclei in vitro reflects the corresponding process in vivo. There are, however, many aspects of this model that show a degree of biological reproducibility. Many characteristics of the RNA transported from isolated nuclei, including poly(A)[1] content (Schumm & Webb, 1974; Smuckler & Koplitz, 1976), size (Smuckler & Koplitz, 1976; Ishikawa et al., 1970a), base content and activity in directing protein synthesis in vitro (Ishikawa et al., 1970a), incorporation into polysomes in reconstructed systems (Ishikawa et al., 1970b), and inclusion in specific RNP particles (Smuckler & Koplitz, 1974; Raskas, 1971; Ishikawa et al., 1969; Sato et al., 1977), point to similarities between RNA transported in vitro and messenger and ribosomal RNA found in the cytoplasm. RNA transport proceeds in vitro with an activation energy of 13 kcal/mol (Clawson & Smuckler, 1978), a value consistent with an energy-requiring process. The constancy of this value under incubation conditions which dramatically alter the species of RNA transported suggests that the system's selectivity is distinct from its energetics. Furthermore, the relationship between facilitated RNA transport and the hydrolysis of high-energy phosphate bonds is of high statistical significance (Clawson et al., 1978). One high-energy phosphate bond is hydrolyzed in the facilitated transport of each nucleotide in transported RNA.

RNA transport in vitro necessarily involves both intranuclear RNA processing and subsequent transport. Other investigators have reported that RNA is processed in the absence of an energy source (Brunner & Raskas, 1972), and the results of our work support this observation. When isolated nuclei were incubated in surrogate cytoplasmic mixtures containing AMPCP (a methylene-blocked analogue of ADP which does not possess a high-energy phosphate bond and does not stimulate RNA transport), nuclear RNA (rapidly labeled with [^{14}C]orotic acid or L-[^{3}H]methionine) was processed but not transported (unpublished data). These findings suggest that it is the translocation of RNA from the nuclear interior which

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[1] Abbreviations used: NE, nuclear envelope; RNP, ribonucleoprotein; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; $NaDodSO_4$, sodium dodecyl sulfate; TKM buffer, 50 mM Tris-HCl (pH 7.4), 25 mM KCl, and 5 mM $MgCl_2$; NTPase, nucleoside triphosphatase; DNP, 2,4-dinitrophenol; BHT, butylated hydroxytoluene; poly(A), poly(adenylic acid).

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requires the hydrolysis of high-energy phosphate bonds. Agutter et al. (1976) have reported a correspondence between RNA transport from SV40-3T3 nuclei incubated in vitro and NTPase activity in isolated nuclear envelopes. Here we report further parallels between in vitro RNA transport and NTPase activity in isolated nuclear envelopes and parallels between in vivo RNA transport effects and nuclear envelope NTPase effects induced by CCl_4 or thioacetamide treatment of rats and present evidence suggesting that the nuclear envelope NTPase activity is distributed along the nuclear envelope surface.

Materials and Methods

Male Sprague-Dawley rats were obtained from the Charles River Breeding Laboratories (Wilmington, DE), and fresh calf liver was obtained from a local slaughterhouse. Rats were starved overnight prior to use.

Treatments. For CCl_4 experiments, rats were given 0.25 mL of CCl_4 + 0.25 mL of mineral oil per 100-g body weight via stomach tube. Controls were given mineral oil. For thioacetamide treatment experiments, rats were given 5 mg of thioacetamide per 100-g body weight via stomach tube. Controls were given water. For in vitro transport studies, RNA was labeled via tail vein injection of 3 μCi of [^{14}C]orotic acid per 100-g body weight 45 min prior to sacrifice. For in vivo transport studies, RNA was labeled by intraperitoneal injection of 3 μCi of [^{14}C]orotic acid per 100-g body weight 90 min prior to sacrifice.

Rat Liver Nuclear Isolation and Nuclear Envelope Preparation. Rat liver nuclei were isolated by sedimentation through a 2.3 M sucrose buffer cushion (50 mM Tris-HCl, pH 7.4; 25 mM KCl; 5 mM $MgCl_2$; 5 mM 2-mercaptoethanol) (Blobel & Potter, 1966; Smuckler & Koplitz, 1974); the nuclear pellets obtained are free of plasma membrane markers.

Rat liver nuclear envelopes were isolated by two independent techniques; for phospholipid analyses, they were isolated via the method of Harris & Milne (1974). This technique did not affect phospholipid or fatty acid distributions (or P_{450} content) but provided variable results for enzymatic activities. For enzymatic assays, rat liver nuclear envelopes were isolated by Monneron's (1974) technique as follows. Purified nuclear pellets were resuspended in 5 mL of 50% sucrose buffer containing 50 mM Tris-HCl (pH 7.4) and 500 mM $MgCl_2$. The viscous suspension was mixed thoroughly and then overlayered with a 30-mL linear 50–20% sucrose buffer gradient containing the same additives. Gradients were centrifuged at 190000g for 2 h at 4 °C in a 60Ti rotor. The envelopes banded at 1.17 g/mL. The portions of the gradients containing the envelopes were removed, diluted with buffer containing 50 mM Tris-HCl (pH 7.4) and 25 mM KCl, and centrifuged at 215000g for 1 h at 4 °C in a 60Ti rotor. The pelleted envelopes were rinsed with and resuspended in 0.88 M sucrose–TKM buffer (50 mM Tris-HCl, pH 7.6; 25 mM KCl; 5 mM $MgCl_2$).

RNA Transport Assays. RNA transport assays were conducted as previously described (Clawson et al., 1978), with calculation of the percentage of RNA released on the basis of total nuclear radioactivity (nearly all of which was Cl_3AcOH precipitable).

Calf Liver Nuclear Isolation and Nuclear Envelope Preparation. Calf liver was homogenized in 6–8 volumes of 0.25 M sucrose–TKM buffer in a Waring blender. Crude nuclear pellets were obtained from the whole homogenate by centrifugation at 2000g for 15 min at 4 °C and were resuspended in twice the original liver volume; sucrose and TKM buffer were added to this suspension to a concentration of 1.7 M sucrose–TKM buffer. Nuclei were purified by sedimentation through a 2.0 M sucrose–TKM buffer cushion at 105000g for 90 min at 4 °C in an SW27 rotor and were resedimented through 2.0 M sucrose–TKM buffer to ensure purity.

Calf liver nuclear envelopes were then prepared from the nuclear pellets by the method of Harris & Milne (1974) with two minor modifications: DNase I (RNase free; Worthington Biochemical, Freehold, NJ) was added to 20 μg/mL with $MgCl_2$ at 0.1 mM and incubation was carried out for 15 min at 20 °C. Although we obtained consistent results with calf liver nuclear envelopes with this procedure, additional DNase treatment led to a selective decline in cytochrome b_5 and NADH-ferricyanide reductase, apparently due to protease activity during incubation.

Measurement of Cytoplasmic RNA. Livers were homogenized in 0.25 M sucrose–TKM buffer, and the homogenate was centrifuged at 6000g for 10 min at 4 °C. RNA in the supernatant was quantitated by using the orcinol reaction (described below) after extraction from Cl_3AcOH-precipitated pellets. Alternatively, RNA was extracted with phenol at 60 °C (Smuckler & Koplitz, 1976), precipitated with 2 volumes of ethanol, resuspended, and quantitated spectrophotometrically by absorbance at 260 nm.

Analytical Methods. Protein concentrations were measured by Bio-Rad protein assays (Bio-Rad Laboratories, Richmond, CA) or by the Lowry method (Lowry et al., 1951) and are expressed on the basis of Lowry measurements, using albumin as a standard. DNA and RNA were solubilized from Cl_3AcOH-precipitated pellets by heating at 90 °C (Schneider, 1945). DNA was measured by the Burton diphenylamine technique (Burton, 1956) with color development overnight at room temperature. Mejbaum's (1939) orcinol technique, as modified by Ceriotti (1955), was used to measure RNA. Lipids were extracted by the method of Folch et al. (1957), and phospholipid was quantitated by the method of Bartlett (1959).

Phospholipid Analyses. Lipids were extracted from purified nuclear envelopes as described by Folch et al. (1957); nuclear envelopes were homogenized in the organic phase with a Polytron homogenizer. After evaporation of the chloroform–methanol solvent and addition of hexane, phospholipids were separated by high-pressure liquid chromatography using a Hewlett-Packard 1084A liquid chromatograph and an Si60 column (silica, 10-μm particle size). Phospholipids were eluted with a linear gradient ranging from 100% solvent A (hexane–propanol–water at 6:8:0.75) at 0 min to 100% solvent B (hexane–propanol–water at 6:8:1.5) at 5 min. The flow rate was 1.5 mL/min, the pressure was 60 bar, and runs were performed at room temperature. The effluent was monitored at 206 nm, fractions containing the various phospholipid species were collected, and phosphorus (and phospholipid) was then quantitated by using our standard calibrations or by direct assay (Bartlett, 1959). The identity of the phospholipids was verified by ascending chromatography on silica gel plates (Sil G-25; Brinkmann, Westbury, NY) in Skipski's solvent (chloroform–methanol–acetic acid–water at 25:15:4:2); phospholipids were stained with rhodamine G, and PS was distinguished from PI by a ninhydrin reaction. PE, PI, PS, and PC were normalized to 100% to facilitate comparisons.

Fatty Acid Analyses. For transesterification reactions, 1 mL of isolated phospholipid solution (containing up to 3 mg of phospholipid) was combined with 1 mL of benzene and 1 mL of methanolic base reagent (3-3080; Supelco, Bellafonte, PA). The mixture was flushed with N_2 in a screw cap vial, incubated at 80 °C for 15 min, cooled to room temperature, and washed with 3 mL of water. Three milliliters of diethyl

ether was added and mixed thoroughly. The top layer was removed, concentrated by evaporation, and examined in a Hewlett-Packard 5830A gas chromatograph using a Supelco column (SP-2330 on 100–120 Chromosorb). The column temperature at the beginning of the run was 170 °C, the flow rate was 20 mL of He per min, and detection was by a flame ionizer. After 16 min, the column temperature was increased to 200 °C for the remainder of the run. Standards were obtained from Sigma Chemical Co. (St. Louis, MO) and Supelco and were run separately and/or admixed with samples. In many experiments, BHT was added to lipid extracts to prevent autoxidation.

Enzymatic Assays. Succinate dehydrogenase (EC 1.3.99.1) was assayed as described by Bachmann et al. (1966). NADH-ferricyanide reductase (EC 1.6.99.3) was assayed as described by Rogers & Strittmatter (1973). Cytochromes b_5, P_{450}, and $a + a_3$ were measured as described by Berezney & Crane (1972), except that for P_{450} assays CO was bubbled through the solutions for 20 s. Spectral readings were obtained with an Aminco DW-2 recording spectrophotometer.

Nucleotide solutions were prepared in 0.88 M sucrose–TKM buffer (to pH 7.5) and stored frozen. [γ-^{32}P]ATP (8 Ci/mmol) or [γ-^{32}P]GTP (18 Ci/mmol) was mixed with unlabeled nucleotide to a specific activity of ~500 cpm/μg of NTP, determined by measuring Cerenkov radiation in water. Solutions with 5′-nucleotides labeled with ^{14}C in the nitrogen base ring (ATP, GTP, GDP, ADP, UTP, CTP, or TTP) were prepared to specific activities near 100 cpm/μg, as assayed by liquid scintillation counting.

NTPase assays were conducted with fresh nuclear envelope suspension or with suspension that had been stored at −80 °C (little loss of NTPase activity occurred). Fifty microliters of nucleotide solution was added to 100-μL aliquots of nuclear envelope suspension (generally containing nearly 20 μg of protein). Reactions were allowed to proceed for periods ranging from 0 to 20 min at 20 °C and were stopped by addition of 1.7 mL of water and perchloric acid to a final concentration of 300 mM [see Clawson et al. (1978)]. After sedimentation of the insoluble white precipitate, Darco G-60 charcoal (acid washed) was added to the supernatant fluid, and the suspension was mixed thoroughly and incubated for 20 min at room temperature. The charcoal was pelleted by centrifugation and then was resuspended in and rinsed with 2.5 mM perchloric acid and repelleted. The supernatant fractions were combined. Nucleotides were removed from the charcoal with successive washes with ammoniacal alcohol (EtOH–H_2O–NH_4OH at 50:48:2). With ^{32}P-labeled nucleotides, NTPase activity was calculated from the percentage of label appearing in the supernatant fraction. Base-labeled nucleotides were analyzed on poly(ethylenimine)-impregnated cellulose plates as previously described (Clawson et al., 1978), except that short-wavelength ultraviolet light was used to locate the species. Energy charge was then calculated as 1 × (fraction of the nucleotide in triphosphate form) plus 0.5 × (fraction of the nucleotide in diphosphate form) and thus ranges between 0 and 1. Linear regression coefficients were calculated from the time course data and are used in comparing activities. For pH dependency assays, nuclear envelope preparations were resuspended in 0.88 M sucrose–TKM buffer at pH 6.0, the pH was adjusted with KOH, and aliquots were withdrawn at the appropriate pH. This procedure did not affect subsequent enzymatic activity at pH 7.5. For inhibitor (and activator) studies, additives were mixed with nuclear envelope suspension 5–10 min before nucleotide was added; solvent blanks were run in parallel.

Histochemical Localization Studies. For localization of NTPase activity, calf liver nuclear envelopes were isolated by the DNase method described and were fixed for 5 min at 0 °C in 4% paraformaldehyde containing 1% calcium chloride in 0.1 M sodium cacodylate buffer, pH 7.4, and washed overnight in buffer at 4 °C. ATPase was detected by a modification of the Wachstein–Meisel technique (Wachstein & Meisel, 1957) with incubation for 90 min at 37 °C in medium containing 0.83 mM ATP, 2.4 mM $Pb(NO_3)_2$, and 10 mM $MgSO_4$ in 80 mM Tris–maleate buffer (pH 7.2). Controls consisted of incubations without substrate or with 5 mM $ZnCl_2$ or 5 mM $CuSO_4$ added to the medium (which produced only a moderate inhibition due to the presence of Mg^{2+}). After incubation, the fractions were recovered by centrifugation, fixed in acetate–Veronal buffer–OsO_4, stained en bloc in aqueous uranyl acetate, dehydrated, and embedded in Epon epoxy resin. Thin sections were cut on a Proter-Blum Sorvall MT-2 microtome, and the sections were examined unstained or lightly stained with lead citrate on a Siemens 101 electron microscope.

Results

In this study, we utilized nuclear envelopes prepared from rat and calf liver. The properties of the rat liver nuclear envelopes could be directly compared with RNA transport results. Calf liver nuclear envelopes could be prepared in much greater quantity, which facilitated more extensive enzymatic analyses and allowed comparison with properties of rat liver nuclear envelopes.

Nuclear Envelope Characterization. Rat liver nuclear envelopes, isolated by Monneron's (1974) technique, consisted of 65% protein, 22% phospholipid, 8% RNA, and 5% DNA (on a dry weight basis). No succinate dehydrogenase was detectable in isolated envelopes, but they contained cytochrome b_5 at 0.262 ± 0.058 nmol/mg of protein, cytochrome P_{450} at 0.10 ± 0.02 nmol/mg, and NADH-ferricyanide reductase at 4.42 ± 0.36 units/mg of protein. Their phospholipid composition was 25.3% PE, 10.8% PI, 3.6% PS, and 60.5% PC. Analysis of fatty acids of the separated nuclear envelope phospholipids indicated distributions similar to those found in PE, PI, and PC microsomal phospholipids, but PS differed with regard to 16:0, 18:0, and 18:2 constituents.

Calf liver nuclear envelopes were isolated by a method based on that of Harris & Milne (1974) and were composed of 70.9% protein, 19.4% phospholipid, 7.6% RNA, and 2.1% DNA. Succinate dehydrogenase activity in calf liver nuclear envelopes was 2 nmol of dichlorophenolindophenol reduced per mg per min. NADH-ferricyanide reductase and cytochrome b_5, P_{450}, and $a + a_3$ contents were 3.3 units/mg, 0.24 nmol/mg, 0.013 nmol/mg, and 0.038 nmol/mg, respectively. The phospholipid composition of the isolated envelopes was 27.9% PE, 5.7% PI, 10.4% PS, and 56.6% PC. Fatty acid analyses of the separated nuclear envelope phospholipids showed distributions similar to those found in microsomal PE, PI, and PC fractions; differences were again found between nuclear envelope and microsomal PS fatty acids, with regard to 18:1 and 18:2 components. These characteristics are in good agreement with previously reported nuclear envelope properties [see, for instance, Fry (1976)].

NTPase Activity. NTPase assays were performed at 20 °C for up to 20 min, since RNA transport from rat liver nuclei in vitro proceeds linearly under these conditions. Use of labeled nucleotides was chosen as a direct assay of the molecules involved and to minimize problems of inorganic phosphorus release from nonnucleotide sources. The use of regression coefficients calculated from the time course data allows more

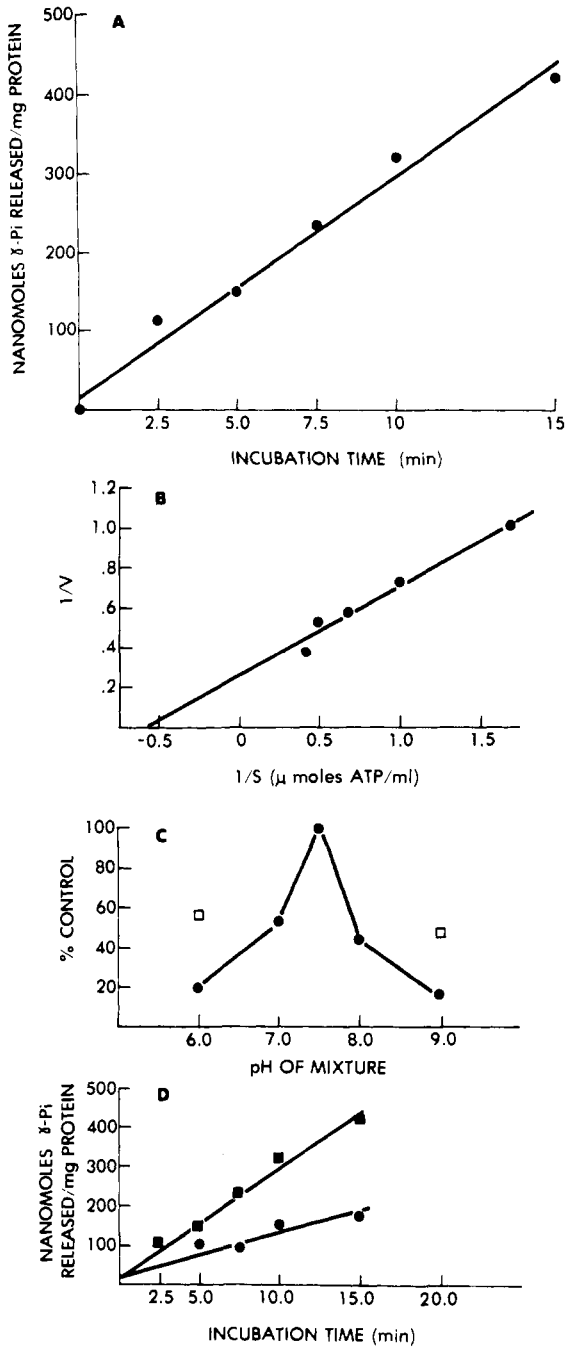

FIGURE 1: Characteristics of the rat liver nuclear envelope NTPase. The NTPase activity in nuclear envelope preparations was linear for at least 20 min at 20 °C over a wide range of protein concentrations. (A) The specific activity of the NTPase activity at 20 °C was 572 ● 66 nmol of γ-P_i released per 20 min per mg of protein. Data are from 10 individual time course experiments. (B) Lineweaver–Burk graph of $1/V$ vs. $1/S$. $1/V$ is expressed relative to the NTPase activity observed at 0.6 mM ATP. Under the conditions used in these experiments, excess Mg^{2+} ranged from 2.6 to 4.5 mM. (C) Relationship between NTPase activity and pH. Aliquots of nuclear envelope preparations at the indicated pH values were incubated for various periods. Linear regression coefficients were calculated from the data and are expressed as a percent of control values. (□) points indicate initial linear rates for NTPase activity at pH 6 and 9. The activity quickly disappeared under these conditions. (D) Inhibition of NTPase activity by quercetin. Nuclear envelope preparations were divided into two groups. Quercetin was mixed with one group (●) at a final concentration of 10 μg/mL, and solvent was added to the other group (■). Regression coefficients indicated that the activity decreased by 75% in the presence of quercetin.

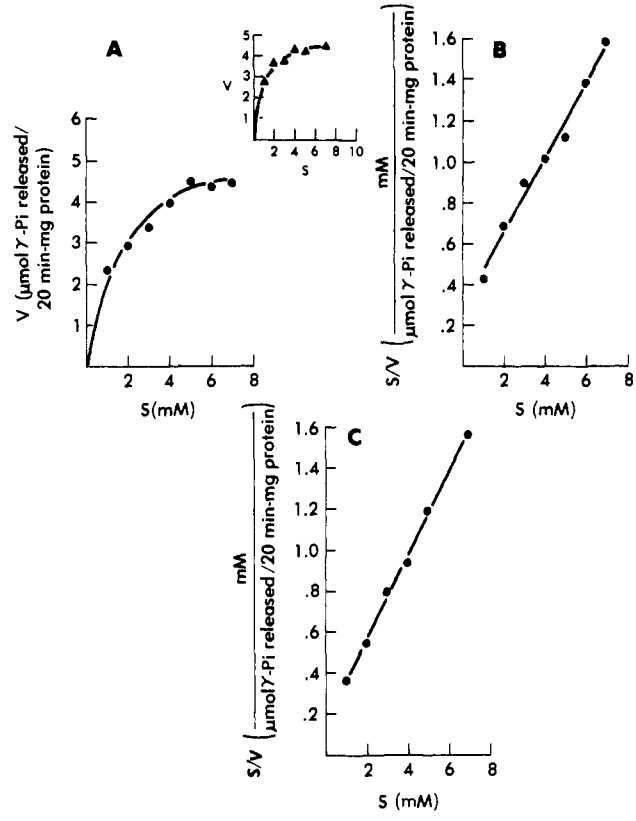

FIGURE 2: Substrate behavior of the nuclear envelope NTPase activity in calf liver preparations. NTPase activity was assayed by using [γ-^{32}P]ATP. (A) Velocity (V) vs. substrate concentration (S) plot of NTPase activity with Mg^{2+} added in 5 mM excess. Inset shows the analogous plot with Mg^{2+} added at 10 mM and the ATP concentration varied. (B) Eadie plot of S/V vs. S, with Mg^{2+} added in 5 mM excess. The data fit very well ($p < 0.007$) with a linear solution; the K_m under these conditions was 1.65 mM. (C) Eadie plot of S/V vs. S, with Mg^{2+} added at 10 mM and the ATP concentration varied. The K_m was reduced under these conditions, and the Eadie plot is again linear.

accurate assessment of NTPase activity and permits detection of changes in linearity. [γ-^{32}P]ATP was added to reaction mixtures containing isolated rat liver nuclear envelopes in 0.88 M sucrose–TKM buffer. After various incubation periods, the amount of [γ-^{32}P]P_i released was quantitated as described. The activity was linear with protein concentration. At 20 °C, 572 ± 66 nmol of γ-P_i was released per 20 min per mg of protein (Figure 1A). This represents an approximate sixfold increase in specific activity as compared to that of the isolated nuclear fraction, and about one-half of the total activity associated with nuclei could be recovered in the envelopes. The specific activity in calf liver nuclear envelopes was 916 nmol of γ-P_i released per 20 min per mg of protein.

We also examined the effects of variations in substrate concentration on the enzymatic activity in rat liver preparations (under our standard RNA transport conditions). The results, displayed on a Lineweaver–Burk graph (Figure 1B), showed a good linear fit ($p < 0.01$) with a slope of 0.45 and a K_m of 1.8 mM ATP. This K_m suggests that the NTPase would be responsive to small changes in intracellular ATP levels, since ATP is present intracellularly at approximately this concentration.

The NTPase activity in calf liver nuclear envelopes was investigated over a more extensive range of substrate concentrations (Figure 2A), with Mg^{2+} added in 5 mM excess with respect to added nucleotide. Under these conditions, Lineweaver–Burk plots also showed a good linear fit. Re-

Table I: Dependence of Calf Liver Nuclear Envelope NTPase Activity on Temperature: Arrhenius Analysis[a]

incubn temp (°C)	sp act. of nuclear envelope NTPase (μmol of γ-P_i released per h per mg of protein)
0	0.427
5	0.521
10	1.209
20	2.748
25	3.893
30	4.882
35	5.624

[a] Release assays were performed with [γ-^{32}P]ATP, and liberated [γ-^{32}P]P_i was quantitated as described. Arrhenius analysis of the results indicates an activation energy of 13.31 ± 0.88 kcal/mol. Substitution of V_{max} values for the activity at various temperatures (estimated from Lineweaver–Burk plots) indicated that this value reflects the true activation energy.

gression coefficients were examined on Eadie plots of S/V vs. S (Figure 2B) to magnify possible departures from linearity. The data fit a linear solution (analysis of fit showed $p < 0.007$), and the K_m was 1.65 mM ATP; curvilinear solution did not improve the fit. In contrast, Agutter et al. (1977) reported nonlinear Eadie plots, but they were able to restore linearity by manipulating incubation conditions. With Mg^{2+} added at 10 mM and the ATP concentration varied between 1 and 7 mM, our Eadie plots were still linear (Figure 2C; the K_m was significantly reduced under these conditions). Similarly, with Mg^{2+} added at 5 mM and ATP concentration varied between 1 and 4 mM (conditions identical with those in experiments titrating RNA transport with ATP concentration) Eadie plots were linear and the K_m was 1.8 mM.

The NTPase activity in nuclear envelopes from both sources was sharply dependent upon pH. Rat liver preparations showed maximal activity near pH 7.5 (Figure 1C). Time course experiments indicated that the activity at pH 7 and 8 was only about half of the maximal activity. At pH 6 and 9, the activity after 15 min was only 20% of the maximal activity, although the initial linear rates were near those found at pH 7 and 8. This pH dependency is not consistent with alkaline phosphatase activity. The amount of inhibition at pH 6 is consistent with alkaline phosphatase activity. The amount of inhibition at pH 6 is consistent with previous data showing that RNA transport at pH 6 is ~75% less than that at pH 7.6 (Clawson & Smuckler, 1978).

The temperature dependence of the NTPase activity in nuclear envelopes was examined at 5 °C intervals between 35 and 0 °C. By use of the Arrhenius relationship, the activation energy was 13.8 kcal/mol for rat liver (data not shown), a value consistent with that for the rate-limiting step in RNA transport (Clawson & Smuckler, 1978) and with that expected for ATPase activity. Similarly, calf liver nuclear envelope preparations yielded an activation energy of 13.3 ± 0.9 kcal/mol (Table I). .

The enzymatic activity with other nucleotides was examined. [γ-^{32}P]GTP was employed in the same way as [γ-^{32}P]ATP. Assays with ^{14}C-base-labeled nucleotides were performed, and the products were analyzed by chromatographic separation on poly(ethylenimine)-impregnated cellulose plates (Clawson et al., 1978). All of the nucleotides tested (ATP, ADP, GTP, GDP, CTP, UTP, and TTP) were hydrolyzed; this result demonstrates a broad substrate specificity (Table II) similar to that for intact nuclei (Clawson et al., 1978), and for this reason the enzymatic activity will be referred to as a nucleoside triphosphatase (NTPase activity, EC 3.6.1.4). Furthermore,

Table II: Metabolism of Nucleotide Additives by Isolated Rat Liver Nuclear Envelopes[a]

nucleotide additive	facilitated RNA transport at 1 mM[b] (% nuclear cpm)	decline in energy charge
GTP	3.0	0.41
TTP	2.9	0.39
ADP	2.4	0.31
GDP	2.2	0.34
UTP	2.1	0.31
CTP	2.0	0.30

Metabolism of Added ADP

incubn time (min)	% of nucleotide recovered as ATP	ADP	AMP
0	1.1	94.6	4.4
10	7.3	45.6	47.1
20	4.2	27.7	68.1

[a] ^{14}C-Base-labeled nucleotide solutions were prepared, incubated at 20 °C at a concentration of 1 mM with nuclear envelope suspension containing 400 μg/mL, and processed and examined on poly(ethylenimine)-impregnated cellulose plates. Energy charge was calculated as described. Standard deviations are 10% or less. [b] RNA transport figures were obtained in standard assays (see Materials and Methods) with 1 mM nucleotide addition. Energy charge declined more rapidly with GDP than with ADP.

the declines in energy charge obtained with the various nucleotide additives paralleled their stimulation of RNA transport in the in vitro system. ADP and GDP stimulated facilitated RNA transport to about the same extent as CTP and UTP. In parallel, the high-energy phosphate bonds present in ADP and GDP were extensively hydrolyzed (Table II), yielding about the same declines in energy charge that were observed with UTP and CTP. In this regard, the nuclear envelopes showed a myokinase-like activity, similar to that observed with whole nuclei (Clawson et al., 1978); 10 min after addition of ^{14}C-base-labeled ADP, over 7% of the nucleotide appeared as ATP (Table II). The energy charge declines reflect the NTPase activity, since the myokinase-like activity does not affect this value.

Nuclear envelopes from calf liver were also able to convert ADP to AMP. Since calf liver nuclear envelopes appear to possess an intact electron transport system (Berezney & Crane, 1972), we tested their ability to utilize the energy charge of exogenously added ADP in the presence of sodium cyanide (NaCN) and of 2,4-dinitrophenol (DNP). The energy charge of added ADP (at 1 mM) was extensively utilized in the presence of these additives; the rate of utilization was 90% of the control rate in the presence of 1 mM NaCN (RNA transport was also 90%) and 100% in the presence of 1 mM DNP. These results strongly suggest that the utilization of ADP by isolated calf liver nuclear envelopes is not dependent on coupled oxidative phosphorylation or on electron transport.

Relevance of Nuclear Envelope NTPase to in Vitro RNA Transport Results. The effects of various compounds on the NTPase activity in rat and calf liver nuclear envelopes were similar to reported effects of these compounds on RNA transport in vitro, as has been demonstrated by the extensive investigations of Agutter et al. (1976, 1977, 1979). Exogenous RNA stimulates ribosomal RNP transport in vitro (Sato et al., 1977), and we found that adding yeast RNA at 200 μg/mL increased NTPase activity in rat liver nuclear envelope by 60% and by 35% in calf liver nuclear envelopes. Agutter et al. (1977) reported similar results with pig liver nuclear envelopes.

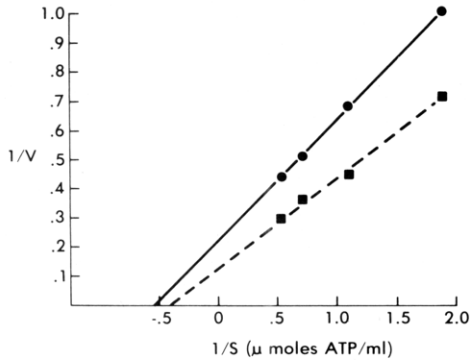

FIGURE 3: Lineweaver–Burk graph of NTPase activity in calf liver nuclear envelopes. $1/V$ is expressed relative to the activity for the control preparation at 0.53 mM ATP. (■) NTPase activity in the standard reaction mixture supplemented with 1 μM cAMP; (●) NTPase activity in the standard reaction mixture. Under these conditions, excess Mg^{2+} ranged from 3 to 4.5 mM.

The activity in rat liver preparations was reduced by 75% by quercetin (Figure 1D), although the amount of inhibition was somewhat variable. Oligomycin (at 200 μg/mL) did not inhibit the activity in either rat or calf liver preparations.

Some new findings regarding effectors of nuclear envelope associated NTPase include effects of NaF and cAMP. NaF at 1 mM inhibited the NTPase activity by 40% in rat liver preparations and by 50% in calf liver preparations, in agreement with the finding (Schumm & Webb, 1978) that NaF (at 10 mM) inhibited RNA transport by ~40%. Schumm & Webb (1978) also reported that cAMP significantly increased RNA transport in resting liver, maximally at concentrations between 1 and 0.1 μM. At these concentrations, cAMP induced a 25–30% increase in rat liver nuclear envelope NTPase [1 mM 2′(3′)-AMP was without effect]. At 1 μM (and with ATP added at 1 mM concentration) it induced a 50% increase in the NTPase activity in calf liver nuclear envelopes. Measurements of the activity in calf liver preparations at various substrate concentrations were analyzed on Lineweaver–Burk graphs (Figure 3). The K_m for the activity was 1.9 mM in the control preparations. With cAMP added at 1 μM, the K_m was 2.4 mM (the difference was not statistically significant) and the V_{max} was increased by 65% over the control value. These results indicate that cAMP modulates the nuclear envelope NTPase activity in a noncompetitive manner.

(*a*) *Histochemical Localization Experiments.* We sought to determine the site of the NTPase activity by using a modified Wachstein–Meisel procedure (Wachstein & Meisel, 1957) for histochemical localization. Under the conditions chosen, no reaction product was observed in preparations incubated without ATP (Figure 4A). Preparations incubated with ATP showed reaction product which was distributed at sites along the nuclear envelope and which was *not* localized to nuclear pores (Figure 4C; see Discussion). Reaction product was trapped within perinuclear cisternae and was often heavier along the nuclear membrane lacking attached ribosomes (presumably inner nuclear membrane). The reaction product was often associated with an amorphous layer along the nuclear envelope (see Figure 4B); the amorphous layer could be removed (or selectively reduced) by RNase treatment.

Since these histochemical results suggest that the nuclear envelope NTPase activity is distributed along the nuclear envelope, we investigated the increase in this activity following thioacetamide-induced nuclear swelling. The results (Table III) show a basic agreement between the increase in nuclear envelope surface area and the NTPase activity, further supporting the contention that the activity is distributed (in some manner) along the nuclear envelope (see Discussion).

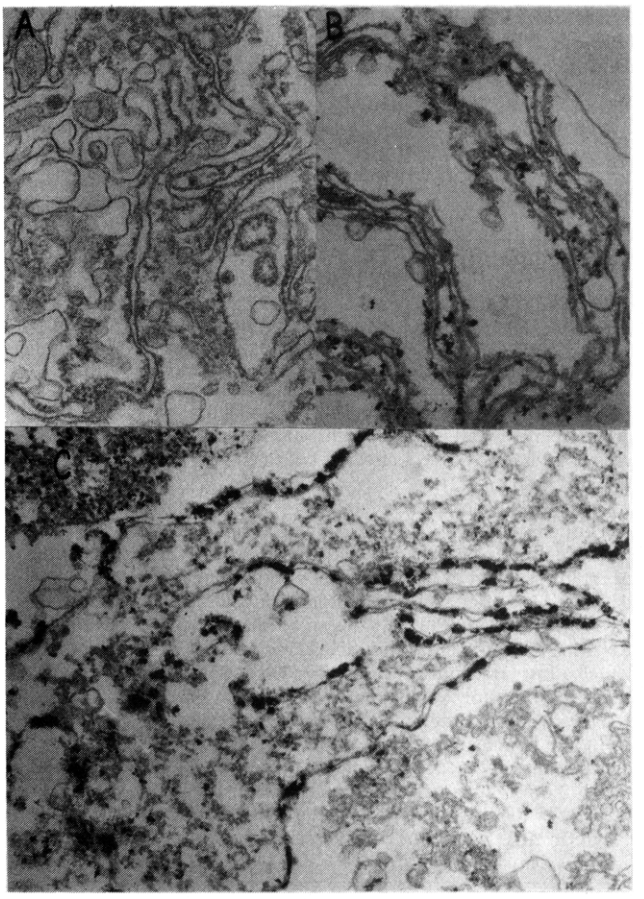

FIGURE 4: Histochemical localization of NTPase activity. Isolated calf liver nuclear envelopes were incubated in a modified Wachstein–Meisel medium. Magnification is 30 000 times. (A) Envelopes incubated in lead-containing medium without ATP; no lead phosphate deposits were observed under these conditions. (B) Envelopes incubated in medium containing lead and ATP and also containing 5 mM $ZnCl_2$; the inhibition was moderate under these conditions (a typical distribution is shown). (C) Envelopes incubated in complete medium containing lead plus ATP. Reaction product was distributed along the nuclear envelope and was deposited within the perinuclear cisternae; it often appeared to be associated with the inner nuclear membrane. In subsequent experiments, inclusion of NaF or quercetin completely blocked reaction product deposition.

Relevance of Nuclear Envelope NTPase to in Vivo RNA Transport Results. The data presented here and by others show that additives which alter RNA transport in in vitro systems similarly alter the nuclear envelope NTPase activity. Although RNA transported in vitro has many characteristics which resemble those of cytoplasmic RNA, little is known about the specificity involved in this simulated system. A more significant test to determine whether nuclear envelope NTPase is involved in the regulation of RNA transport was needed. We posed the question "Do perturbations of the biological system which produce alterations in RNA transport in vivo also produce parallel changes in the nuclear envelope NTPase activity?"

To answer this question, we examined both in vivo RNA transport and nuclear envelope NTPase activity with rat liver at various intervals following CCl_4 or thioacetamide intoxication. The results (Tables III and IV) demonstrate that a parallel exists between these two phenomena. Following CCl_4 treatment, there is a dramatic decline in both nuclear envelope NTPase activity and in vivo RNA transport, as assessed by measuring the specific activity of cytoplasmic RNA. We point

Table III: Parallelism between in Vivo RNA Transport, Nuclear Envelope NTPase Activity, and Nuclear Envelope Surface Area following Thioacetamide Treatment of Rats[a]

period following treatment (h)	sp act. of cytoplasmic RNA (cpm/A_{260} unit)	sp act. of nuclear envelope NTPase[b] (nmol of γ-P freed per 20 min per mg)	nuclear surface area[c] (% control)
control	461 ± 127	572 ± 66	100
8	214 ± 111[e]	460 ± 40[d]	125
18	150 ± 26[f]	853 ± 85[e]	125
48	608 ± 154[d]	1628 ± 198[f]	185
72	529 ± 107[g]	781 ± 165[g]	150
96	553 ± 120[g]	718 ± 73[g]	140

[a] Rat liver RNA was prelabeled by ip injection of 3 μCi of [^{14}C]-orotic acid per 100-g body weight 90 min prior to sacrifice, at various periods of time following treatment of rats with 5 mg of thioacetamide per 100-g body weight. Total cytoplasmic RNA content was approximately 100, 95, 130, 130, and 130% of the control content at 8, 18, 48, 72, and 96 h, respectively. At 16 h following treatment, there is a dramatic increase in the percentage of rapidly labeled cytoplasmic RNA containing poly(A); the decreased specific activity presumably involves decreased nuclear RNA synthesis and the well-known modifications in ribosomal RNA manufacture and processing. With our in vitro system, which allows transport of messenger RNP (Sato et al., 1977), an enhanced transport is observed at 16 h (Clawson et al., 1980). [b] Assays were conducted at 20 °C as described under Materials and Methods. Values shown are means ± standard deviations from multiple measurements. [c] Control nuclear envelope surface area was 240 μm^2. Measurements were taken from Olason & Smuckler (1976). [d] Significant at $p < 0.05$. [e] Significant at $p < 0.01$. [f] Significant at $p < 0.001$. [g] Not significant at $p < 0.05$; the 96-h data are significant at $p < 0.06$.

Table IV: Parallelism between in Vivo RNA Transport and Nuclear Envelope NTPase Activity following CCl_4 Treatment of Rats[a]

period following treatment (h)	sp act. of cytoplasmic RNA (cpm/A_{260} unit)	sp act. of nuclear envelope NTPase[b] (nmol of γ-P freed per 20 min per mg)
control	487 ± 60	572 ± 66
2	319 ± 28[d]	NM[g]
4	374 ± 5[c]	130 ± 5[e]
8	560 ± 44[f]	784 ± 121[d]
18	724 ± 96[d]	738 ± 61[c]

[a] Rat liver RNA was prelabeled by ip injection of 3 μCi of [^{14}C]-orotic acid per 100-g body weight 90 min prior to sacrifice, at various periods of time following treatment of rats with 0.25 mL of CCl_4 + 0.25 mL of mineral oil per 100-g body weight. Total cytoplasmic RNA content was approximately 110, 110, 115, and 95% of the control content at 2, 4, 8, and 18 h, respectively. [b] Assays were conducted at 20 °C as described under Materials and Methods. Values shown are means ± standard deviations of four measurements. [c] Significant at $p < 0.05$. [d] Significant at $p < 0.01$. [e] Significant at $p < 0.001$. [f] Not significant at $p < 0.05$. [g] NM, not measured.

out that variations in the total cytoplasmic RNA following this treatment are relatively small (see footnotes to Table IV). Following thioacetamide treatment, the nuclear envelope NTPase activity and RNA transport are reduced for a longer period but both increase later, with both reaching maximal values at 48 h after treatment. The specific activities of cytoplasmic RNA at 48, 72, and 96 h after treatment represent conservative estimates of increased transport, since livers from animals at these times contain more cytoplasmic RNA (see footnotes to Table III). The increase in nuclear surface area at 8 h appears to precede the increase in nuclear envelope NTPase, which may be significant because this phase of the nuclear swelling appears to be passive. In turn, the increase in nuclear envelope NTPase activity is evident before any increase is observed in in vivo RNA transport. Coincidently, however, there is a dramatic rise in the percentage of rapidly labeled cytoplasmic RNA which contains poly(A) at 16 h following treatment (from 4.6 ± 0.9% in control to 12.8 ± 2.9% in treated samples). Transport results using our in vitro assay conditions, which allow mRNA transport (Sato et al., 1977), demonstrate an increased transport at this time (Clawson et al., 1980). The decreased appearance of label at this time point may relate to reduced nuclear RNA synthesis (82% of control synthesis) and to the well-known alterations in ribosomal RNA manufacture and processing.

Discussion

We have described an NTPase associated with isolated calf and rat liver nuclear envelopes. Many parallels exist between the level of this enzymatic activity and the rate of in vitro RNA transport. Like RNA transport, nuclear envelope NTPase shows a broad substrate specificity, and similar results have been reported for pig liver nuclear envelopes (Agutter et al., 1977). In general, our results with regard to the in vitro situation are in accord with the excellent characterizations of Agutter et al. (1976, 1977, 1979). New findings reported here include the effects of NaF and cAMP at physiological concentrations. NaF, which has been shown to inhibit RNA transport in vitro, inhibited the nuclear envelope NTPase. cAMP increases RNA transport in vitro, and our results suggest that it induces this increase by modulating the activity of the rate-limiting RNA translocation NTPase associated with the nuclear envelope. Failure to find cAMP stimulation at 5 mM (Agutter et al., 1979) may relate to the fact that the stimulation of in vitro RNA transport is apparently lost above 10 μM (Schumm & Webb, 1978).

The appearance of rapidly labeled RNA in the cytoplasm after the short interval monitored (90 min) affords a relatively direct assessment of nucleocytoplasmic RNA transport (degradation of labeled RNA was not observed and shorter intervals showed greatly reduced transport). Using this simple assay, we have now demonstrated the parallelism between in vivo RNA transport and nuclear envelope NTPase activity. The nuclear envelope NTPase may exert a considerable influence on the rate of nucleocytoplasmic RNA transport in the in vivo setting.

Although our results in vitro are in general agreement with those of Agutter et al., some notable differences exist. The most serious discrepancy involves results presented in Arrhenius plots. We reported (Clawson & Smuckler, 1978) an activation energy of 13 kcal/mol for RNA transport, and herein we report values of 13.3 and 13.8 kcal/mol for nuclear envelope NTPase activity (using [γ-^{32}P]ATP). The activation energy for RNA transport is in good agreement with the in vivo data presented by Nagel & Wunderlich (1977) in the upper temperature domain and with that mentioned by Stuart et al. (1977) [see Clawson & Smuckler (1978)]. Furthermore, these values are in the range expected for ATPase activity [see, for example, Dean & Tanford (1978)]. However, Agutter et al. (1979) presented an Arrhenius plot for NTPase activity in SV40-3T3 nuclear envelopes with an approximate activation energy of 3 to 4 kcal/mol. This discrepancy suggests that the inorganic phosphate release they measured is not wholly attributable to NTPase activity over the temperature range. Concerning their Arrhenius graph for ATP-stimulated RNA transport, the y axis is based upon conversion of ^{3}H counts per minute to disintegrations per minute (dpm). Since the values they report are very low (at temperatures below ~12 °C the dpm are less than 200), the significance of these results

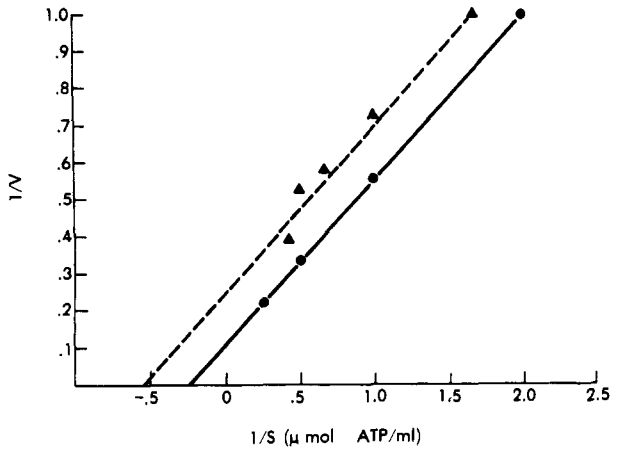

FIGURE 5: Lineweaver–Burk plots of facilitated RNA transport and nuclear envelope NTPase activity in rat liver preparations. For facilitated transport, $1/V$ is expressed relative to the rate of facilitated transport at 0.5 mM ATP. For nuclear envelope NTPase activity, $1/V$ is expressed relative to the rate of ATP hydrolysis at 0.6 mM ATP. (▲) Nuclear envelope NTPase activity; (●) facilitated RNA transport.

is obscure. In fact, their Arrhenius plot for RNA transport could be interpreted as a linear plot from 37 to ~12 °C; the lower temperature portion of their plot, which is parallel to the plot for release in the absence of energy source, could reflect RNase activity (and low transport activity), since the activation energy is equivalent to that we have reported for RNase activity (Clawson & Smuckler, 1978).

Another notable difference between the system employed by Agutter et al. and that employed by us concerns the effects of diphosphate additives. ADP and GDP are effective in stimulating RNA transport in the rat liver system (Clawson et al., 1978). Using chromatographic separation of base-labeled nucleotides (Table II), we have now demonstrated that the nuclear envelope NTPase utilizes the energy charge of the diphosphates (through a myokinase-like activity) in relation to the ability of these additives to stimulate RNA transport. In contrast, Agutter et al. (1977) report that diphosphate additives are utilized by isolated pig liver nuclear envelopes at a rate which is less than 2% of that for ATP.

Lineweaver–Burk graphs for facilitated RNA transport and for NTPase in rat liver nuclear envelopes (Figure 5) have identical slopes (K_m/V_{max} = 0.45), with the substrate curve for the NTPase activity displaced to lower concentrations. Although interpretation of this relationship is difficult, it seems to agree with previous work in this system (Clawson et al., 1978) showing that about half of the phosphate bond hydrolysis observed with whole nuclei is not involved in facilitated RNA translocation. Thus, it appears that the NTPase activity has a lower K_m than facilitated RNA transport because the nuclear envelopes lack the competing hydrolytic processes that occur in whole nuclei. This result differs from that of Agutter et al. (1976); they reported similar K_m values for RNA transport and nuclear envelope NTPase with SV40-3T3 nuclei, although their later work (Agutter et al., 1977) suggests that this relationship may have been improperly estimated because the K_m for NTPase activity was extrapolated by assuming linear kinetics for results possibly nonlinear in nature. In a subsequent paper (Agutter et al., 1979), they also note the presence of a considerable intranuclear NTPase activity. The differences between their results and ours may arise from the in vitro conditions chosen, for instance, the much high nuclear protein concentrations we employ in our RNA transport assays.

Our histochemical studies demonstrate that the reaction product formed by the NTPase activity is trapped within the perinuclear cisternae and that the activity is not localizable to nuclear pore complexes. Ishikawa et al. (1978) have similarly reported that they were unable to demonstrate a Mg^{2+}-ATPase at the nuclear pores. Investigators working with sections from intact cells have demonstrated a considerable ATPase activity at nuclear pores (Klein & Afzelius, 1966; Yasuzumi & Tsubo, 1966; Franke, 1974), although in these studies reaction product was also distributed along the inner nuclear membrane. The lack of major lead phosphate deposits at nuclear pores in our study does not conflict with these studies. The dense patches of reaction product observed outside nuclear pores in sections from intact cells were located at some distance from the pores, making it likely that the reaction product would have diffused away under the conditions we utilized. It seems clear, however, that exclusive localization of NTPase activity to pore complexes in nuclear envelope preparations may depend upon removal of the RNA lining the inner nuclear membrane. The stimulation of NTPase activity by exogenous RNA and inhibition by RNase treatment may arise through association and dissociation (respectively) of RNA with membrane and/or membrane components. Furthermore, the stimulatory activity of RNA may arise through a "substrate" effect, if the nuclear envelope NTPase requires an RNA chain.

Further support for the contention that the NTPase is distributed along the nuclear envelopes can be derived from other sources. For instance, while Agutter et al. (1977) report a stimulation of nuclear envelope NTPase by poly(G) with pig liver nuclear envelopes, Coffey et al. (1974) have demonstrated that poly(G) interacts with the entire nuclear envelope surface area, forming a layer upon it. Additionally, as shown, thioacetamide-induced increases in nuclear envelope surface area parallel increases in nuclear envelope NTPase specific activity induced by the treatment (Table III). It may be important that the passive phase of nuclear swelling is not associated with an increase in nuclear envelope NTPase activity.

Nucleotide additives stimulate RNA transport from isolated rat liver nuclei, and the activation energy for RNA transport reflects an energy requirement, presumably for translocation of RNA from the nucleus and necessarily through the nuclear envelope, which may therefore be the location of phosphate bond hydrolysis. Since there is a close correspondence between phosphate bond hydrolysis and RNA transport (Clawson et al., 1978), it appears that an NTPase activity plays some role in moving RNA from the nuclear interior to the cytoplasm. The similarity between RNA transport and nuclear envelope NTPase activity strongly suggests that the nuclear envelope NTPase activity functions in this way, and the in vivo data presented further suggest that regulation of this activity may be an important regulatory element in the control of nucleocytoplasmic RNA transport.

Many aspects of the relationship between phosphate bond hydrolysis, the nuclear envelope, and RNA transport are not clear. However, our functional model postulates that the RNA chain is translocated by the length of one nucleotide for each high-energy phosphate bond hydrolyzed. Our histochemical results further suggest that the RNA may move along the inner nuclear membrane (or through it). The point of export is not known, although penetration through the nuclear pore is the most plausible site.

References

Agutter, P., McArdle, H., & McCaldin, B. (1976) *Nature (London) 263*, 165.

Agutter, P., Harris, J., & Stevenson, I. (1977) *Biochem. J. 162*, 671.
Agutter, P., McCaldin, B., & McArdle, H. (1979) *Biochem. J. 182*, 811.
Bachmann, E., Allman, D., & Green, D. (1966) *Arch. Biochem. Biophys. 115*, 153.
Bartlett, G. (1959) *J. Biol. Chem. 234*, 466.
Berezney, R., & Crane, F. (1972) *J. Biol. Chem. 247*, 5562.
Blobel, G., & Potter, V. (1966) *Science 154*, 1662.
Brunner, M., & Raskas, H. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3101.
Burton, K. (1956) *Biochem. J. 62*, 315.
Ceriotti, D. (1955) *J. Biol. Chem. 214*, 59.
Clawson, G., & Smuckler, E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5400.
Clawson, G., Koplitz, M., Castler-Schechter, B., & Smuckler, E. (1978) *Biochemistry 17*, 3747.
Clawson, G., Koplitz, M., Moody, D., & Smuckler, E. (1980) *Cancer Res. 40*, 75.
Coffey, D., Barrack, E., & Heston, W. (1974) *Adv. Enzyme Regul. 12*, 219.
Dean, W., & Tanford, C. (1978) *Biochemistry 17*, 1683.
Folch, J., Lees, M., & Sloane-Stanley, G. (1957) *J. Biol. Chem. 226*, 497.
Franke, W. (1974) *Int. Rev. Cytol., Suppl. 4*, 71.
Fry, D. (1976) in *Subnuclear Components* (Birnie, G., Ed.) p 59, Butterworth, Boston, MA.
Harris, J., & Milne, J. (1974) *Biochem. Soc. Trans. 2*, 1251.
Ishikawa, K., Kuroda, C., & Ogata, K. (1969) *Biochim. Biophys. Acta 179*, 316.
Ishikawa, K., Kuroda, C., Ueki, M., & Ogata, K. (1970a) *Biochim. Biophys. Acta 213*, 495.
Ishikawa, K., Ueki, M., Nagai, K., & Ogata, K. (1970b) *Biochim. Biophys. Acta 213*, 542.
Ishikawa, K., Sato-Odani, S., & Ogata, K. (1978) *Biochim. Biophys. Acta 521*, 650.
Klein, R., & Afzelius, B. (1966) *Nature (London) 212*, 609.
Lowry, O., Rosebrough, N., Farr, A., & Randall, R. (1951) *J. Biol. Chem. 193*, 265.
Mejbaum, W. (1939) *Hoppe-Seyler's Z. Physiol. Chem. 258*, 117.
Monneron, A. (1974) *Philos. Trans. R. Soc. London, Ser. B 268*, 101.
Nagel, W., & Wunderlich, F. (1977) *J. Membr. Biol. 32*, 151.
Olason, D., & Smuckler, E. (1976) *Arch. Pathol. Lab. Med. 100*, 415.
Raskas, H. (1971) *Nature (London), New Biol. 233*, 134.
Rogers, M., & Strittmatter, P. (1973) *J. Biol. Chem. 248*, 800.
Sato, T., Ishikawa, K., & Ogata, K. (1977) *Biochim. Biophys. Acta 474*, 536.
Schneider, W. (1945) *J. Biol. Chem. 161*, 293.
Schumm, D., & Webb, T. (1974) *Biochem. J. 139*, 191.
Schumm, D., & Webb, T. (1978) *J. Biol. Chem. 253*, 8513.
Smuckler, E., & Koplitz, M. (1974) *Cancer Res. 34*, 827.
Smuckler, E., & Koplitz, M. (1976) *Cancer Res. 36*, 881.
Stuart, S., Clawson, G., Rottman, F., & Patterson, R. (1977) *J. Cell Biol. 72*, 57.
Wachstein, M., & Meisel, E. (1957) *Am. J. Clin. Pathol. 27*, 13.
Yasuzumi, G., & Tsubo, I. (1966) *Exp. Cell Res. 43*, 281.

Incubation of Exogenous Fatty Acids with Lymphocytes. Changes in Fatty Acid Composition and Effects on the Rotational Relaxation Time of 1,6-Diphenyl-1,3,5-hexatriene†

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ABSTRACT: Lymphocytes were incubated with various long chain fatty acids bound to albumin. Incubation for 20 h with unsaturated fatty acids resulted in uptake into the neutral lipids and phospholipids. The addition of concanavalin A enhanced the uptake. With the unsaturated fatty acids the rotational relaxation time of 1,6-diphenyl-1,3,5-hexatriene (DPH) was decreased, while with saturated fatty acids there was only a very small effect. The effect on the rotational relaxation times with unsaturated fatty acids was found to be due to the formation of lipid droplets in the cytoplasm. When plasma membrane free of lipid droplets was prepared, there was no effect on the rotational relaxation time despite incorporation of either linoleate or palmitate into the membrane phospholipids. Phospholipid liposomes prepared from plasma membranes obtained from cells which had been cultured with and without exogenous linoleate gave identical rotational relaxation times regardless of the history of the cells from which they were obtained. It is concluded that although the fatty acid composition of lymphocyte plasma membrane can be modified by exogenous fatty acids, there is little effect on the degree of order of the membrane phospholipid fatty acyl chains as monitored by the effects on the rotational relaxation time of DPH.

An increasing interest has been directed toward the elucidation of the functional roles of lipid in animal cell membranes. The starting point for investigations has often been to study how the membrane lipid composition of certain cells could be varied in response to the addition of exogenous fatty acids. The phospholipid fatty acids of various cell types have been successfully modified in culture after supplementation with both saturated and unsaturated fatty acids. The cell which has been most commonly used is the fibroblast (Williams et al., 1974; Glaser et al., 1974; Ferguson et al., 1975; Doi et al., 1978; Spector et al., 1979), while other cells which have been

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