Energy Utilization and RNA Transport: Their Interdependence†

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ABSTRACT: The interdependence of RNA transport and the metabolism of nucleotide additives was investigated. Rat-liver RNA was radioactively labeled in vivo for 45 min before isolation of liver nuclei, and the concentration dependence of RNA transport on nucleotide additives was determined. In a parallel investigation, using nucleotide additives labeled in the base moiety, the distributions of label in the tri-, di-, and monophosphate forms were examined after various intervals of incubation. Analysis of results revealed that RNA transport was linearly related to the decline in energy charge of nucleotide additives, with high statistical correlation. Kinetic analysis of labeled-nucleotide metabolism led to a simple schematic model for pathways for the utilization of high-energy phosphate bonds, and predictions of the scheme were confirmed by studies examining the effects of nucleotide analogues upon RNA transport. Data concerning inhibitors and chelators intimated that multiple avenues of inhibition and stimulation may potentially influence RNA transport. On the basis of previous data and the results presented in this communication, we conclude that nucleocytoplasmic RNA transport is dependent upon high-energy phosphate-bond hydrolysis and that nucleotides do not stimulate RNA transport via a simple chelation mechanism.

Stimulation of in vitro RNA transport by nucleotide addition has been reported (Schneider, 1959; Ishikawa et al., 1969; Smuckler & Koplitz, 1974; Schumm & Webb, 1975), but the nature of phosphate ester involvement in the release process is unknown. Some investigators have viewed nucleotide participation in a physical sense, claiming that enhancement of RNA transport occurs by chelating divalent cations (Chatterjee & Weissbach, 1973; Sauermann, 1974) known to inhibit transport (Ishikawa et al., 1969). Others have considered nucleotide participation to be a biological function which supplies the energy necessary for the transport process (Ishikawa et al., 1969; Smuckler & Koplitz, 1974). We found that RNA transport in vitro had an activation energy (E_a)[1] of 13 kcal/mol (Clawson & Smuckler, 1978). Initial linear rates of RNA transport increased with temperature over the domain of 0–35 °C. The activation energy for RNA transport was shown to differ significantly from that for passive transfer of RNA, further indicating the energy-requiring nature of the process. Here we demonstrate with an in vitro rat-liver system that the utilization of the energy charge of nucleotide additives is linearly related to RNA transport, with a very high correlation. We propose a simple scheme for defining nucleotide participation in RNA transport in vitro.

Materials and Methods

Experimental Animals. Male pathogen-free Sprague-Dawley rats were obtained from the Charles River Breeding Laboratories and kept in our vivarium for at least 5 days before experimentation.

RNA Labeling. Preceding the experiments, the rats were starved overnight (15 h) and then given injections of [^{14}C]-orotic acid (New England Nuclear, 28 mCi/mmol) via the tail vein at a dosage of 3 μCi/100 g of body weight. The animals were sacrificed after 45 min of labeling.

Preparation of Nuclei. Livers were quickly taken from the rats and homogenized in sucrose buffer (0.25 M RNase-free sucrose, 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 5 mM mercaptoethanol) using Teflon–glass homogenizers, then mixed with 2 volumes of 2.3 M sucrose buffer (with the same additions), layered over a "cushion" of 2.3 M sucrose buffer, and centrifuged at 95 000g for 75 min at 5 °C with an SW27 rotor in a Beckman L5-65 ultracentrifuge (Blobel & Potter, 1966; Smuckler & Koplitz, 1974). The pelleted nuclei were resuspended in 0.88 M sucrose–TKM buffer (0.88 M RNase-free sucrose, 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 25 mM KCl) and diluted to a protein concentration of 7.4 mg/mL.

Transport Assay. Nuclei at a final protein concentration of 3.7 mg/mL were incubated with 0.88 M sucrose buffer, purified RNase inhibitor (Gagnon & de Lamirande, 1973), and concentrations of nucleotide as specified (e.g., see Figure 1). The nuclei were incubated in the different mixtures for 20 min at 20 °C (under these conditions, transport is linear). Reactions were terminated by the addition of ice-cold sucrose buffer, and the nuclei were sedimented by centrifugation at 800g for 8 min.

For assaying released labeled RNA, the supernatant was withdrawn and precipitated in 10% trichloroacetic acid. The precipitated material was collected by centrifugation at 800g for 30 min, the pellet was dissolved in 5 N NaOH, and radioactivity was measured by liquid scintillation counting. Supernatant radioactivity at 0 time was subtracted to determine the amount released—"facilitated transport" is the additional amount of transport obtained over that of preparations incubated without nucleotide. Total radioactivity was measured in an aliquot of nuclear suspension and was used here as a percentage standard. Total Cl_3AcOH-precipitable and soluble radioactivity was monitored throughout incubation. The amount of Cl_3AcOH-insoluble material (in the presence of RNase inhibitor) remained constant for 30 min.

Isolation and Analysis of Nucleotide Additives. For analysis of nucleotide metabolism in this in vitro RNA transport

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[1] Abbreviations used are: RNP, ribonucleoprotein; Cl_3AcOH, trichloroacetic acid; ATP, ADP, and AMP, adenosine tri-, di-, and monophosphates; AMPCP, methylene-blocked ADP; AMPCPP, α,β-methylene-blocked ATP; AMPPCP, β,γ-methylene-blocked ATP; EDTA, ethylenediaminetetraacetic acid; PCA, perchloric acid; E_a, activation energy; TKM buffer, 50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$; ATPase, adenosine triphosphatase; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

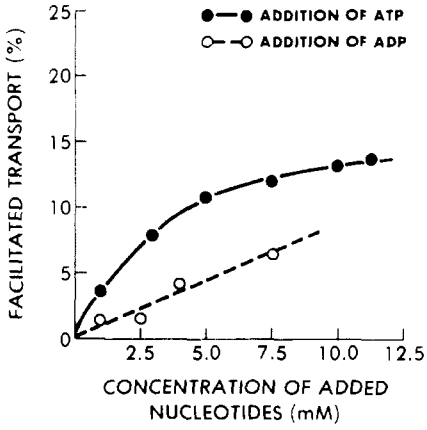

FIGURE 1: Facilitated RNA transport obtained by the addition of nucleotides: (●) ATP addition; (○) ADP addition. Incubations were performed for 20 min at 20 °C and transport was assayed as described. Zero-time values and the rate of transport without nucleotide additive were subtracted to obtain facilitated transport.

system, unlabeled liver nuclei were isolated. Nucleotides labeled with ^{14}C in the nitrogen base ring were added to the mixtures (at varying concentrations up to 8.0 mM) containing 3.7 mg/mL nuclear protein, 0.88 M sucrose buffer, and purified RNase inhibitor. Labeled nucleotide additives were prepared as a mixture of 0.1 mg/mL [8-^{14}C]adenosine diphosphate (ADP) or [8-^{14}C]adenosine triphosphate (ATP) (both from New England Nuclear, 20 μCi/mL) and the appropriate amount of unlabeled nucleotide and diluted 1:6 in the final brei.

After incubation for specified periods, the reaction was stopped by the addition of 4 volumes of ice-cold 0.88 M sucrose-TKM buffer. One-tenth volume of 3 M $HClO_4$ was added, and the ingredients were mixed thoroughly, incubated on ice for 15 min, and centrifuged at 800g. The supernatant was then collected. The precipitate was washed with 1 mL of 0.3 M $HClO_4$ and centrifuged; subsequently, the supernatant was withdrawn and combined with the first one. For adsorption of the nucleotides to charcoal, specially prepared Darco G60® was added to the supernatant. Next the suspension was mixed thoroughly, incubated at room temperature for 20 min, and centrifuged. The supernatant was discarded. The charcoal was washed with 2.5 mM perchloric acid (PCA), the wash was discarded, and the nucleotides were eluted from the charcoal with 2 mL of NH_4OH-H_2O-C_2H_5OH (2:48:50). The mixture was centrifuged and the supernatant was carefully collected. The charcoal was washed with 1 mL of ammoniacal-ethanol twice, and the washes were combined with the original eluate. The samples were then lyophilized and redissolved in a small amount of water. Preliminary experiments carried out with standard nucleotides indicated recovery of greater than 97%.

Polyethylenimine-impregnated cellulose plates (PEI plates, Baker-Flex, 20 × 20 cm) were washed with 50% methanol by ascending chromatography and air-dried. Samples (0.35 OD_{260} unit) and standards were spotted on the plates and dried. The chromatograms were developed with 1 M LiCl in water by ascending chromatography for the time necessary for the solvent to move 18 cm (approximately 2 h). The plates were again dried thoroughly and exposed to X-ray film (Kodak X-Omat R®) for 2 days, and the films were processed. The sites of the radioactive spots were marked on the backs of the PEI plates, and the spots were identified by comparison with standards, cut out, and placed in scintillation vials. The samples were then eluted with 0.7 M $MgCl_2$, 20 mM Tris-HCl, pH 7.4, for 30 min and counted in Aquasol after removal of the plate segment. Typically, recovery of label from the inital addition to incubation mixtures through this procedure exceeded 94%.

Results

In the rat-liver system utilized, RNA is released to surrogate cytoplasm containing ATP, predominantly in the form of 40-45S ribonucleoprotein (RNP) (Ishikawa et al., 1969; Smuckler & Koplitz, 1974). We undertook experiments to test whether other nucleotides could also stimulate RNA release and found that an analogous transport occurs when nuclei are incubated in surrogate cytoplasm containing ADP. The distribution of transported RNA in sedimentation profiles and in polyacrylamide gel electrophoresis is similar to that released with ATP. Nucleus-restricted RNA was examined, and again the distributions in the two incubation mixtures were comparable (data not shown) (manuscript in preparation). This basic similarity between the transported and nucleus-restricted fractions following ATP and ADP stimulation suggests that the same selective transport controls may be operative. Since we have demonstrated that the activation energy for RNA transport is the same in the presence of ATP or ADP (Clawson & Smuckler, 1978) and that ADP in higher concentrations stimulates RNA transport to the same extent as ATP (Smuckler & Koplitz, 1974), we decided to examine the metabolism of the nucleotides during in vitro incubation.

To determine the relationship between RNA transport and nucleotide additives, we assayed the level of transport with various concentrations of added nucleotide (Figure 1). RNA transport increased sharply up to 7.5 mM ATP addition and increased only slowly thereafter. Titration with ADP addition revealed a basically linear relationship in the lower concentration range (as shown).

As well as assessing the effects of the adenine-ribose-containing nucleotides on RNA transport, we assayed the effects obtained upon adding the mono-, di-, and triphosphate species of the nucleotide series containing the different bases (G, T, C, U) and containing deoxyribose moieties (e.g., dATP, etc.) rather than the ribose moieties. Fundamentally, the number of high-energy phosphate bonds afforded by the additive is the critical factor in the magnitude of transport stimulation (aside from minor differences in specificity), with adenine-ribose nucleotides being the most effective. For example, at 7.5 mM concentrations, we obtained 99, 90, 85, 80, and 85% of ATP-stimulated transport with CTP, dATP, dCTP, TTP, and dGTP, respectively (UTP and dUTP resulted in significantly lower values, and pyrophosphate did not stimulate RNA transport, indicative of some base specificity).

Our data indicate that RNA transport is directly related to the decrease in "energy charge" in this system (Table I) (energy charge is defined as 1.0 × (fraction of nucleotide in triphosphate form) + 0.5 × (fraction of the nucleotide in diphosphate form) (Atkinson, 1968). The energy charge of the system is thus normalized to values between 0 (each nucleotide has no high-energy bonds) and 1 (each nucleotide has two high-energy bonds) and serves, when related to concentration, as a measure of the quantity of high-energy bonds utilized.

When the reduction in energy charge (as related to a concentration of 0.5 mM nucleotide) was graphed vs. the percentage of facilitated transport obtained (Figure 2), a linear correspondence was observed—regardless of whether the adenine nucleotide was added as a diphosphate or triphosphate. Statistical correlative analysis yielded a product-moment correlation coefficient of 0.984 between the decrease in energy charge with added ATP and the percent of RNA transported,

TABLE I: Distributions of Label in Nucleotide Additives.

	concn (mM)	0 time (%) ATP	0 time (%) ADP	0 time (%) AMP	20 min (%) ATP	20 min (%) ADP	20 min (%) AMP
added ATP	0.5	92.5	7.5	0	36.9	40.6	22.4
	1.0	92.0	8.0	0	54.6	35.6	9.8
	2.0	91.8	8.2	0	62.0	31.2	6.8
	4.0	90.6	9.4	0	71.9	24.2	3.9
added ADP							
	0.5	0.72	98.4	1.03	9.79	37.0	52.2
	1.0	0.48	98.0	1.57	12.2	46.4	41.4
	2.0	1.21	96.4	2.43	12.3	56.2	31.4
	4.0	0.51	97.4	2.09	10.3	66.1	23.6

	concn (mM)	decline in energy charge (E_c)[a] with above additions: with ATP, 0 time	with ATP, 20 min	with ATP, E_c decline	with ADP, 0 time	with ADP, 20 min	with ADP, E_c decline
	0.5	0.962	0.572	0.390	0.493	0.283	0.210
	1.0	0.960	0.724	0.236	0.49	0.354	0.136
	2.0	0.959	0.776	0.183	0.492	0.404	0.088
	4.0	0.951	0.840	0.111	0.487	0.433	0.054

[a] E_c = (fraction of nucleotide as triphosphate) + 0.5 × (fraction of nucleotide as diphosphate).

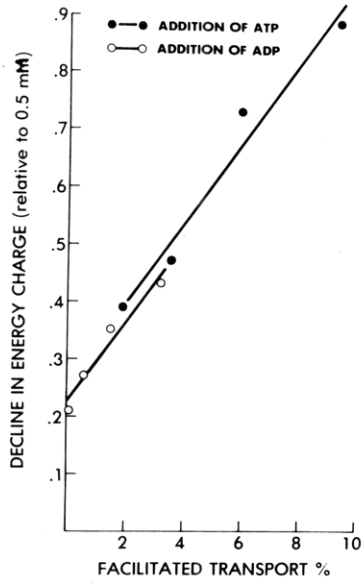

FIGURE 2: Linear relationship between the utilization of energy charge, nucleotide additive, and RNA transport obtained. RNA transport was assayed as described. Nucelotide hydrolysis was assayed during a 20-min incubation, and the decline in energy charge was calculated. The decline in energy charge with various nucleotide concentrations was normalized to the equivalent decline with 0.5 mM nucleotide additive: (●) ATP; (○) ADP.

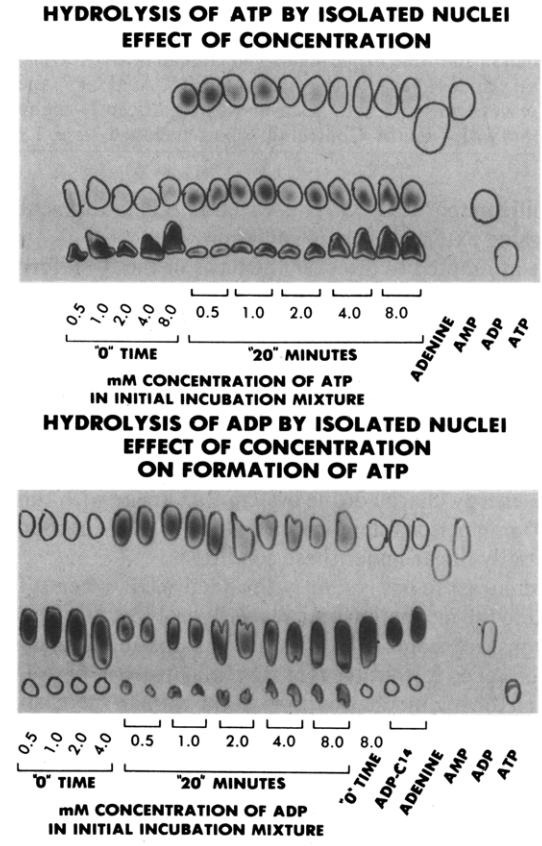

FIGURE 3: Hydrolysis of ATP or ADP by isolated rat-liver nuclei: effect of concentration. Unlabeled nuclei were incubated with ^{14}C-labeled ATP or ADP as described. At the indicated times, they were removed by centrifugation. PCA-precipitable material was removed from the supernatant by adding 2% PCA. The nucleotides from the PCA-soluble fraction were adsorbed to activated charcoal and eluted with ammoniacal-alcohol. The isolated products were then separated by thin-layer chromatography, and the plates were autoradiographed and viewed under ultraviolet light. Spots were scraped from the thin-layer sheets and their radioactivity was measured. Control experiments yielded nucleotide recoveries of 94.3 ± 4.2%.

with a linear-regression coefficient of 0.070 ± 0.009 unit increase in energy-charge decline/% transported. Similar analysis with added ADP resulted in a correlation coefficient of 0.980 and a regression coefficient of 0.069 ± 0.009. These findings demonstrate an extremely high reciprocity between the utilization of energy charge and RNA transport.

A series of experiments was undertaken to determine the fate of nucleotide additives during incubation. Radioactive nucleotide (labeled in the nitrogen base ring) and nuclei were added to the incubation brei and the mixture was incubated at 20 °C (see Materials and Methods). After various intervals, samples were withdrawn and the labeled nucleotides separated into their mono-, di-, and triphosphates (Figure 3, Table I). Whether nucleotide was added as a diphosphate or triphosphate, an extensive redistribution of label within the nucleotide series occurred after 20 min. When ATP was added to the preparation, significant amounts were found as ADP and AMP after incubation. Unexpectedly, when labeled ADP was added,

TABLE II: Kinetics of Redistribution of High-Energy Bonds (ADP Added at 1.0 mM Concentration).

incubat time (min)	nucleotide (%) ATP	ADP	AMP	energy charge
0	0.4	99.6	0	0.502
2.5	17.3	58.2	24.5	0.464
5	19.3	47.2	33.5	0.429
10	18.4	42.8	38.8	0.398
20	13.2	37.7	49.1	0.320

TABLE III: Corrected RNA Transport Obtained by Addition of Nucleotides or Analogues.[a]

additive	concentration 7.5 mM	15 mM
ATP	20.8 ± 1.1	31.1 ± 5.0
AMPPCP	14.9 ± 5.8	26.1 ± 5.9
AMPCPP	18.4 ± 6.2	26.4 ± 5.5
ADP	16.6 ± 3.6	24.9 ± 4.9
AMPCP	10.4 ± 4.9	14.2 ± 5.8
EDTA	11.9	12.6
AMP	4.5 ± 1.3	9.2 ± 1.6

[a] Transport was assessed as described, and values are corrected for transport obtained at 0 time. The values listed above (±SD) represent the mean of multiple experiments (no change in Cl_3AcOH-soluble material was observed). With ATP, AMPPCP, AMPCPP, and ADP, in all cases transport with 15 mM was significantly higher than transport with 7.5 mM. Control transport averaged 7.5 ± 1.5%.

a significant portion (10%) appeared as ATP after incubation, indicating extensive phosphorylation.

We attempted to discover the basis of the ATP formation from ADP by means of an investigation of the kinetics of ADP metabolism; nucleotide distribution was ascertained at different periods after adding labeled ADP to the incubation mixture (Table II). A dramatic redistribution of label was evident during the initial 2.5 min of incubation, with >17% of the labeled nucleotide appearing as triphosphate. Although this redistribution occurred almost immediately, the decrease in the energy charge of the system was linear with time over the 20-min period (significance >0.987). RNA transport was essentially linear under these conditions.

In contrast to our results with added ADP, when ATP was added to the incubation brei, it was hydrolyzed at a rate which was constant from 2.5 to 20 min of incubation. The observed rate of ADP formation (via direct hydrolysis of ATP) decreased during incubation, consistent with an increasing rate of ADP–ADP exchange as the ADP concentration was elevated.

We wished to further survey nucleotide hydrolysis, using additives having methylene-blocked phosphate bonds; α,β-methylene-blocked and β,γ-methylene-blocked ATP analogues were therefore tested for stimulation of RNA transport. When these analogues were added to the incubation mixture (Table III), RNA transport (and energy-charge utilization) was equivalent to that of the unblocked nucleotides. These results are analogous to those presented by other investigators (Raskas & Rho, 1973) and indicate that energy for transport may be obtained via hydrolysis of high-energy phosphate bonds in either position. It was expected that methylene-blocked ADP (AMPCP) would not stimulate RNA transport, since no high-energy bond is provided. In agreement, AMPCP (Table III) disclosed only minor stimulation of RNA transport at 7.5 mM, and the rate of such transport was lower than that observed with ADP. When the concentrations of AMPCP and ADP were raised to 15 mM, AMPCP stimulated no further transport, whereas ADP increased transport by an additional 60%. The effects obtained with AMPCP mimicked those obtained with EDTA—at a concentration of 7.5 mM EDTA, transport was lower than with 7.5 mM ADP; at 15 mM EDTA, no further stimulation of transport ensued. Thus, we attribute the meager transport stimulation afforded by AMPCP to the chelation of divalent cations. Under these experimental conditions, chelation effects were distinguishable from energy effects due to different patterns of RNA transport—namely, saturation at a lower percentage of transport (as mentioned, see Table III) and partial additivity with energy effects. Regarding additivity, RNA transport with ATP plus EDTA addition at low concentrations, for example, is slightly greater than transport with equivalent ATP addition alone (not shown). This intimates that different types of stimulation (and inhibition) may exist.

TABLE IV: Effects of Inhibitors on RNA Transport and Nucleotide Metabolism (Energy-Charge Decline).[a]

additive	ATP-contain. transp system (% control) E-c[b] decline	RNA transp	ADP-containing transp system (% control) E-c decline	RNA transp
PCMB	85	197	55	240
NaF	92	125	50	100
$CuSO_4$	15	0	2	5
EtOH	80	78	80	75
CN	92	100		100
rotenone	80	67	94	90
oligomycin	73	55	95	78

[a] Standard deviations are approximately 10% of the values given. [b] E-c = energy charge.

In an effort to perturb nucleotide metabolism in this system, we tested many compounds,[2] including *p*-chloromercurobenzoate (PCMB), sodium fluoride (NaF), silver nitrate ($AgNO_3$), copper sulfate ($CuSO_4$), ethanol (EtOH), sodium cyanide (NaCN), rotenone, oligomycin, hydrogen peroxide (H_2O_2), zinc chloride ($ZnCl_2$), amobarbital, sodium azide, and anaerobic incubation conditions (see Table IV). Our results with the rat-liver system may be summarized as follows: Copper sulfate and zinc chloride drastically inhibited both RNA transport and nucleotide hydrolysis. Silver nitrate inhibited RNA transport and nucleotide hydrolysis to differing degrees—with the addition of ATP, hydrolysis was inhibited about 10%, while transport was inhibited 35%; with the addition of ADP, hydrolysis was 65% and transport was inhibited 80%. However, this latter additive ($AgNO_3$) modified zero time values, and so these results may be of little significance. Sodium fluoride inhibited nucleotide hydrolysis but had little or no effect on RNA transport (the inhibition of nucleotide hydrolysis was greater when ADP was added than with the addition of ATP). *p*-Chloromercurobenzoate doubled the amount of RNA transported, whereas it had an inhibitory effect on nucleotide hydrolysis; again, inhibition of nucleotide hydrolysis was greater with added ADP than with ATP. (Zero-time values were much higher with PCMB.) Rotenone induced a moderate inhibition of RNA transport and nucleotide hydrolysis. Sodium cyanide had little effect on RNA

[2] Concentrations: Rotenone, 30 and 300 mM; oligomycin, 50 and 500 μg/mL; NaCN, amobarbital, and sodium azide, 1, 10, and 100 mM (each at all three concentrations); $CuSO_4$, 1 and 5 mM; EtOH, 66 μL/mL; $ZnCl_2$, 5 mM; $AgNO_3$, 1 mM; NaF, 1 mM; PCMB, 5 mM.

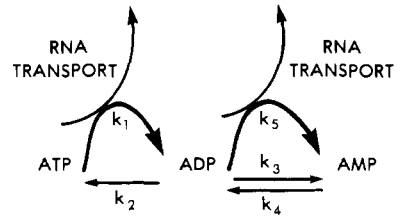

FIGURE 4: Schematic model for nucleotide interconversion and/or hydrolysis during in vitro RNA transport assay.

transport and nucleotide hydrolysis. Oligomycin inhibited RNA transport, in good agreement with previous observations by other workers (Agutter et al., 1976), and nucleotide hydrolysis to a slightly lesser extent; inhibition was greater with added ATP than ADP. The oligomycin, however, was provided in EtOH, and for this reason EtOH was tested for effects. Inhibition was observed with EtOH (although somewhat less than with oligomycin in EtOH, suggesting that oligomycin may not be the sole active ingredient in this case). Hydrogen peroxide had little effect on RNA transport (nucleotide hydrolysis was not monitored). Anaerobic incubation did not significantly affect RNA transport. Sodium cyanide, amobarbital, and sodium azide had little effect on transport under anaerobic conditions, demonstrating only negligibly decreased transport (except at the highest concentrations, which induced a slight increase in transport).

Discussion

We have interpreted our data on the basis of a simple schematic model for nucleotide interconversion and/or hydrolysis (Figure 4). The principal premises of the model are as follows: (1) An exchange reaction may occur between ADP molecules—that is, ADP + ADP $\rightleftharpoons$ ATP + AMP. (2) In this system, the decline in energy charge is due to its utilization in the RNA transport process. (3) The energy for RNA transport (E_a = 13 kcal/mol) is provided by the hydrolysis of high-energy phosphate bonds. Such hydrolysis can occur via k_1 or k_5. (4) Compared to the other constants involved, k_4 is small.

These assumptions are based upon the observed character of the system. Rapid redistribution of the label when ADP was added, with a concurrent lack of fluctuation in the energy charge of the system, indicates that an ADP exchange reaction had taken place. Moreover, the kinetics of ADP formation after ATP addition support this contention. Initially, ADP exchange should be small after the addition of ATP (due to low concentration of the former nucleotide), but as ADP concentration rises the rate of ADP exchange would be expected to increase and hence the *rate* of ADP formation should decline, as we observed. The energy required to transport the large amount of RNA delivered to surrogate cytoplasm and the fact that protein and nucleic acid syntheses do not proceed under such conditions suggest that the RNA transport component may require the lion's share of the energy utilized (in the liver system). The similarity between ATPase activation energies and the activation energy for transport is compatible with the assumption that phosphate-bond energy is utilized in transport. Also, the reported close association between nuclear envelope triphosphatase activity and RNA transport (Agutter et al., 1976) and the notably high correlation between decline in energy charge and RNA transport, as recorded here in the rat-liver system, support this assumption. AMP (or any of the monophosphate nucleotides) exhibited little transport stimulation or phosphorylation upon addition to the incubation brei.

TABLE V: Rate Constants (k_2 or k_3) for ADP Exchange Reaction as Calculated Using ADP Data and Possible Pathways for Hydrolysis.[a]

time period (min)	assume k_1 occurs	assume k_5 occurs
calcd rate const assuming that overall disapp of ADP is 2nd order		
0-2.5	0.16	0.13
2.5-5	0.28	0.19
5-10	0.13	0.07
10-20	0.13	0.04
calcd rate const assuming that overall disapp of ADP is 1st order[b]		
0-2.5	0.12	0.09
2.5-5	0.16	0.08
5-10	0.07	0.02
10-20	0.08	0

[a] All calculations are based on the assumption that ADP exchange is second order; $-d[ADP]/dt = k[ADP]^2$. Units are 1/mM-min. [b] The observed disappearance of ADP basically follows second-order kinetics. However, at longer incubation times, ADP disappearance may acquire some first-order character, and for this reason both forms were used in rate-constant calculation. The similarity of results indicates that assumptions regarding the nature of ADP disappearance do not greatly influence the interrelationships between calculated exchange-rate constants.

On the basis of the scheme presented in Figure 4 and the data in Table II regarding ADP disappearance, the supposition that the process represented by k_1 occurs implies a given rate for the ADP exchange reaction, while assuming that the k_5 process occurs indicates a different rate. The exchange rate must be second order in respect to ADP concentration; that is, $-d[ADP]/dt = k[ADP]^2$. When we used these data to calculate rate constants (Table V), we found that this boundary condition was satisfied when the process denoted by k_5 operated for the inital 5-min period, with the process denoted by k_1 active thereafter.

Thus, it appears that when ADP is added to the incubation mixture the nucleotide is at first directly hydrolyzed to supply the energy for RNA transport, with simultaneous ADP-ADP exchange. As the concentration of ATP increases via this exchange, the process designated by k_1 predominates.

It has been contended (Chatterjee & Weissbach, 1973) that nucleotide stimulation of RNA transport is indirect, based upon the chelation of divalent cations which inhibit transport (Ishikawa et al., 1969). There are strong arguments against such an indirect physical process, however. Stimulation of RNA transport by chelating agents (e.g., EDTA or AMPCP) follows a different saturation pattern (under the conditions of this study) than stimulation of transport by a high-energy-bond source such as ADP, ATP, and the phosphate-bond-containing methylene-linked analogues. As mentioned, EDTA in combination with ATP stimulates greater transport than an equivalent addition of ATP. We have reported (Clawson & Smuckler, 1978) an activation energy of 13 kcal/mol for RNA transport, a value in close concordance with measured activation energy of functional ATPase activity (see, for example, Raison, 1973; Bertoli and co-workers, 1973). Other investigators (Agutter et al., 1976) have found good agreement between k_m values for nuclear envelope triphosphatase activity (determined by means of phosphate liberation) and RNA transport, further suggestive of the hydrolysis of high-energy phosphate bonds in the transport process. In this communication, we have reported data demonstrating a linear correlation between RNA transport levels and the decline in energy

charge of nucleotide additives. En masse, these data strongly oppose nucleotide enhancement of RNA transport by simple chelation of inhibitory divalent cations.

Our results with inhibitors indicate that many of them concurrently affect RNA transport and nucleotide hydrolysis, with slight variations probably attributable to differences in individual experiments. Some inhibitors, however, appear to uncouple RNA transport and nucleotide hydrolysis. Such an example is PCMB, a sulfhydryl-blocking agent, which moderately inhibits nucleotide hydrolysis, while increasing the amount of RNA transported. The inhibition of nucleotide hydrolysis was greater with the addition of ADP than with added ATP. This may be related to the fact that PCMB is an inhibitor of the muscle enzyme myokinase, which catalyzes ADP exchange reactions (analogous effects are observed with NaF, also a myokinase inhibitor), or to the fact that PCMB alters the ATPase activity of myosin molecules (Levy et al., 1962). There are, however, alternative explanations, since PCMB may inactivate ribonuclease inhibitor and since we obtained greatly increased zero-time transport values (220% of control values); such considerations do not apply to NaF. This differential inhibition of hydrolysis while maintaining or increasing transport may be associated with altered efficiency of transport, modifications in processing/catabolism of nuclear RNA, changes in the types of RNA transported, or other mechanisms. The activity of this kind of inhibitor (sulfhydryl-blocking agents) apparently involves a different mechanism than that of the other electron-transport inhibitors.

Another category of inhibitors would seem to be represented by inhibitory additives containing divalent cations, including $ZnCl_2$ and $CuSO_4$. In the case of these inhibitors, the inhibition of transport and energy-charge utilization is drastic and does not depend upon the type of nucleotide added (ATP or ADP, etc.) nor the tissue of origin—e.g., thymus and kidney. With such inhibitors, inhibition of nucleotide hydrolysis is always closely related to the inhibition of RNA transport. The most reasonable mechanism of divalent cation activity would be via alterations in RNA, RNP, and/or nuclear structure, with shrinkage of nuclear volume up to 50% (unpublished experiments; Leake et al., 1972), and Mg^{2+} has been reported to modify mRNP structure (Favre et al., 1975). The partial additivity of chelation and energy-stimulating effects at low concentrations of energy source and chelators is also suggestive of a different mode of divalent cation activity.

In addition, lowered pH causes significant inhibition of RNA release (Ishikawa et al., 1969; Clawson & Smuckler, 1978), and adding spermidine to the incubation brei also results in reduced release (Schumm & Webb, 1975). Elevated hydrogen-ion concentrations and spermine have been shown to induce extensive shrinkage of nuclear volume (Leake et al., 1972) and changes in chromatin structure. In general, factors which elicit nuclear shrinkage also inhibit RNA transport—perhaps accounting for the widely varying levels of transport in various mixtures of rat-liver nuclei (Smuckler & Koplitz, 1974; Schumm & Webb, 1975; Sato et al., 1977).

The relationship of in vitro RNA release to the in vivo processes is not altogether clear. In the vitro liver system, because of the lack of protein and nucleic acid syntheses and the absence of cell-sap factors influencing energy-charge utilization, we were able to make the approximation that the decline in energy charge in this system is due to the hydrolysis of high-energy phosphate bonds in the RNA transport process. In this connection, divergent energy-utilization relationships have been demonstrated in rat kidney and thymus, and it appears that the foregoing assumption is not universally applicable. The occurrence of these processes in vivo precludes such an approximation.

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