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Inhibition of Intractable Nucleases with Ribonucleoside–Vanadyl Complexes: Isolation of Messenger Ribonucleic Acid from Resting Lymphocytes†

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ABSTRACT: Human lymphocyte lysates prepared by detergent treatment of intact, normal resting cells contain ribonucleases that are insensitive to many inhibitors commonly used with eucaryotic cells. Phenol-extracted ribonucleic acid (RNA) obtained directly from unfractionated cytoplasm is sometimes degraded, but after fractionation of the cytoplasmic material by sucrose density gradient centrifugation, the polyadenylated RNA, in particular, is inevitably destroyed. An extensive survey of ribonuclease inhibitors, undertaken as a consequence, indicated that the complexes formed between the oxovanadium ion and the four ribonucleosides were unique in their ability to suppress lymphocyte nuclease activity. It proved possible to isolate intact ribosomal RNA and polyadenylated messenger RNA from lymphocyte cytoplasm fractionated on sucrose gradients when 2.5 mM each of the four ribonucleoside–vanadyl complexes was used throughout the procedure. The data showed that the size distribution of poly(A)-bearing RNA remained unchanged, with a peak at ~16 S under denaturing conditions, regardless of whether the mRNA was originally associated with polysomes or was nonpolysome bound. The cytoplasmic RNAs were completely free of contamination by either intact nuclear RNA or nuclear fragments. Furthermore, exogenous globin mRNA mixed with lymphocytes and reisolated together with endogenous cytoplasmic polyadenylated RNA was fully translatable only when ribonucleoside–vanadyl complexes were employed during the preparation. The use of this inhibitor should therefore be considered for all tissues in which ribonucleases impede isolation of intact RNA.

The isolation of intact RNA from most animal cells relies on the use of exogenous ribonuclease inhibitors. Many substances including diethyl pyrocarbonate, polyvinyl sulfate, heparin, bentonite, macaloid, an assortment of ribonucleotides, sodium dodecyl sulfate, and proteinase K have been employed routinely for this purpose (Poulson, 1977). Recent investigations, however, have revealed that pancreatic ribonuclease (Jones, 1976) as well as other nucleases in crude cell homogenates (Gray, 1974; Egberts et al., 1977) retains enzymatic activity in the presence of these agents. Although the studies were carried out under test conditions removed from those actually confronted in preparative situations, in all cases the warning implicit in these findings is unmistakable. The ability to obtain intact RNA from HeLa cells (Milcarek et al., 1974), hen oviducts (Schimke et al., 1974), or fibroblasts (Johnson et al., 1974), for example, is probably a fortuitous event stemming from low endogenous levels of nucleases that are sensitive to one or more of the substances listed above. It is also possible that subcellular compartmentalization maintains the separation between RNA and RNases more effectively during lysis of some cells than during disruption of others. Clearly, the ability to carry out quantitative studies of RNA, particularly under conditions in which protein denaturation and deproteinization are incompatible with the aim of the experiment, depends upon a restricted choice of biological material. Those systems in which the nucleases are known to be intractable (Spradling et al., 1975; Cooper & Kay, 1969) receive little attention.

In order to work with resting lymphocytes, it was necessary to overcome this problem. Toward this end, we have tested a number of ribonuclease inhibitors for their ability to protect RNA in crude lysates of these cells. The criteria for choosing suitable inhibitors were the following: (1) the substance must be compatible with cell lysis techniques in which subcellular organelles are to be purified; (2) RNA must not leak out of the nuclei; (3) cytoplasmic components such as ribonucleoprotein particles and polysomes should be unaffected; and (4) RNA must remain undegraded in disrupted cells during sucrose gradient centrifugation of cytoplasm, a technique requiring several hours in the presence of proteins. Of the inhibitors tested, only one related set, the complexes formed between $VOSO_4$ and each of the four ribonucleosides, proved satisfactory. The use of the mixed vanadyl complexes not only facilitated the isolation of structurally intact, translatable RNA but also increased the yield of polyadenylated mRNA from resting lymphocytes fourfold.

Materials and Methods

Materials. Iodoacetic acid, EGTA,[1] diethyl pyrocarbonate, and the ribonucleoside 2′,3′-monophosphates (cyclic) of adenine, guanine, cytosine, and uracil were purchased from Sigma. Other compounds and their suppliers were as follows: polyuridylic acid, Schwarz/Mann; vanadyl sulfate, Fisher; proteinase K, E. Merck Darmstadt; ribonucleoside 3′,5′-bis-

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[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

phosphates and 7-methylguanosine, P-L Biochemicals; and heparin, 150 USP units/mg, Hynson, Westcott and Dunning, Inc. Formamide was obtained from Eastman and partially purified with Dowex 50 and Norit according to the method of Tibbetts et al. (1973). Wheat germ came from General Mills. Globin mRNA was supplied by Miles.

Preparation of Cells and Labeling Procedures. Resting lymphocytes were purified from the venous blood of unselected normal donors and cultured at 37 °C by using published methods (Berger, 1979). For short periods of time, cells at 10^7/mL were labeled with [^{3}H]uridine (Amersham, 20–30 Ci/mmol) as described elsewhere (Berger & Cooper, 1975); for overnight labeling, the cells were maintained at 2×10^6/mL. Specific details, such as the concentration of the radioactive precursors and the duration of exposure, are indicated in the figure legends.

Cell Fractionation and Preparation of RNA. Our method for separating cytoplasm from nuclei of washed lymphocytes is a modification of published procedures (Berger & Cooper, 1978; Berger & Birkenmeier, 1979): cells were resuspended in 1.5 mL of low-salt buffer containing 10 mM NaCl, 3 mM magnesium acetate, and 20 mM Tris-HCl at pH 7.4; a nuclease inhibitor was added when indicated; and cell lysis was accomplished by the addition of 0.5 mL of lysing buffer made by supplementing low-salt buffer with 5% sucrose (w/w) and 1.2% Triton N101 (w/w) (Rohm and Haas). After thorough mixing, nuclei were removed by centrifugation at 2200 rpm for 3 min in a Sorvall RC-3 centrifuge. All procedures were performed at 4 °C.

When cytoplasm was to be used for further fractionation on sucrose gradients, it was necessary to reduce the volume of the lysate. Accordingly, washed cells in 0.3 mL of low-salt buffer with ribonucleoside–vanadyl complexes at 10 mM (see below) were disrupted by the addition of 0.1 mL of lysing buffer. The same techniques for removing nuclei were employed. A 0.4-mL sample of cytoplasm was then sedimented on a 10–40% linear sucrose gradient in low-salt buffer containing 10 mM ribonucleoside–vanadyl complexes (see below) at 40000 rpm ($195000g_{av}$) for 165 min at 4 °C as described by Cooper et al. (1976). An SW 41 rotor and a Beckman L 565 ultracentrifuge were used. Our methods were slightly modified for high-salt sucrose gradients. The linear gradients were 10–30% sucrose in buffer composed of 10 mM Tris-HCl, 50 mM magnesium acetate, and 0.5 NaCl at pH 7.4 made 10 mM in mixed vanadyl complexes (see below) and 5 mM in magnesium chloride. The protocols for cell lysis and for centrifugation remained unchanged. Gradients were collected by upward displacement with 50% sucrose using an ISCO Model 185 density gradient fractionator equipped with a Model UA 5 absorbance monitor, a Model 568 fraction collector, and a Model 613 recorder. Approximately 40 0.3-mL fractions were obtained from each 12-mL gradient.

RNA was obtained directly from the isolated unfractionated cytoplasm as described previously (Berger & Cooper, 1975) or from intact cells in the presence of 2% bentonite by using published methods (Cooper & Kay, 1969). In all cases, phenol was redistilled and stored at −20 °C in the presence of 0.1% 8-hydroxyquinoline prior to use. RNA was stored at −20 °C as an ethanol precipitate (Berger & Cooper, 1975).

RNA was also obtained from fractionated cytoplasm. In some cases the desired fractions were first precipitated by using the standard methods for RNA (Berger & Cooper, 1975). Then they were recovered by centrifugation, redissolved in 10 mL of buffer containing 50 mM sodium acetate and 10 mM EDTA at pH 5.1, made 1% in sodium dodecyl sulfate, and treated with an equal volume of phenol as described previously (Berger & Cooper, 1975). In other cases the appropriate sucrose-containing gradient fractions served as the entire aqueous phase for a phenol extraction (Berger & Cooper, 1975). In both cases bentonite was omitted. Poly(A)-bearing molecules were obtained by chromatography on oligo(dT)–cellulose (Collaborative Research) (Berger & Cooper, 1975) or on poly(U)–Sepharose (Pharmacia) (Lindberg et al., 1972), as indicated in the text.

When the polyadenylated RNAs were required for translational purposes, they were washed by repeated precipitations. Toward this end, the original ethanol precipitates were redissolved in a large volume of H_2O and reprecipitated at −55 °C for 15 min with 0.1 M KCl and 2 volumes of ethanol. Precipitates were recovered by centrifugation at 10000 rpm for 30 min in a Sorvall RC-5 centrifuge. After three cycles, the RNA was redissolved in a small volume (0.5–1 mL) of H_2O in order to determine the absorbance at 260 nm and was precipitated again. The final precipitate was partly resuspended in 70% ethanol and recovered by centrifugation. The mRNA was then dissolved in H_2O.

Fractionation of RNA According to Size. Deproteinized RNA was fractionated with an L 565 Beckman ultracentrifuge in an SW 41 rotor on 11.6-mL linear gradients containing 8–30% sucrose (w/w) in buffer composed of 10 mM sodium acetate, 1 mM magnesium acetate, and 0.1 M NaCl at pH 5.1 (MAC buffer). When denaturing conditions were required, the sample was dissolved in buffer composed of 3 parts 3 mM Tris and 3 mM EDTA mixed with 7 parts formamide at pH 7.4 and heated for 5 min at 60 °C. The denatured RNA was then centrifuged with the same equipment on a 4.6–22% (w/w) linear sucrose gradient in the same buffer (Brown & Suzuki, 1974).

Substrate RNA for nuclease assays was prepared by labeling cells overnight, extracting RNA from intact cells, ethanol-precipitating it, and sedimenting the redissolved material for 16 h at 22000 rpm in a sucrose gradient made with MAC buffer. Unless specified, all samples sedimenting more rapidly than 28 S were combined with those sedimenting between 18 and 28 S. The pooled fractions, composed primarily of hnRNA and polyadenylated hnRNA, were reserved as ethanol precipitates for subsequent use.

Ribonuclease Assays. Measurements of nuclease activity were performed at pH 7.4 with the cytoplasm from $(2.0–2.5) \times 10^7$ cells in a total volume of 1 mL containing 20 mM Tris-HCl, 10 mM NaCl, 3 mM magnesium acetate, 1.25% sucrose, 0.3% Triton N101, inhibitors as indicated, and labeled hnRNA as substrate. Reactions were stopped by precipitating an aliquot from each sample with cold 10% trichloroacetic acid for 10 min and recovering the precipitate on a GF/C glass fiber filter. Radioactivity on the filters was determined by scintillation spectrometry. In some experiments, the inhibitors were included in the cell suspension. Subsequently, lysing buffer was added to disrupt the cells and the nuclei were removed by centrifugation. In other experiments, inhibitors were added to isolated cytoplasm. In both cases the assays were initiated by the addition of labeled hnRNA, in a small volume of H_2O, to the cytoplasm.

Preparation of Ribonucleoside–Vanadyl Complexes. The method for making vanadyl complexes as described by Lienhard et al. (1971) was modified to produce a mixture of the compounds at higher concentration. A solution containing 0.5 mmol each of the four ribonucleosides in a total volume of 8 mL was heated in a boiling water bath until all material dissolved. The solution was flushed with nitrogen gas while

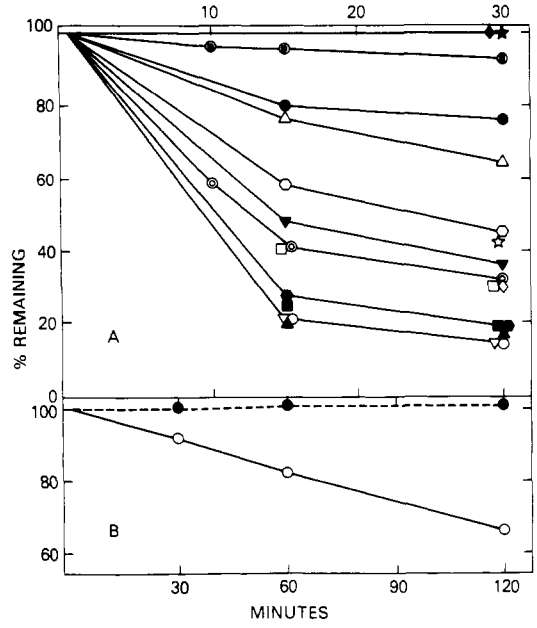

FIGURE 1: Effect of inhibitors on the degradation of ^{3}H-labeled RNA by lymphocyte nucleases. (A) The rate of acid solubilization of ^{3}H-labeled RNA containing 2×10^4 cpm was measured at 37 °C in the presence of cytoplasm from 2.5×10^7 cells. Potential inhibitors of ribonucleases were included as follows: no additions (○, ◎, ◇, and ☆); 10 mM vanadyl complexes (⊙, ●, ♦, and ★); 0.1% sodium dodecyl sulfate (△); 1 mg/mL poly(U) (○); 0.1 M iodoacetate (▼); 25 mM EGTA, 25 mM $MgCl_2$, and 15 mM NaCl (□); 20 μg/mL proteinase K (●); a mixture of 2.5 mM each of the ribonucleoside 3′,5′-bisphosphates of adenosine and guanosine (■); 10 mM 7-methylguanosine (▽); a mixture of 1.25 mM each of the ribonucleoside 2′,3′-monophosphates (cyclic) of adenosine, cytidine, guanosine, and uridine (▲). Both the inhibitors and the labeled RNA used as substrate were added after removal of the nuclei except for ◇, ♦, ☆, and ★. In these cases the inhibitors, but not the substrate, were added to the intact cells. (B) Same as for (A) at 0 °C. All experiments in (A) and (B) were performed with cells from the same donor with the exception of ⊙, ♦, ★ and their respective controls, ○, ◇, and ☆; separate donors were used for each pair.

1 mL of 2 M $VOSO_4$ (vanadyl sulfate) was added. The pH was adjusted to ~6 with the dropwise addition of 10 N NaOH and finally to 7 with 1 N NaOH, under nitrogen, with stirring, in the water bath. The formation of the complex is indicated by a change in color from blue, characteristic of $VOSO_4$, to green-black, characteristic of the complex. The volume was adjusted to 10 mL, and the entire sample was stored frozen in small aliquots. The resultant solution, a 1:1 ribonucleoside–vanadyl complex, was 200 mM. No attempt was made to separate the complexes from Na_2SO_4 produced as a byproduct.

Cell-Free Protein Synthesis. Wheat germ extract was prepared as described by Roberts & Paterson (1973). The S-30 fraction was used without preincubation for translation at 25 °C for 60 min. The reactions were run in a total volume of 50 μL containing the following compounds: wheat germ extract, 22.2 A_{260} units/mL; Hepes, 24 mM at pH 7.6; potassium chloride, 0.1 M; magnesium acetate, 3 mM; dithiothreitol, 2 mM; ATP, 1 mM; GTP, 0.1 mM; creatine phosphate, 8 mM; creatine phosphokinase, 4 μg/mL; 5-adenosylmethionine, 2 μM; spermine, 40 μM; 19 amino acids, 30 μM each; and [^{3}H]leucine (50 Ci/mmol from Amersham), 2 μM. The reaction was terminated by incubating a 20-μL aliquot in 0.2 mL of 0.3 N potassium hydroxide at 37 °C for 1 h, and the products were precipitated with an excess of 10% trichloroacetic acid. Samples were recovered on Whatman GF/C glass fiber filters which were washed exhaustively with 5% trichloroacetic acid. Protein synthesis was determined by subjecting the filters to liquid scintillation spectrometry.

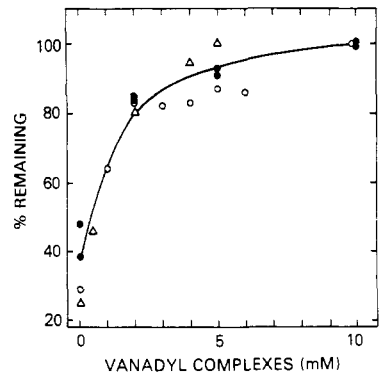

FIGURE 2: Effect of vanadyl complexes on the degradation of ^{3}H-labeled RNA. Each assay contained the cytoplasm from 2×10^7 cells in buffer as described under Materials and Methods and vanadyl complexes where indicated, in a volume of 1 mL. The cells were resuspended and lysed in the presence of the stated total concentration of an equimolar mixture of the four ribonucleoside–vanadyl complexes in one example; in the other, the guanosine–vanadyl complex was added after removal of the nuclei. The disappearance of labeled RNA fractions sedimenting between 18 and 28 S obtained from intact cells as indicated under Materials and Methods was measured initially and after 20 or 30 min at 35 °C. The amount remaining is expressed as a percent of the initial value for each sample. All samples contained ~35 000 cpm of substrate RNA at zero time. Each symbol represents a different donor. Lysis with mixed vanadyl complexes, assay at 30 min (○, ●); guanosine–vanadyl complex added to cytoplasm, assay at 20 min (△).

Results

Inhibition of Nucleases in Lymphocyte Lysates. Intact RNA can be extracted from resting human lymphocytes with phenol, provided the whole cells are exposed directly to the solvent in the presence of 0.5–1% sodium dodecyl sulfate and 2–3% bentonite; attempts at obtaining undegraded RNA from isolated cytoplasm have been largely unsatisfactory. Although excellent results have been achieved at times with either diethyl pyrocarbonate (Berger & Cooper, 1975) or rat liver ribonuclease inhibitor (Berger & Cooper, 1978; Cooper et al., 1976), these methods have never been reproducible on demand. The difficulty is the result of nucleases present in lymphocyte cytoplasm. At 37 °C the degradation of exogenous radioactive hnRNA added to a lymphocyte lysate is extensive (50–80% in 30 min) and varies with the donor (Figure 1A; ○, ◎, ◇, and ☆), whereas at 0 °C (Figure 1B, ○) the rate of degradation is slower but nevertheless detrimental to obtaining intact molecules. As illustrated in Figure 1, most potential nuclease inhibitors were incapable of preventing the degradation of RNA despite their ability to retard nuclease action. Only the equimolar mixture of the four ribonucleoside–vanadyl complexes at 10 mM protected the exogenous hnRNA substrate. At 0 °C the protection was apparently complete during a 2-h period, whereas at 37 °C the extent of protection varied with the donor, from complete recovery of the RNA as acid-insoluble material in many experiments to a loss of almost 20% in 15 min in one case. The vanadyl complexes protected the substrate RNA from degradation regardless of whether they were added to the isolated cytoplasm or to the intact cells just prior to lysis. Since the latter procedure theoretically provides protection from nuclease action as soon as the cellular RNA is at risk, it was chosen for all subsequent preparations.

As shown in Figure 2, concentrations of ribonucleoside–vanadyl complexes below 10 mM were not entirely effective. Protection of exogenous labeled RNA was incomplete, ranging from ~65% recovery of acid-insoluble radioactivity at 1 mM to ~90% at 5 mM. At 10 mM, 100% of the exogenous RNA

remained after 30 min in this series of experiments. Figure 2 also shows that the guanosine–vanadyl complex, alone, was capable of protecting exogenous hnRNA added to lymphocyte cytoplasm. A comparison of the four ribonucleoside–vanadyl complexes used individually at 10 mM revealed no differences; all of them virtually completely inhibited lymphocyte nucleases (data not shown). However, it should be understood that recovery of all radioactivity in RNA in experiments such as these does not guarantee that the RNA is intact. Nevertheless, substances which *are* capable of preserving RNA in an intact state must meet this minimum standard.

Two substances commonly used for ribonuclease inhibition are absent from the compounds tested in Figure 1A: heparin and diethyl pyrocarbonate. Heparin at 2 mg/mL and diethyl pyrocarbonate at 2 μl/mL in the isolated cytoplasm of resting lymphocytes completely prevent acid solubilization of labeled hnRNA under the experimental conditions described in Figure 1A (data not shown). Nevertheless, these compounds were eliminated from the search for a suitable inhibitor because heparin, acting as a detergent, and diethyl pyrocarbonate, acting as a solvent, ruptured the nuclei of resting lymphocytes. Furthermore, diethyl pyrocarbonate reacts with the adenine rings of mRNA (Henderson et al., 1973), rendering faithful translation of affected molecules impossible. In addition, heparin, as indicated below, does not completely prevent nuclease action in lymphocytes when more sensitive assays are employed.

Effect of Ribonucleoside–Vanadyl Complexes on Nuclear Leakage. Since many procedures for preparing specific RNAs make use of cell fractionation as a preliminary purification technique, it was necessary to determine whether the distinction between cytoplasmic and nuclear RNAs could be preserved when cell lysis occurred in the presence of vanadyl complexes. Toward this end, experiments were designed to see if hnRNA$^+$ poly(A) or degraded fragments of nuclear material contaminated the cytoplasm after detergent lysis. Cells were labeled for 10 min with [^{3}H]uridine at 37 °C, washed in ice-cold minimal essential medium, and lysed in the presence and absence of vanadyl complexes. Poly(A)-bearing molecules were obtained as detailed under Materials and Methods. Under these conditions of labeling, radioactive polyadenylated mRNA is unable to reach the cytoplasm since approximately 10 min are required for synthesis of the poly(A) "tail" and transport of the finished mRNA out of the nucleus (Darnell et al., 1971). Any radioactive polyadenylated RNA observed in the cytoplasm in such experiments would reflect nuclear leakage of whole molecules or fragments. As illustrated in parts B and D of Figure 3, virtually no labeled poly(A)-bearing RNA was found whether or not the vanadyl complexes were used. The only labeled RNA present in abundance in the cytoplasm was tRNA (parts A and C of Figure 3). Therefore, there is no evidence of labeled high molecular weight nuclear material in the cytoplasm in either experiment. However, the effect of the vanadyl complexes on the integrity of unlabeled cytoplasmic RNA is clearly shown; in Figure 3A in the presence of the inhibitor complexes, 4S tRNA and 18S and 28S rRNAs are sharp symmetrical absorbance peaks, whereas in Figure 3C, in the absence of the vanadyl complexes, the cytoplasmic RNAs are degraded beyond recognition. Clearly, vanadyl complexes are instrumental in preserving the structural integrity of RNA.

That large quantities of hnRNA and hnRNA$^+$ poly(A) were labeled in 10 min is shown in Figure 4. The 45S ribosomal precursor RNA is superimposed on a broad band of hnRNA (Figure 4). Polyadenylated hnRNA is also apparent (Figure

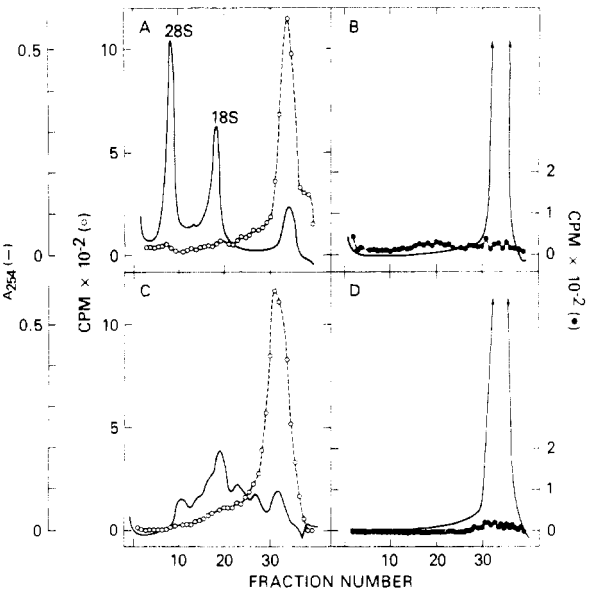

FIGURE 3: Distribution of radioactive cytoplasmic RNA after 10 min of labeling. Resting lymphocytes were labeled for 10 min with 100 μCi/mL [^{3}H]uridine. RNA was extracted from the cytoplasm of 4 × 10^8 cells with phenol in the presence (A and B) and absence (C and D) of 10 mM mixed ribonucleoside–vanadyl complexes. Columns of oligo(dT)–cellulose were used to separate poly(A)-bearing RNA (B and D) from all other cytoplasmic RNA (A and C). Separations of RNAs were carried out in linear sucrose gradients made with MAC buffer. Samples were centrifuged for 16 h at 32000 rpm. Points represent total radioactivity in each gradient fraction. The solid line is the absorbance profile at 254 nm. Carrier tRNA in poly(A)-bearing samples is responsible for the prominent absorbance peak at 4 S.

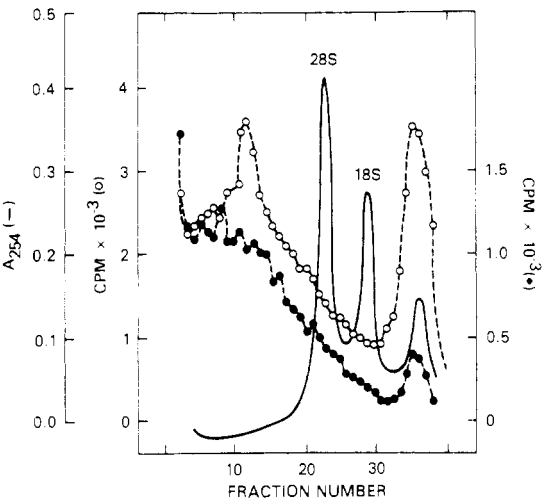

FIGURE 4: Distribution of radioactive RNA in whole cells after 10 min of labeling. Resting lymphocytes were labeled as detailed in Figure 3. RNA was extracted directly from 1.6 × 10^8 intact cells (without vanadyl complexes) and chromatographed on poly(U)–Sepharose to obtain nonadherent (○) and poly(A)-bearing (●) RNA. The solid line represents the absorbance at 254 nm of the nonadherent fraction. There was no measurable absorbance in polyadenylated RNA (not shown). Details of the gradient preparation appear in the legend to Figure 3. Samples were centrifuged at 22000 rpm for 16.5 h. When vanadyl complexes were used in the extraction (in addition to bentonite), the pattern was almost identical (not shown).

4). Since there was virtually no cytoplasmic poly(A)-bearing material evident in either case and since radioactivity in polyadenylated hnRNA was abundant, these data indicate that nuclear leakage during cell lysis is highly unlikely in our preparations regardless of the presence of the inhibitor.

Effect of Ribonucleoside–Vanadyl Complexes on Ribosomal Particles. The use of vanadyl complexes is not only

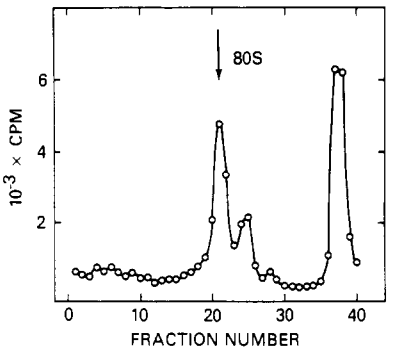

FIGURE 5: Fractionation of lymphocyte cytoplasm on a sucrose gradient. Cytoplasm from 1 × 10^8 resting lymphocytes labeled overnight with 10 μCi/mL [^{3}H]uridine was prepared by resuspending washed cells in 0.3 mL of modified low-salt buffer (10 mM Tris-HCl and 12 mM Mg^{2+} at pH 7.4) made 10 mM in mixed vanadyl complexes and lysing them by the addition of 0.1 mL of the same buffer made 1.2% (w/w) in Triton N101 and 5% (w/w) in sucrose. After removal of the nuclei by sedimentation, the cytoplasm was layered on a 10–40% linear sucrose gradient in modified low-salt buffer made 10 mM in vanadyl complexes. The gradients were centrifuged at 40 000 rpm for 165 min at 4 °C in an SW 41 rotor. Points represent trichloroacetic acid insoluble material in 50-μL aliquots. Sedimentation is from right to left.

compatible with cell lysis techniques but is also compatible with fractionation of the cytoplasmic components on sucrose density gradients. When low-salt sucrose gradients made with 10 mM vanadyl complexes throughout were charged with cytoplasm containing the complexes (Figure 5), the ratio of 40S/60S ribosomal subunits was the same as that illustrated in published profiles produced without the benefit of these substances (Cooper & Braverman, 1977). The ratio of polysomes to 80S monomers (monosomes plus free ribosomes) differed; polysomes increased slightly at the expense of monomers regardless of whether the complexes were used only in the lysis step or in the gradients as well [Figure 5 and Cooper & Braverman (1977)]. [The increase in polysomes at the expense of monosomes is more clearly seen in high-salt gradients (parts A and B of Figure 6).] However, with 10 mM vanadyl complexes included in low-salt gradients, it is necessary to adjust the concentration of Mg^{2+} to 12–14 mM to accommodate the binding of this ion by the inhibitors. It should also be noted that the ribonucleoside–vanadyl complexes at 10 mM are incompatible with either absorbance measurements at 260 nm or the use of EDTA. In the former case, the complexes themselves absorb, and in the latter, EDTA dissociates the complexes almost instantaneously.

Size Distribution of RNA from Fractionated Cytoplasm. When cytoplasmic components are separated on sucrose gradients, endogenous nucleases remain in contact with ribonucleoprotein particles for more than 3 h at 4 °C. Under these conditions RNA, particularly mRNA, is often damaged unless vanadyl complexes are used throughout all procedures. Examples of the efficacy with which these inhibitors protect RNA in the presence of native enzymes during sucrose gradient centrifugation, as described under Materials and Methods, are shown in parts A and B of Figure 7. In Figure 7A, RNA was recovered from a recombined high-salt gradient by phenol extraction and fractionated into nonpoly(A)-bearing and poly(A)-bearing material on columns of poly(U)–Sepharose. The profile of the nonadherent fraction is illustrated (Figure 7A). There is no evidence of degradation of the 18S and 28S rRNAs or 4S tRNA and no accumulation of low molecular weight labeled fragments at the top of the gradient. Figure 7B illustrates the size distribution of poly(A)-bearing RNAs recovered from low-salt gradients after phenol extraction and

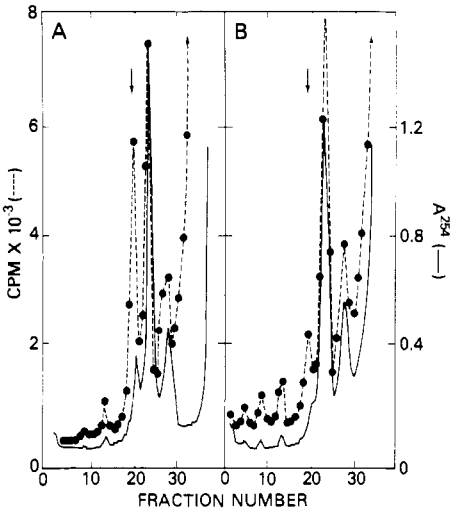

FIGURE 6: Effect of ribonucleoside–vanadyl complexes on the yield of polysomes. Resting lymphocytes were labeled overnight with 7.5 μCi/mL [^{3}H]uridine. The cells were lysed in the absence (A) and the presence (B) of 10 mM mixed vanadyl complexes. In each case the cytoplasm from 10^8 cells was centrifuged on a high-salt sucrose gradient made without either the vanadyl complexes or the 5 mM $MgCl_2$ specified under Materials and Methods. (These materials were omitted in order to allow direct viewing of the absorbance profile.) Fractions of 0.3 mL were assayed for radioactivity by counting a 50-μL aliquot directly in Aquasol. The arrow marks the position of the 80S monosome peak. Sedimentation is from right to left. That all peaks sedimenting at or faster than 80 S represent species actively engaged in protein synthesis has been shown (Cooper, 1979).

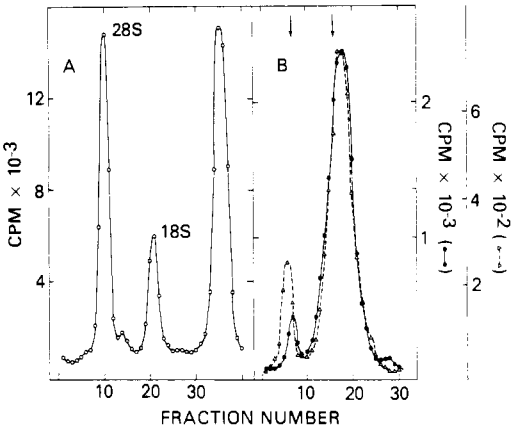

FIGURE 7: Recovery of RNA from fractionated cytoplasm. Cytoplasm from 1 × 10^8 cells labeled overnight with 10 μCi/mL [^{3}H]uridine was sedimented on either a high-salt or low-salt sucrose gradient containing 10 mM mixed vanadyl complexes. Details of the buffer composition, time, and centrifugal force for both types of gradients are given under Materials and Methods. After fractionation, the appropriate areas of the gradient were recombined and extracted with phenol. RNA was precipitated, redissolved, and finally separated into the nonadherent (rRNA and tRNA) and the adherent [poly(A)-bearing RNA] fractions on columns of either poly(U)–Sepharose or oligo(dT)–cellulose. The size distribution of the recovered RNA was determined as follows. (A) Nonadherent RNA from an entire high-salt gradient was sedimented at 30 000 rpm for 16.5 h on a sucrose gradient in MAC buffer. (B) Poly(A)-bearing RNAs from low-salt gradients were centrifuged at 40 000 rpm for 22 h at 25 °C on formamide–sucrose gradients as described under Materials and Methods: (●) polyadenylated RNA from an entire gradient; (△) nonpolysomal polyadenylated RNA obtained from cytoplasm sedimenting more slowly than 80 S monomers. The arrows from left to right mark the positions of the peak fractions of 28S and 18S RNA, respectively, run on a separate gradient.

chromatography on oligo(dT)–cellulose. Both the total cytoplasmic polyadenylated RNA obtained by combining an entire gradient and the nonpolysome-bound polyadenylated mRNA obtained by combining those fractions sedimenting more slowly

than 80 S were assayed. The size distributions were identical. Under denaturing conditions the poly(A)-bearing RNA sedimented with a peak at ~16 S. Once again, no low molecular weight labeled material was found. This behavior is characteristic of polyadenylated RNA obtained directly from the cytoplasm with phenol (Berger & Birkenmeier, 1979) in the presence of vanadyl complexes. The rather prominent peak of 28S ribosomal RNA contaminating the mRNA is characteristic of separations performed on oligo(dT)–cellulose.

When lymphocyte cytoplasm was prepared in the presence of the ribonucleoside–vanadyl complexes and centrifuged on either low-salt or high-salt sucrose gradients containing 1.75 mg/mL heparin throughout (instead of the complexes), the recovered mRNA was partially degraded. On formamide–sucrose gradients, the bulk of the polyadenylated material sedimented at 9 S (data not shown). The use of heparin as a nuclease inhibitor was therefore discontinued.

The yield of poly(A)-bearing RNA was compared after cell lysis in which either 1 μL per mL of diethyl pyrocarbonate or 10 mM vanadyl complexes were included in the disruption medium. Both RNA samples were obtained from phenol extracts of cytoplasm by absorbing them to oligo(dT)–cellulose. The results indicated that fourfold more poly(A)-containing RNA was obtained by using the vanadyl complexes for the inhibition of ribonuclease. Obviously, diethyl pyrocarbonate was inadequate. It is interesting to note, however, that the half-life of labile polyadenylated mRNA measured with diethyl pyrocarbonate (Berger & Cooper, 1975) was identical with that measured with ribonucleoside–vanadyl complexes despite the pronounced difference in recovery.

Translation of Lymphocyte mRNA. Although the mRNA obtained from resting lymphocytes appears to be intact when assayed in formamide–sucrose gradients, final proof of the integrity of the molecules requires translation of the polyadenylated material. Unfortunately, the small amount of mRNA present in resting lymphocytes is translated with low efficiency. Therefore, in order to demonstrate the validity of our preparative procedures with ribonucleoside–vanadyl complexes, we performed a more rigorous experiment. A known amount of globin mRNA was added to intact lymphocytes in our standard low-salt buffer with and without 10 mM ribonucleoside–vanadyl complexes. The cells were lysed, and poly(A)-bearing RNA was obtained from the cytoplasm as described under Materials and Methods. The resultant molecules were compared with control globin mRNA molecules which had never been in contact with lymphocyte cytoplasm. Thus, not only could we monitor the recovery of globin mRNA by using absorbance as an assay but also we could assess the ability of the mRNA which was recovered to stimulate protein synthesis. As shown in Table I, the globin mRNA recovered from lymphocyte cytoplasm stimulated protein synthesis only when ribonucleoside–vanadyl complexes were used in its preparation. Furthermore, the stimulation by globin mRNA was virtually identical with that of the lymphocyte-free control. Thus, exposure to lymphocyte cytoplasm in the presence of the complexes is not deleterious to globin mRNA. The losses in overall yield of globin mRNA which were incurred were predominantly technical difficulties with poly(U)–Sepharose columns. It is interesting to note that the loss of globin mRNA never exposed to lymphocytes was 66% after poly(U)–Sepharose chromatography in the absence of carrier RNA. It can therefore be inferred from these experiments that the lymphocyte mRNAs themselves are recovered in intact form when ribonucleoside–vanadyl complexes are employed in their preparation.

Table I: Translation of Exogenous Globin mRNA Recovered from Lymphocyte Lysates[a]

sample no.	globin mRNA	lymphocyte lysate	ribonucleoside–vanadyl complexes	protein synthesis (cpm)
1	–	–	–	679
2	+	–	–	9538
3	–	+	+	1369
4	+	+	+	11638
5	+	+	–	1190

[a] The ability of globin mRNA to direct protein synthesis after exposure to lymphocyte cytoplasm was measured as follows. 5×10^7 resting lymphocytes were resuspended in 1.5 mL of low-salt buffer containing 10 mM ribonucleoside–vanadyl complexes and 5 μg of globin mRNA where indicated. After cell lysis with Triton N101 and removal of nuclei by centrifugation, RNA was extracted with phenol as described under Materials and Methods. Poly(A)-bearing molecules were obtained with columns of poly(U)–Sepharose. The recovered, washed, polyadenylated RNA was redissolved at 50 μg/mL based on the absorbance at 260 nm. It was subsequently tested for its ability to support protein synthesis by the addition of 1 μg (20 μL) to a wheat germ cell-free protein-synthesizing system. Overall recoveries of globin mRNA were respectively 52% for sample 4 and 46% for sample 5. In the absence of globin mRNA in sample 3, a recovery of 53% was obtained based on the addition of trace amounts of labeled lymphocyte cytoplasmic RNA^+ poly(A) to the buffer in which the intact lymphocytes were resuspended. Polyadenylated RNA obtained from sample 3 was redissolved accordingly and tested for the ability of 20 μL to direct protein synthesis. Controls include endogenous protein synthesis in the wheat germ system (sample 1) and protein synthesis directed by globin mRNA (sample 2). The latter was initially dissolved in the formamide buffer used for elution of mRNA from poly(U)–Sepharose and subsequently washed by the repeated ethanol precipitations comprising our RNA washing regimen.

Whereas the ribonucleoside–vanadyl complexes are useful for producing intact RNA, these substances cannot be used to inhibit nucleases during the assay for protein synthesis itself. At 10 mM, translation in the wheat germ system was virtually eliminated regardless of whether sodium hydroxide or potassium hydroxide was used in the titration step required for preparation of the complexes. Thus, the complexes and not Na^+ are the source of the inhibition. Other cell-free protein-synthesizing systems have not been investigated.

Discussion

The existence of nucleases in the cytoplasm of resting lymphocytes has made routine preparation of RNA extraordinarily difficult. Methods that are adequate for other cell systems are simply ineffective with lymphocytes. For example, heparin, used in preparing ovalbumin mRNA from chick oviduct (Schimke et al., 1974), causes immediate nuclear rupture in lymphocytes and is therefore not suitable for studies of cytoplasmic mRNA. As shown in Figure 4, undegraded RNA can be obtained from intact resting lymphocytes with phenol and bentonite as an RNase inhibitor, but bentonite added to cell lysates fails to protect lymphocyte mRNA from degradation. Furthermore, ribosomes are adversely affected by this material (Hsiao, 1968). The ribonucleoside 3′,5′-bisphosphates were completely effective against ribonuclease in a postribosomal supernatant from Ehrlich ascites cells (Egberts et al., 1977) whereas the ribonucleoside 2′,3′-monophosphates (cyclic), depending on the base, completely protected exogenous RNA from degradation in homogenates of the leaves of the French bean plant (Gray, 1974). As demonstrated in Figure 1, neither of these substances proved useful in disrupted lymphcytes. Moreover, rat liver ribonuclease inhibitor (Roth, 1958), which was highly active against pancreatic ribonuclease A, rarely protected lymphocyte mRNA completely, even at 0 °C.

The inhibition of ribonuclease by transition-state analogues composed of complexes between vanadyl sulfate and ribonucleosides was first demonstrated by Lienhard, using the uridine–vanadyl complex to retard the hydrolysis of uridine 2′,3′-monophosphate (cyclic) by purified pancreatic ribonuclease (Lienhard et al., 1971). Since that time, these analogues have been used successfully in more complex mixtures. The guanosine–vanadyl complex in molar proportions of 10:1 (2 mM guanosine and 0.2 mM vanadyl sulfate) completely inhibited both the soluble and microsomal enzymes from French bean leaves when yeast RNA was used as substrate (Gray, 1974). The same concentration included during homogenization of the leaves markedly increased the yield of polysomes. In resting lymphocytes the concentration of a 1:1 complex necessary to produce complete inhibition was 50-fold higher. Like the bean, the yield of polysomes from lymphocytes increased when ribonucleoside–vanadyl complexes were used during cell lysis. The Ehrlich ascites system provides yet another example of the use of these substances since the ascites nucleases were inhibited by ribonucleoside 5′-monophosphate–vanadyl complexes using poly(A), poly(U), or an RNA fraction transcribed from calf thymus DNA as substrate (Egberts et al., 1977).

In resting lymphocytes, we have purified structurally intact mRNA by using 10 mM mixed ribonucleoside–vanadyl complexes at all stages of preparation. The product is free of contamination by nuclear RNA. We have also recovered intact mRNA from sucrose gradients of lymphocyte cytoplasm. It is not possible to obtain enough mRNA from human resting lymphocytes to test its ability to stimulate protein synthesis in a cell-free system in any meaningful way. However, our evidence indicates that the isolation of poly(A)-bearing mRNA in the presence of vanadyl complexes *does* give rise to functional molecules. For example, globin mRNA added to intact resting lymphocytes and subsequently reisolated together with lymphocyte polyadenylated mRNA retained full activity when translated. Furthermore, these methods have been used by us to isolate mRNA from virus-treated lymphoid cells. When translated in Xenopus oocytes, this RNA gave rise to copious amounts of interferon, proving that interferon mRNA was extracted in a functional state (Berger et al., 1979). Thus, the accumulated experience with ribonucleoside–vanadyl complexes has established these compounds as excellent ribonuclease inhibitors which should serve well in a wide variety of preparative situations.

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