STUDIES ON MESSENGER RNA IN *Artemia salina* CYSTS

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ABSTRACT

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By Diana K. Filner

A poly dT-cellulose chromatography system was developed to detect and isolate poly A-containing RNA (presumed to be messenger RNA) from total RNA preparations. The poly dT-cellulose was prepared and its retention properties characterized using total RNA extracted from Novikoff ascites cells. Approximately 1.5% of Novikoff cell RNA interacted as poly A RNA. Of this material around 7% was T_1 RNase-resistant (poly A) as assayed by sephadex G-50 or poly dT-cellulose chromatography. Preliminary results for *Artemia salina* indicated that 0.4% of the total RNA extracted from developing cysts contained poly A sequences, and that the percent in dormant cysts is not zero.

Attempts were also made to isolate mRNA in polysome preparations, made from incubated *Artemia* cysts. Up to 50% of the 260nm absorbing material in a nominal ribosome-polysome pellet was RNase-sensitive and sedimented as polysome area material (PAM) in a sucrose gradient. However spectral examination and EDTA sensitivity of this material revealed a high degree of contamination.

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TO LEE WHO ENCOURAGED, PHILIP WHO ENDURED, AND TO DANIEL BAER AND DAVID HOWARD WHO WILL APPRECIATE

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ABBREVIATIONS

A	adenosine
ATP	adenosine triphosphate
C	cytidine
DOC	deoxycholate
DNA	deoxyribonucleic acids
EDTA	ethylenediamine tetraacetic acid
G	guanosine
G.L.C.	gas-liquid chromatography
HnRNA	heterogeneous nuclear RNA
mRNA	messenger RNA
PAM	polysome area material, as assayed on a sucrose gradient
poly A	homopolymer of adenylic acid
poly U	homopolymer of uridylic acid
poly dT	homopolymer of deoxythymidylic acid
poly A RNA	RNA covalently bound to poly A
RNA	ribonucleic acids
rRNA	ribosomal RNA
RNase	pancreatic ribonuclease, specific for pyrimidines
RNP	ribonucleoprotein
SDS	sodium dodecyl sulfate
T_1	Takadiastase, type 1, a ribonuclease specific for guanylic acid
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl) aminomethane
U	uridine

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INTRODUCTION

The species Artemia salina, (Crustacea, Phyllopoda, subclass Branchiopoda, order Anostraca) commonly known as the brine shrimp, presents a potentially interesting organism for the study of morphogenesis and cell differentiation, particularly in terms of control mechanisms. Embryonic development can proceed via two pathways after fertilization occurs in the ovisac of adult females. Meiosis and blastulation are apparently normal, but gastrulation either occurs normally with the release of the free-swimming nauplius larvae from the ovisac or it may be arrested indefinitely by the formation of a cyst shell (Finamore and Clegg, 1969). Morris and Afzelius (1967) have extensively studied the structure of this shell. It was found to be composed of a 1μ thick hypochlorite-sensitive chorion of maternal origin which is at least 50 % protein in composition. Below the chorion lies an outer cuticular membrane of unknown origin that is resistant to most reagents including hypochlorite and trypsin; and an embryonic cuticular layer containing chitin and protein.

The encapsulated cysts are believed to be at least partially dehydrated in the ovisac and then released to the saline medium in which the species lives. The cysts float on the water surface and are eventually blown to shore where they are further dehydrated. It is thought that complete desiccation dries and consequently destroys an outermost cellular membrane covering the chorion, which may be responsible for maintaining minimal dehydration during the trip to shore (Morris and Afzelius, 1967). This "air drying" activates the gastrulae which are then dormant only because they lack water

(Dutrieu, 1960).

Emerson (1963) found no evidence for oxygen consumption in this encysted dormant state, which may last up to 23 years without affecting the cysts' viability. One can rehydrate the cysts at 0 - 4° without inducing resumption of metabolism, a situation which makes it possible to biochemically analyze the dormant cyst (Iwasaki, 1964; Clegg et al., 1967), but metabolic activity is resumed within five minutes of addition of water at 28° (Finamore and Clegg, 1969).

A rehydrated encysted gastrula stage embryo undergoes morphogenesis and after 8 - 15 hours emerges as a prenauplius larva which is still bound by a hatching membrane (Finamor and Clegg, 1969). The prenauplius continues to develop into a free-swimming larva resembling a tiny shrimp which then undergoes about 14 molts in as little as two weeks to reach adult stage (Anderson, 1967).

The hydrated encysted embryo of the brine shrimp is quite remarkable in that metabolism may still be reversibly halted by complete redesiccation (Finamore and Clegg, 1969) and in that considerable cell differentiation occurs in the cyst capsule stage without any evidence for cell division (Nakanishi et al., 1962). The last feature is especially interesting since it impliesthat translational changes can be studied independently of cellular variations due to the mitotic cycle.

The feature of prime concern in this study is the appearance of the protein synthesis apparatus during the first 5 - 10 minutes of hydration in dormant cysts apparently (but not definitely) devoid of pre-formed polysomes. Hultin and Morris (1968) found no evidence for

pre-formed polysomal structures in electron micrographs of dormant gastrulae, but they did find that the cytoplasm was densely packed with free ribosomes and a lesser amount of membrane-bound ribosomes. They studied cell-free amino acid incorporation with microsomal fractions, but the levels of incorporation seem too low to be of any real significance (20 -50 cpm) and their techniques did not rule out bacterial contamination. The free ribosomes were capable of incorporating phenylalanine in a reaction dependent upon poly U as template, with low endogenous levels of incorporation (around 2500 cpm over background per 50 ug RNA) and they concluded that free or bound polysomes were at best present at very low levels in dormant cells, although potentially active ribosomes were abundant.

Clegg and Golub (1969) found low amounts of RNAase-sensitive A_{260} absorbing materials (presumably polysomes) sedimenting more rapidly than 81 S monosomes in both dehydrated and rehydrated dormant cysts maintained at 4°. Based on qualitatively convincing sucrose gradient profiles both of extract and the cell-free amino acid incorporating system made from the extract, they defined these materials as polysomes. They were, however, reading RNAase-dependent differences in the 0.04 - 0.08 A_{260} range and in the 2 - 20 cpm range of radioactivity. Cell-free protein synthesis in homologous systems fell within the 50 - 100 cpm/mg protein range, except when additional poly U was added (1200 cpm/mg protein). The rapidly sedimenting A_{254} material as assayed by sucrose gradient analysis was often not completely sensitive to RNAase digestion. Other studies (Finamore and Clegg, 1969) showing low levels of polysomes in extracts

made from dormant cysts also exhibit very low levels of incorporation.

Clegg and Golub (1968) found a relatively dramatic increase in so-called polysomal material after incubating prehydrated cysts three minutes at 28°, and after only 15 minutes in non-prehydrated cysts. As the cysts are incubated at 28°, polysomes detected by sucrose gradient analysis increase rapidly; protein synthesis activity in vitro increases concomitantly. The question, therefore, becomes one involving the mechanism of rapidly implementing protein synthesis in an organism almost completely devoid of preformed polysomes. Specifically, what may be the developmental significance of any real polysomes present in dormant cysts, and, given a population of apparently active ribosomes, what is the source of the mRNA found in the polysomes formed so soon after resumption of metabolism ? Is it stored in some manner in the dormant cysts or is it formed rapidly upon rehydration and incubation ?

One would suspect that any preformed polysomes in the dormant cysts may represent a random selection of protein synthesis activity. However, they may represent specific synthetic activity involved in the formation of the cuticular membranes , which Morris and Afzelius (1967) believed to be of embryonic origin. If the latter case is true, it may be possible to synthesize specific proteins from cell-free systems using dormant cyst polysomes, provided an adequate quantity of polysomes could be isolated.

The question of mRNA stored in dormant Artemia cysts invokes the possibility of "masked messenger" or Spirin's "informosome" (Spirin, 1966), a RNA-protein complex which is suggested as the transportable form of messenger RNA or as the RNAase-resistant non-ribosome

bound form which can be stored in the cytoplasm (Lee and Brawerman,1971). Recent developments in RNA chemistry have produced more evidence in support of the existence of such template active ribonucleoprotein (RNP) particles (Toennesen and Toenne, 1973; Infante and Nemer,1968; Perry and Kelley, 1968). Heterogeneous nuclear RNA (HnRNA) proposed as precursor to mRNA (Jelinek et al, 1973; Imaizumi et al, 1973), is found almost entirely in RNP particles (Lukanidin, 1972); rapidly labeled RNA is released from polysomes by EDTA treatment as RNP particles (Adesnik et al.,1972).

The association of both HnRNA and mRNA-bound poly A sequences with HnRNA cleaving enzyme and poly A synthetase (Niessing and Sekeris, 1973) and with poly A protector and cleaving enzymes (Rosenfeld et al., 1972; Blobel,1973) suggests that the protein portion of RNP particles is indeed strongly linked with the fate of the RNA portion. One might expect to find any mRNA or precursor for mRNA stored in dormant cysts as a RNP particle.

It was the purpose of this work to investigate the possible existence of a fraction of non-ribosome bound DNA-like RNA in dormant cysts and if found to determine whether or not it is mRNA, and if possible to isolate a quantity of it to use in more detailed characterization. This project seemed especially feasible since it was reported that the dormant cyst is free of RNase (Warner,1971).

The first prerequisite for such a study is an operational definition of mRNA. By strictest definition mRNA must be characterized by the ability to direct the polymerization of amino acids in specific sequences, i.e. the synthesis of proteins (Zomzely-Neurath and

Moore, 1973). With the exceptions of globin messenger, which is essentially pure in red blood cell extracts, and of myeloma heavy chain messenger, which is apparently specifically precipitated by myeloma protein 5563 H_2L2 (Blobel, 1973), potential templates have not been isolated to purity. Total message is isolated, usually using poly A techniques that will be discussed later, and proteins synthesized in vitro are identified by gel electrophoresis (Stevens and Williamson, 1973; Zomzely-Neurath and Moore, 1973) or by immunochemical precipitation (Schutz et al., 1970).

Generally, incorporation of labeled amino acids into acid precipitable counts in a cell-free system has been accepted as indicative of the presence of non-specific mRNA, but one must consider that other RNAs may have activity also or may activate endogenous mRNA of the ribosomal system (Kruh et al., 1964; Scheffer et al., 1964).

Historically, however, mRNA has been defined as rapidly-labeled RNA with a high turnover rate that is associated with polysomes (Lee and Brawerman, 1971a; Clever and Storbeck, 1970). It has a DNA-like nucleotide composition (Evans and Lingrel, 1969). Such RNA has been shown to be released from polysomes by EDTA treatment (Adesnik and Darnell, 1972; Penman et al., 1968) or by puromycin-induced termination of polypeptides (Blobel, 1973). There is great danger in using this criterion as sole defining condition for mRNA however, as non-RNase-sensitive RNA contaminants have frequently been observed co-sedimenting with polysomes in sucrose gradients (Plagemann, 1969; Kabat and Rich, 1969; Perry and Kelley, 1968). The contaminating particles can be distinguished from polysomes by equilibrium gradient

centrifugation (Olsnes, 1970; Penman et al., 1970); and by lowering of their density gradient S-value by ionic detergent treatment, but not by EDTA (Olsnes, 1970; Penman et al, 1968).

Within the last few years it has been found possible to identify and isolate mRNA as defined by one or more of the above criteria, on the basis of covalently linked polyadenylic acid sequences. The poly A sequences have been shown to be covalently linked to the 3'-OH end of the mRNA (Nakazato et al, 1973) in essentially all eukaryotic and viral RNA's tested thus far (Green and Cartas,1972), except for histone message (Jelinek et al, 1973). No poly A has been found in E. coli (Green and Cartas, 1972). Poly A RNA has been found associated with both membrane-bound (Faust et al, 1973) and free polysomes (Jelinek et al., 1973; Adesnik et al., 1972), with both strands of double-stranded (in vitro) SV-40 specific RNA (Aloni, 1973); with 34 - 39 % of mitochondrial RNA of Lettré Ehrlich ascites tumor cells (Avadhani et al., 1973). Furthermore, 4.8 % of rabbit embryo total RNA was found to be poly A RNA (Schultze et al., 1973). Poly A RNA isolated from polysomes is usually found to represent 2 - 3.5 % of polysomal ^{32}P counts (Schutz et al., 1970; Swan et al., 1972; Green and Cartas, 1972). Between 12 and 20 % of a poly A RNA fraction is found to be RNAase resistant, depending on the species and length of labeling period (Faust, 1973; Nakazato et al., 1973). Yeast rRNA has less than 0.04 % which fits the poly A criteria (McLaughlin et al., 1973).

At least 20 % of HnRNA has also been shown to contain poly A sequences 150-250 nucleotides long (Greenberg and Perry, 1972; Jelinek et al., 1973). Nakazato et al. (1973) propose that no more than 40 % of HeLa cell HnRNA has poly A. HnRNA has for some time been

thought to be a precursor of mRNA (Darnell, 1968); it has now been rather conclusively shown to be so, based on poly A data (Greenberg and Perry, 1972; Jelinek et al., 1973). Nakazato et al. (1973) have recently found an additional poly A sequence of approximately 20 nucleotides in HeLa cell HnRNA that is not at the 3'-OH terminus.

Except for yeast with about 50 nucleotides (McLaughlin et al., 1973), most of the poly A sequences are 4-5 S, 150 -250 nucleotides long (Adesnik et al., 1972; Edmonds et al., 1971; Lee et al., 1971b) and seem to be relatively homogeneous in size in any given species, as judged by gel analysis. However, Stevens and Williamson (1973) found two distinct gel bands for cytoplasmic immunoglobulin heavy chain mRNA that had been specifically precipitated with 5563 H_2L_2 protein. The two fractions differed at least in the length of their poly A sequences. Swan et al. (1972) also found two poly A RNA fractions differing in the length of poly A sequences, using poly dT cellulose chromatography on myeloma (MOPC-41) membrane-bound polysome RNA. These two poly A RNA fractions may represent two stages of adenylation.

The poly A sequences seem to be added after transcription of the HnRNA is completed (Jelinek et al., 1973; Philipson et al., 1971). Poly A synthetases have been shown, as early as 1960 by M. Edmonds. Niessing et al. (1973) have demonstrated the presence of synthetase in ascites cell nuclei and Haff and Walker (1973) have found two distinct poly A polymerases in yeast nuclei. Polymerase I is active in low salt and is dependent on primer without poly A; polymerase II is active in high salt and requires poly A primer. These enzymes may account for

the suggestion of Slater et al. (1972) that mRNA's transcribed before fertilization in sea urchin eggs may have short poly A sequences attached (type I enzyme has been active) while longer stretches of poly A are added after fertilization in the absence of concomitant transcription (type II enzyme has been active). Such a two-stage adenylation process would provide a convenient control point in organisms which pass through a dormant stage for postponing any function dependent on the presence of poly A.

Various functions have been proposed for the poly A sequences: 1) control of transport of mRNA to the cytoplasm (Darnell,1971b; Lee et al., 1971b); 2) recognition signals for cleaving of HnRNA to create mRNA (Edmonds et al.,1971); 3) control the efficiency of mRNA utilization - Sussman's "ticketing" hypothesis (Sussman,1970); 4) directing activity of binding of ^{3}H-met-tRNA to ribosomes (Rosenfeld et al.,1972). An influence of poly A on translational activity is suggested by the report of Swan et al. (1972) that poly A RNA fractions with longer poly A residues are more active in directing protein synthesis <u>in vitro</u> than the RNA fraction with shorter poly A segments. It is most likely that the total mechanism in a normal cell will prove to be a combination of all of these functions.

Since neither the function of poly A in normally developing cells nor the mechanism of reversible dormancy in <u>Artemia salina</u> cysts are known it would be difficult to make a prediction concerning poly A RNA in dormant cysts. Dormant cysts are apparently devoid of preformed polysomes but do exhibit protein synthesis activity within five minutes of onset of metabolism, a fact which indicates either that the dormant organism contains a substantial pool of free mRNA or that

extensive transcription is initiated immediately in the incubated cyst. The latter case seems least likely since the cysts contain a nucleotide pool lacking in ATP and heavily weighted toward guanine-containing nucleotides; are incapable of de novo purine synthesis (Finamore and Clegg, 1969); are impermeable to nucleotides or their immediate precursors; have, at best, only inactive RNase (Warner, 1969). It is much more likely that Artemia cysts do contain a population of preformed mRNA or of mRNA-precursor in the form of HnRNA. In either of these cases the RNA may be protected and/or inactivated in the dormant cyst by specifically bound proteins or by adenylation or lack of adenylation. Whatever the state of mRNA stored in dormant cysts, one may assume on the basis of data obtained from the eukaryotic species examined so far that mRNA in incubated metabolising cysts do contain poly A segments.

In summary, the purpose of this work was to look for an RNA species in dormant brine shrimp that could be defined as mRNA by one or more of the criteria discussed. All experiments based on radioactive incorporation were immediately ruled out as there are reports, not supported by published data, that brine shrimp are impermeable to all commonly used radiolabeled compounds except $^{14}CO_3^{-2}$ (Warner and Finamore, 1965). The major effort of this work was directed toward isolating polysomes from rehydrated incubated cysts. It was hoped that the polysomes could be dissociated to yield ribosomal subunits and the RNA-template fraction. The latter would be extremely useful in defining the sensitivity of a cell-free protein synthesis system assay for any presumed mRNA isolated from dormant cysts. An mRNA from incubated cysts would also have intrinsic interest with respect to its composition, i.e. protein content, and to its other biological characteristics. Determination of these would

facilitate the search for mRNA in dormant cysts.

Difficulties were encountered in the isolation of polysomes from incubated cysts; consequently when reports of poly A RNA appeared, effort was directed toward isolating the mRNA via poly A techniques. Specifically poly dT-cellulose was made and characterized and an experiment was run to determine whether poly A-linked RNA exists in dormant or incubated cysts.

MATERIALS AND METHODS

Unless noted, all chemicals used were reagent grade. All water used was double distilled or deionized distilled.

Part I: Polysome Isolation

Pretreatment of Cysts

All procedures were carried out at 4°. Dry cysts, 25 grams, (Longlife Aquarium Products) were pre-hydrated for 3 h in a 2 % NaCl solution to which had been added 0.6 ml concentrated Wescodyne (West Chemicals Co.) per 100 ml solution. The cysts were drained by decantation and subsequently soaked in 100 ml freshly prepared antiformin (100 ml fresh commercial bleach, 7.8 g NaOH, 3.2 g Na_2CO_3, diluted 7:93 with water) for 30 minutes. The dechorinated cysts were then drained and rinsed thoroughly with sterile 2 % NaCl. During the rinsing process, the cysts were decanted away from the heavy residues and stones always found in commercial batches of dry cysts. The sterile cysts were then hydrated for 6 h in sterile 2 % NaCl to which 1 % urea had been added to insure complete removal of residual antiformin. Prior to incubation, the cysts were drained and rinsed with a total of 2 liters sterile 2 % NaCl after which excess liquid was aspirated off.

Incubation of Cysts

Five g drained cysts were added to 800 ml sterile growth medium (0.428 M NaCl, 0.0094 M KCl, 0.0254 M $MgSO_4$, 0.0227 M $MgCl_2$, 0.0014 M $CaCl_2$, 0.0005 M $NaHCO_3$) resembling the composition of water in the Great Salt Lake region (Warner and McLean, 1968), in sterile 2800 ml Fernbach flasks. 1000 units Penicillin G per ml medium, 17 % Zephiran (Winthrop Labs) to make the medium 10^{-4} % in Zephiran and 0.1 g streptomycin were added and the flasks were inc-

ubated at 28° on an Eberbach reciprocal shaker at slow speed.

Cysts were collected on a Buchner funnel and rinsed with 500 ml water at room temperature. Cysts were either immediately placed in ice cold grinding buffer for polysome extraction or wrapped in the filter paper and aluminum foil, immediately frozen in a dry ice-acetone bath and stored at -70°. One flask was allowed to continue incubation as a control for normal development of the cysts and as a control for contamination.

Polysome Preparation

All procedures were carried out as quickly as possible at 4°. Solutions containing sucrose were pre-treated with diethyl pyrocarbonate (ethoxyformic anhydride, or Baycovin from Bayer, Inc.) at 4 drops per 200 ml solution, heated 1 h to drive off the reagent, and stored at 4°. All glass equipment was treated with 1 M KOH to insure freedom from RNAase contamination.

Incubated cysts from two flasks were added to 5 volumes (50 ml) 10 % sucrose-buffer solution. The buffer used was 0.01 M Tris HCl, pH 7.4; 0.01 M $MgCl_2$, 0.25 M KCl, 0.007 M beta mercaptoethanol (TKM_h buffer) unless designated differently. This high ionic strength buffer stabilizes polysomes and increases their concentration relative to monosomes (Heywood et al, 1968). 0.1 % Nonidet P-40 (NP-40, Shell Oil Co.), a nonionic detergent, was added to the homogenizing buffer when noted. The cysts were then broken in Dounce homogenizers using first 2-3 passes in a 40 ml capacity homogenizer with an "A" pestle which had been hand-ground to give a minimal passage to the cysts, and then with one pass, down only, in a 12 ml homogenizer with a normal "A" pestle. The use of the Dounce

homogenizer results in gentle breakage of the cells with minimal rupture of the nuclei (Penman et al. ,1963). Such treatment rendered the cysts 80 -90% broken as judged by visual examination. The homogenate was centrifuged in a Sorvall centrifuge, SS-34 rotor, for 30 minutes with the rotation increased from 2,000 to 10,000 g after 10 minutes, and then to 14,000 g after 15 additional minutes with a 5 minute spin at 14,000 g. This procedure was designed to minimize mechanical damage to the polysomes (Evans, 1970). The top layer of orange lipid material was aspirated and 70-80% of the remaining supernatant was drawn off as the 14,000 g supernatant.

Discontinuous gradient centrifugation

Five ml of 0.6 M sucrose-buffer was layered over 5 ml 1.6 M sucrose-buffer and pre-cooled 2-4 hours. TMK_h was used unless otherwise noted. Approximately 15 ml of 14,000 g supernatant was layered over the gradients and centrifugation carried out for 7 h at 28,000 rpm (or equivalent) in a type 30 rotor in a Beckman L2 ultracentrifuge. The supernatant was aspirated, the clear pellet at the bottom was rinsed 3 times with 0.5 ml portions of ice cold 10% sucrose solution and dissolved in 500 ul of the same solution (1X pellet).

Polysome Assay by Continuous Sucrose Gradient Centrifugation

Linear gradients (4.4 mls) from 15 to 40 % sucrose were poured at room temperature over a 0.2 ml cushion of 40% sucrose. Gradients were equilibrated for at least 2 h at 4° before use. Unless noted, 0.5 ml of sample was layered over the gradients and centrifugation was carried out for 50 minutes at 38,000 rpm in the Beckman SW 39 rotor at 4°. Gradients were either collected by hand or run through a Gilford 0.2 cm flow cell mounted in a Gilford spectrophotometer, using a Buchler polystaltic pump to pull

the solutions through the system, and a Sargent recorder to trace the changes in absorbancy.

Calculation of Yield

The yield of 260 nm absorbing material sedimenting in the polysome area of the 15 - 40 % gradient will hereafter be referred to as polysome area material or PAM. The polysome area is defined as the section of the gradient below the level of the ribosome peak. The ribosome peak represents around 30 - 90 % of the 260 nm absorbing material in Artemia extracts and sediments almost at the same rate as E. coli ribosome standards. The PAM was quantitated as the percent of total absorbancy falling below the lower margin of the ribosome peak, as extrapolated to the baseline of the gradients. Total absorbancy at 260 nm is taken as the sum of the absorbancies above baseline in the monosome and polysome regions. In calculation of PAM yield, the baseline was established by the absorbancy in the polysome area of an identical sample which had been treated with 5 ul pancreatic RNAase for 10 minutes at 0 - 4° prior to centrifugation.

When the automatic flow cell system was used, background absorbancy was observed for blank gradients, i.e. with .5 ml portions of 10 % sucrose solution applied in lieu of sample. This level of true background absorbancy was used as baseline for calculating percent PAM remaining after RNAase treatment. Areas were computed by weighing cut out graph areas.

EDTA Sensitivity of PAM

1 X pellets were dissolved in 0.01 M Tris HCl, pH 7.4, 0.01 M

$MgCl_2$, 0.1 M KCl, 0.02 M EDTA, 0.005 M dithiothreitol and analyzed over 15 - 40 % sucrose gradients made in TEK_h buffer (0.01 M Tris HCl pH 7.4, 0.01 M EDTA, 0.25 M KCl).

Part II: Poly dT-Cellulose Chromatography

Techniques for isolating poly A-linked RNA's are based on the following characteristics of poly A (Raskas and Bhaduri, 1973): 1) poly A is resistant to pancreatic and T_1 RNAases but not to T_2 RNAase; 2) it binds to millipore filters in high but not in low salt; 3) the poly A segment clipped from the RNA by T_1 RNAase migrates as 6 -7 S material in polyacrylamide gel electrophoresis; 4) it has few or no uridine residues. Various methods of isolation have been used, including collection on millipore filters (Raskas and Bhaduri, 1973); retention on poly dT-cellulose (Edmonds et al, 1971a), poly dT-sepharose (Adesnik et al.,(1972a) or poly U-cellulose (Kates, 1970); and poly U hybridization followed by hydroxyapatite chromatography to separate out the double and triple stranded U:A complexes (Avadhani et al., 1973). These techniques are dependent on proper initial extraction of the RNA (Perry et al., 1972). The poly A-protein complex property of the RNA results in apparent selective loss of poly A RNA when high ionic strength buffers are used at pH 7 (Lee et al., 1971b). It is believed that this RNA aligns perpendicular to the phenol-aqueous interface with the poly A - protein section in the phenol phase and the remaining nucleotides in aqueous phase. The result is either shearing of the poly A sequences with their loss in the phenol, or loss of the entire poly A RNA at the interface which is normally discarded(Perry et al., 1972). Endonucleolytic cleavage also leads to loss of poly A sequences from poly A RNA during isolation (Adesnik et al., 1972a). These problems can be overcome by phenol extracting

at neutral pH at 60 ° (Edmonds, 1971a); or by extracting at pH 9, room temperature or below; or by using a chloroform: phenol mixture at pH 7 (Perry et al. ,1972; Schutz, 1970). Although mRNA molecules may not all include covalently bound poly A sequences, and although those that do may have sequences of different lengths (McLaughlin et al., 1973), extraction techniques based on the unique properties of poly A have thus far proved to be, by far, the most successful methods for isolating RNA fractions from eucaryotic cells that can direct the synthesis of specific proteins in vitro.

RNA Preparation from Novikoff Cells

Male Sprague Dawley rats were injected intraperitoneally with 0.5 ml Novikoff ascites cells. Seven or eight days later they were killed by ether inhalation and ascites fluid was collected by syringe, after opening the abdominal cavity surgically. Fluid volume was measured and cells were collected by 8 minutes of centrifugation at 150 -164 g, 4°. The supernatant was aspirated and the cells were washed twice with 1 fluid volume of ice cold 1 % NaCl solution. The layer of red blood cells on top of the straw-colored ascites cells was aspirated and one fluid volume of 0.1 M acetate buffer pH 5.0 (0.1 M NaAcetate pH 5,0, 0.001 M EDTA, 0.5 % sodium dodecyl sulfate) and an equal volume of phenol reagent (275 ml distilled, water-saturated phenol, 0.25 g 8 hydroxyquinoline, 35 ml m-cresol) was added. The mixture was vortexed intermittently at room temperature for 20 minutes and then hand-shaken in a 65° water bath for 10 minutes. The 65° phenol method results in an increase in RNA yield with less degradation and less DNA contamination (McClean and Warner, 1971). The solution was chilled one h and then centrifuged for 35 minutes at 25,400 g.

The phases were separated and reextracted at 65° for 10 minutes; the aqueous phase by an equal volume of phenol reagent with 0.1 % sodium dodecyl sulfate, and the phenol phase by acetate buffer. These extraction mixes were chilled and centrifuged as above. Aqueous layers were combined and RNA precipitated by two volumes of acetone at -20°.

The precipitate was collected at 12,000 g in 20 minutes, rinsed with absolute ethanol, dissolved in 10 ml 0.1 M acetate buffer, pH 5.0 without sodium dodecyl sulfate and reextracted with 1 volume of phenol reagent at 4°. The phases were separated by spinning at 12,000 g for 30 minutes and the aqueous layer cleared by spinning it at 20,000 g for 30 minutes. The RNA was precipitated from the aqueous phase by the addition of two volumes of absolute ethanol at -20°. Residual phenol was removed by three washes with 1.5 volumes ethyl ether, -20°. The RNA was precipitated overnight in absolute ethanol and redissolved in the high salt buffer to be used of the poly dT cellulose column.

RNA Preparation from Artemia salina

5.5 g portions of prepared cysts were incubated for 8 hours in 2 L growth medium with forced aeration at room temperature before being collected, frozen and ground as below. 11.5 g portions of cysts were frozen immediately in dry ice-acetone and then ground in an ice-chilled mortar and pestle. 10 ml 0.1 M acetate buffer pH 5.0 that was 1.2 % in sodium dodecyl sulfate and 10 ml phenol reagent were added to the slurry and the mix was ground for an additional 2 minutes. An additionl 20 ml of buffer and of phenol reagent were added and the entire mix was shaken by hand at room temperature for 5 minutes. The mix was then incubated for 30 minutes in a 65° water bath with occasional swirling

and subsequently chilled on ice for 1 h. The aqueous phase was removed after 50 minutes of centrifugation at 12,000 g and the RNA was precipitated by two volumes acetone, -20°. The RNA was further treated as with the Novikoff ascites cell RNA.

Preparation of Poly dT-Cellulose

Two batches of poly dT-cellulose were prepared according to the method of Gilham (1964). Thymidine 5' monophosphate (2 mmoles) as the sodium salt (Rayle Labs) was converted to the free acid by passing it over a Dowex-50 column in the H^+ form. The eluate was evaporated to dryness at least 3 times in the presence of 10 ml dry pyridine. Five g Whatman column chromedia CF1 fibrous cellulose powder #11011, predried over P_2O_5 in an evacuated desiccator at 100° was used. The poly dT-cellulose was stored at 4° under aqueous solution of 1 mM NaN_3. Batch II was made with the following alterations: 1) no glass beads were added, 2) 1.5 ml dry dimethylformamide (Aldrich Labs) was added to the reaction, 3) an additional 0.8 g dicyclohexylcarbodiimide (Aldrich) was added to the second step, 4) cellulose had been dried over KOH at 80° and appeared white in contrast to the brown color of batch I.

Columns were made by packing a quantity of poly dT-cellulose in 5 ml luer-lok syringes lined on the bottom with a small glass wool plug. New columns were washed with 1 M NaCl until background absorbancy fell below 0.03 at 260 nm. Prior to each sample application, the columns were washed with 50 ml water and 50 ml 1 M NaCl at room temperature, and then with 25 ml buffer A (0.1 M NaCl, 0.01 M Tris HCl pH 7.5) at 4°. The column was temperature equilibrated at 4° for 2 h

and the column head was brought to a minimum volume before adding the RNA sample in 500 - 900 ul buffer A. Unless noted, the poly dT-cellulose and sample were well mixed at least twice during the 30 minutes following application of sample, taking care to leave a 0.5 cm layer of packed cellulose undisturbed at the bottom of the column.

Fraction Collection During Poly dT-Cellulose Chromatography

Fractions were collected manually as follows, using acid-washed, tared tubes for collection: A_1 - the head from the settled column plus two 1 ml rinses of buffer A (0.1 M Tris HCl pH 7.5, 0.1 M NaCl); A_{xx}- remainder of 50 ml Buffer A wash at 4°. The column was then taken to room temperature and allowed to equilibrate for 30 minutes. Approximately 10 ml of buffer A were allowed to wash through at room temperature, the first 1 - 2 ml forming fraction A_w. The volume at the top of the column was brought to a minimum and buffer B (0.01 M Tris HCl, pH 7.5) at room temperature was used for the next elution step. The first 3 ml of Buffer B eluant formed fraction B. Fractions were kept on ice as much as possible and frozen at -20° for future use as soon as the A_{260} units had been determined.

Columns were regenerated immediately by washing with an additional 25 ml Buffer B at room temperature and with 10 ml of 0.001 M NaN_3. Columns were used repeatedly with no loss of poly A retaining capacity. In later experiments 0.1 M NH_4HCO_3 was substituted for Buffer A and 0.01 M NH_4HCO_3 for Buffer B in order to reduce residual salt in the lyophilized samples to be used for base analysis. There was no change in the retention characteristics when NH_4HCO_3 buffers were used.

T_1-RNAase Digestion of Fractions from Poly dT-Cellulose Chromatography

Selected samples (approximately 10 A_{260} units/ml) were treated with 0.1 volume of T_1-RNAase (100 units/ml) in 0.05 M Tris HCl pH 7.5, 0.05 M NH_4HCO_3 for 20 minutes at 37°. Fractions were brought to 0.1 M NH_4HCO_3 before applying to Sephadex G-50 columns or reapplying to poly dT-cellulose columns in the normal fashion. Fractions from these poly dT-cellulose runs were lyophilized in preparation for analysis on G-50 or for base analysis by G. L. C.

Preparation of RNA Samples for G.L.C. Analysis; Enzymatic Digestion

Samples were precipitated by addition of 2 ml ice cold 5 % trichloroacetic acid and collected on Whatman 3 mm filter paper (2.6 cm) presoaked in 5 % trichloracetic acid. Filters were washed with 6 - 8 ml cold 5 % trichloroacetic acid, 4 - 5 ml cold ethyl alcohol, and at least 2 ml of cold ethyl ether before drying under a lamp.

Dry filters were incubated in 25 ml Erlenmeyer flasks with 1.5 - 2 ml of enzyme solution at 37° for approximately 3 days, including at least 24 h of shaking on a Dubnoff shaker. The enzyme solution used was either Enzyme I (0.05 mg bacterial alkaline phosphatase (BAPSF, salt fraction, Worthington)/ml; 2.0 ug pancreatic RNAase (Sigma)/ml; .05 mg Crotalus venom phosphodiesterase (Worthington)/ml, all in 0.01 M $(NH_4)_2CO_3$, 0.001 M $MgCl_2$,)or Enzyme II (0.1 mg BAPSF/ml, 2 ug pancreatic RNAase/ml, 0.5 mg venom phosphodiesterase/ml in 0.01 M $(NH_4)_2CO_3$, 0.001 M $MgAcetate_2$, 0.001 M NaN_3).

Reaction solutions were removed by pipet and filter and flask were rinsed with water. Combined rinses were lyophilized from water repeatedly until residual salts were minimal. The samples were stored

lyophilized until needed for G.L.C. analysis.

Preparation of Samples for G.L.C. Analysis, Alkaline Hydroylsis

Samples of standard synthetic polymers or nucleotides were treated with 1 N KOH for 24 hours at 37° at concentrations of 5 - 8 A_{260}/ml. The solutions were made slightly acidic with 1 N perchloric acid and centrifuged at high speed after 30 minutes, in a clinical centrifuge, to collect the precipitate. The supernatant was made slightly alkaline with 1 M$(NH_4)_2CO_3$ pH 8.75 and 60 ul alkaline phosphatase was added to each supernatant. The reactions were incubated 7 h at 45 ° and then lyophilized several times from water to remove residue.

Base Ratio Analysis by Gas-Liquid Chromatography (G.L.C.)

Lyophilized samples were analyzed on a Hewlett-Packard F & M 402 High Efficiency Gas Chromatograph, according to the method of Pike (1971). The column was composed of 2 % OV-17 on silanized Gas Chrom Z. Samples were treated with 100 ul of N,O-Bis(Trimethyl silyl)acetamide (Regis Chemical Co.) for 2 h at 120° before injecting 3.9 ul of trimethylsilyl derivative into the column at 200°. Results were analyzed by weighing cut out peaks from one chromatograph.

Gel Electrophoresis

Selected samples were analyzed by gel electrophoresis using a vertical electrophoresis cell, model EC 470, E. C. Apparatus corp.). 2 % acrylamide-0.5 % agarose gels were prepared according to the procedure of Dingman and Peacock (1968), using cyanogum 41 (Fisher Scientific Co.), a prepared mixture of acylamide and N,N'-methylene-bis acrylamide in 19:1 proportion, and Seakem agarose (Bausch and Lomb).

Samples were applied with bromphenol blue dye markers and run 1 h at 250 V, 4°. Gels were stained with Stains-All (Stock No. 2718, Eastman Organic Chemicals) and the results were recorded by polaroid photography.

RESULTS

Part I: Isolation of Polysomes

There are formidable biological difficulties encountered when working with Artemia salina cysts obtained from commercial sources. Such cysts are apparently randomly collected from shores of various salt lakes and pressure sealed in cans without regard for sand and other environmental contaminants. The biological state of such cysts could be expected to vary with time on shore, weather conditions, etc. Indeed, both hatching efficiency and incubation period for the cysts varied considerably (5 - 90 % hatching, first hatching 11 to 20 hours after commencement of incubation) depending on the particular can and the length of time it had been opened. Fungal contamination, classified as Scopulariopsis brevicolus by Dr. Ellingboe of the Michigan State University Botany Department, was difficult to avoid; it often developed after hatching commenced even when the most stringent sterile precautions were observed, unless Zephiran at 1 ppm was included in the growth media. One might almost suspect that there was perhaps an occasional fungal spore present within the cyst shells which was released when hatching occurred. The hatching controls for batches used in these experiments exhibited some hatching after 15 hours, 20 % after 20 hours and 80 - 90 % after 36 hours. There was no visual evidence for either fungal or bacterial contamination after 5 days' incubation at 28°.

Breaking the tough cyst walls open with a Dounce homogeniser was physically quite difficult and the amount of cysts that could be homogenised during any reasonable period was thereby limited to 15 grams. The use of detergents (NP-40 or DOC) did not facilitate this

process noticeably. It was hoped that a certain amount of experimental consistency could be obtained, without sacrificing yield, by storing large batches of incubated cysts at -185° and using portions for several homogenizations.

Effects of freezing

Cyst homogenates were made at various times from the same batch of incubated cysts, designated P^{24}, and subjected to various periods of -185° storage. Freshly prepared homogenate (1 X pelleted) exhibited 48 % of ribosomal material as PAM. There appeared to be only slight decrease in PAM as a function of storage either of incubated cysts (43 % in frozen cyst homogenate) or 1 X pellet at -185° (45 % in frozen pellet made from fresh cysts, 36 % in frozen pellet from frozen cysts) for up to 6 weeks. Time at 4° does seem to lead to loss of this material (13 % after 6 hours at 4°). There was generally more variability in PAM between preparations from different incubated cyst batches (30 - 53 %) than between preparations from the same batch exposed to -185° storage at various stages, and large batches of incubated cysts stored at -185° were routinely used in these experiments.

Yields

The yield of 260 nm absorbing material in the 14,000 g supernatant was generally 70 - 100 A_{260} units per gram of drained incubated cysts. Of this, 7 - 10 % was found to pellet through discontinuous gradients. The yield of PAM, as determined from continuous gradient centrifugation of 1 X pellet material, was either in the 0 - 10 % range or the 30 - 50 % range, depending on fungal contamination of the incubated cysts and on cyst viability. Typical profiles for preparations in the

Figure 1. Sucrose Density Gradient Profiles of Polysome Preparation from Incubated *Artemia* cysts: With or Without Treatment with Pancreatic RNAase.

1X pellet material prepared as described in text from cysts that had been incubated for 12 h at 28° and then stored at -185° for 3 weeks prior to homogenization: _____ 4.2 A_{260} without RNAase treatment and _ _ _ _ _ 4.2 A_{260} treated with 5 ul pancreatic RNAase for 10 minutes at 0 - 4°. Samples layered on 15 - 40 % sucrose gradients in 0.01M Tris pH 7.4, 0.01M $MgCl_2$, 0.25M KCl and centrifuged at 38,000 for 45 minutes. Gradients were collected by the flow cell system; top of gradient is to the right of the scan.

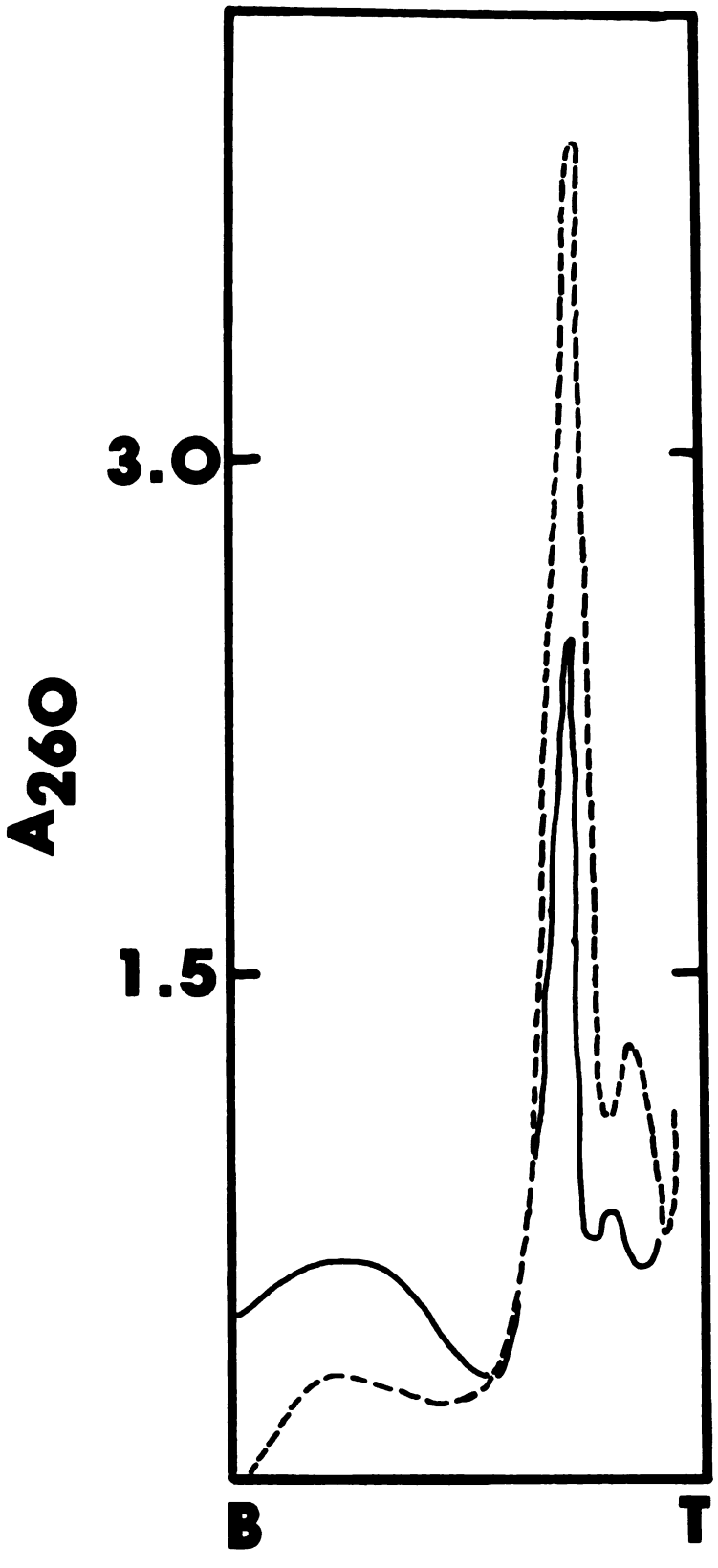

3.0
1.5
A260
B
T

Figure 2. Sucrose Gradient Resolution of Polysomes from Artemia Cysts Incubated 12 h at 28°.

1X pellet was prepared as described from non-frozen cysts. TMK_h homogenization buffer (0.25 M KCl) included 0.1% NP-40. A 3.7 A_{260} sample was applied to a 15-40% sucrose gradient and centrifugation carried out at 32,000 rpm for 1.5 h. Gradient was collected by flow cell system; top of gradient is to right of scan.

Figure 3. Sucrose Gradient Profiles of a Polysome Preparation from Incubated Artemia Cysts; EDTA Sensitivity of PAM, With or Without Treatment with Pancreatic RNAase.

Cysts were incubated for 12 h at 28° and homogenized in 0.01 M tris pH 7.4, 0.1 M KCl, 0.01M $MgCl_2$, 0.02 M EDTA, 0.001 M Dithiothreotol, 10% sucrose.

_____ $^{14}A_{260}$ of 1X pellet material and

___ ___ ___ $^{14}A_{260}$ of pellet material treated with RNAase for 10 minutes at 4° were layered on 15 -40% sucrose gradients in 0.01 M tris pH 7.2, 0.25 M KCl, 0.01M EDTA. Centrifugation was at 38,000 rpm for 2 h. Gradients were collected by flow cell system; top of gradient is to right of scan.

Profiles were similiar for 1X pellet material after storage at -185° for 4 days.

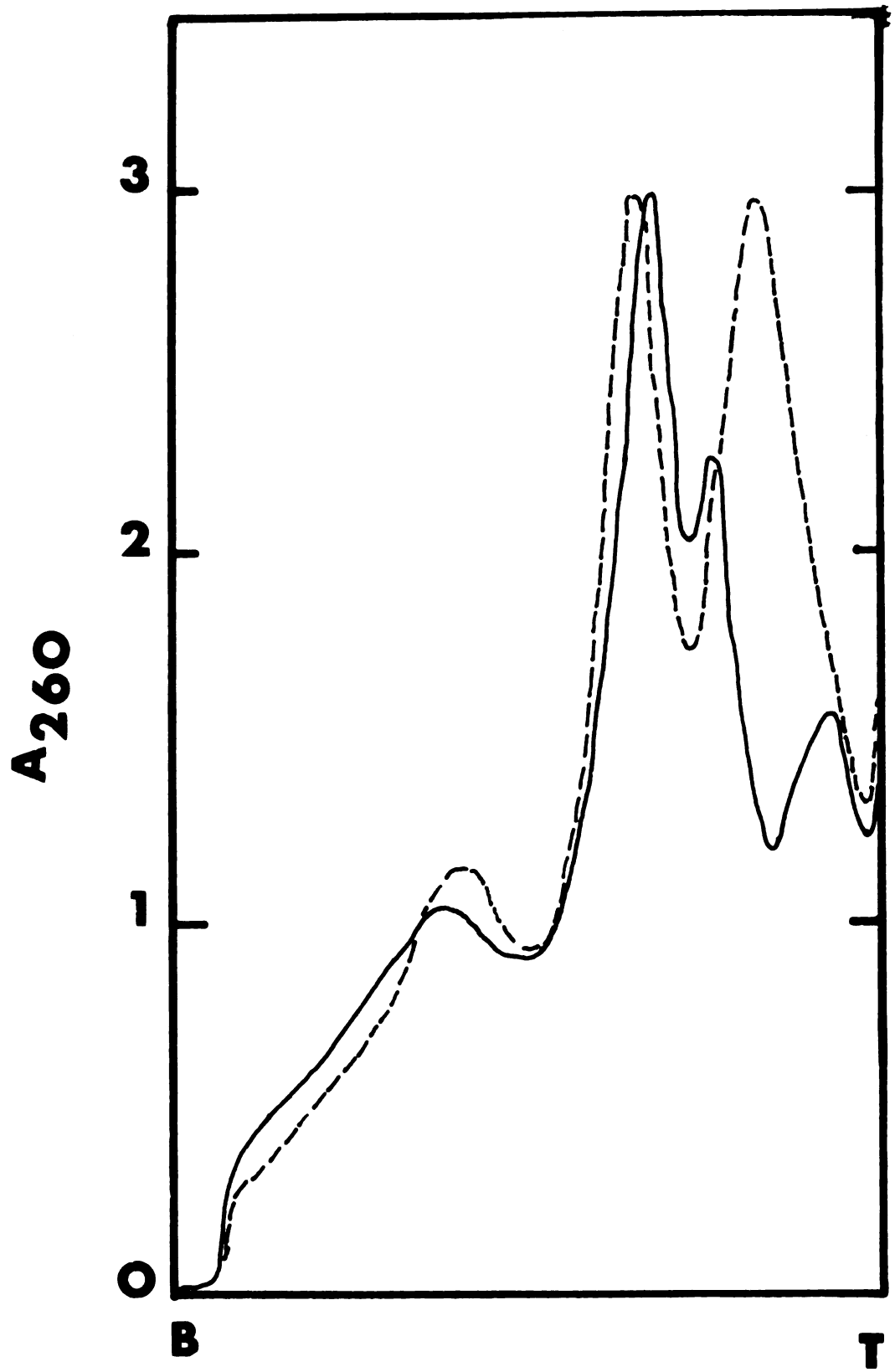

3
2
1
0
A_{260}
B
T

Figure 4. Typical Absorption Spectra Observed for Various Fractions During *Artemia* Polysome Isolation.

Type A spectrum: observed in most 14,000 g supernatants and many 1X pellet samples.

Type B spectrum: observed in many 1X pellets, a few 14,000 g supernatants and in all PAM samples.

Type C spectrum: observed for fractions 1,2, and 3 of figure 5 sucrose gradient scan.

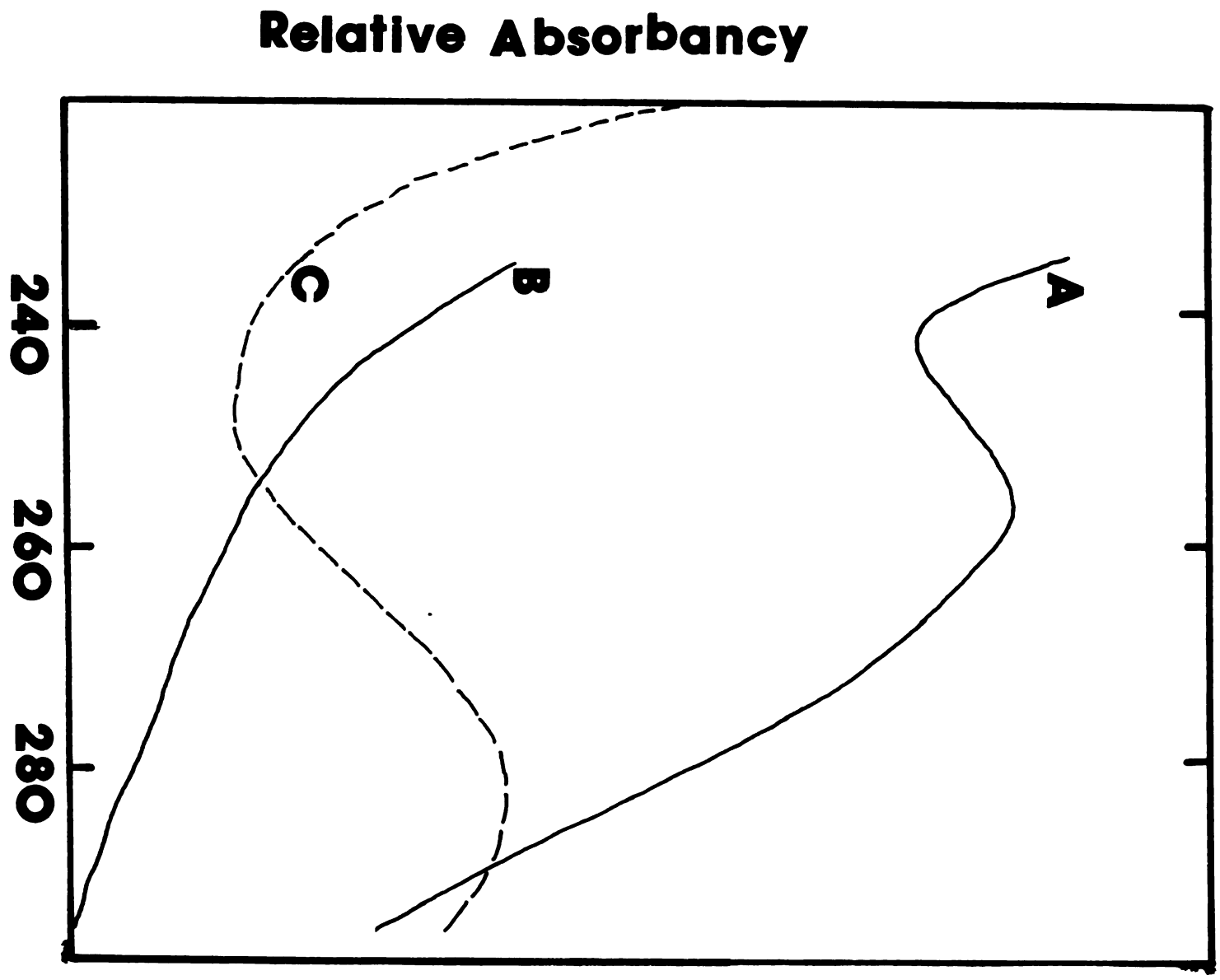

Relative Absorbancy
240
260
280
A
B
C

Figure 5. Sucrose Gradient Profile of Artemia Polysome Preparation P_{25}: RNAase and EDTA - Sensitivity of 1X Pellet Material.

Polysome preparation P_{25} exhibited type B spectrum for both 14,000 g supernatant and 1X pellet material. Fractions marked 1,2, and 3 exhibited protein spectra (type C spectrum, figure 4). Fraction 4 exhibited a type B spectrum(figure 4).

1X pellet was prepared in 0.01 M Tris pH 7.3, 0.05 M KCl, 0.01M $MgCl_2$, 0.005 M dithiothreotol. Samples, each 2.9 A_{260}, were centrifuged at 38,000 rpm for 1.5 h on 15-40 % sucrose gradients made in 0.01 M tris pH 7.4, 0.01 M KCl, 0.01M $MgCl_2$ (TMK) _____; made in TMK brought up to 0.02 M EDTA _____ _____ _____; or made in TMK with pre-treatment with RNAase as described in methods _____._____.___

upper range are shown in Figure 1 (43 % PAM). Very similar profiles have been obtained from continuous gradient centrifugation of 14,000 g supernatant (36 %). Some evidence for gradient resolution of polysome peaks was seen in flow cell profiles of the freshly prepared P^{24} 1 X pellet (Figure 2) and even in P^{24} 1 X pellet stored at -185°C for 16 hours prior to gradient analysis. The latter resolution was blurred but recognizable.

EDTA Sensitivity of PAM

EDTA treatment usually resulted in complete disappearance of the ribosomal peak in sucrose gradient profiles of Artemia extracts and in the appearance of two slower sedimenting peaks assumed to represent subunits. As can be seen in Figure 3, there is PAM under certain conditions which is neither EDTA-sensitive nor RNAase-sensitive. The percent PAM in EDTA goes from 23 % to 17 % after RNAase treatment as calculated using a blank sucrose gradient profile to establish baseline.

Spectral Examination

Figure 4 illustrates typical spectra of homogenate fractions. The abnormal spectra which display a high degree of end absorption (type B spectrum) were observed in at least 3 different batches of cysts (P^{10}, P^{25}, P^{26}) for 1 X pellet material and in batch (P^{26}) in the crude 14,800 g supernatant. In all these cases, 260 nm absorbancy profiles of 15 - 40 % linear sucrose gradients differed from the typical profiles (Figure 1) in that they indicated ample PAM, a minimal ribosome peak, a significant peak of material sedimenting faster than that of the presumed ribosomes. RNAase treatment moved essentially all of these 260 nm absorbing materials to the top of the

the gradient; EDTA treatment resulted in two peaks sedimenting slower than ribosomes (Figure 5). The spectrum of the heaviest of these EDTA peaks was checked once and found to have extremely high end absorption.

There did not appear to be any correlation between these unusual spectra and any particular experimental condition, including the use of NP-40, which exhibits high absortion at 275 nm and below 265 nm. The apparent biological condition of the cysts varied from preparation to preparation, in that the lipid fraction was either salmon colored (common) or bright red-orange. In all experiments in which 1 X pellet material gave abnormal spectra, a layer of pigmented material (salmon colored) was found over the clear 1 X pellet on the bottom of the centrifuge tube. This top layer was removed as well as possible before dissolving the 1 X pellet. It was found on one examination to have an abnormal (type B) spectrum with slight absorption at 257 nm superimposed.

In this work it was only possible to take spectra of continuous sucrose gradient fractions when manual dripping was employed. In no case (6 homogenizations) was an RNA spectrum found for PAM fractions, even for one cyst batch where the 14,000 g supernatant and 1 X pellet spectra, and the sucrose gradient profile itself (58 % PAM) had all been normal. In one cyst batch (P^{25}, which had abnormal 14,000 g supernatant and 1 X pellet spectra) spectra from three representative fractions of the unusual sucrose gradient all gave protein spectra (type C spectrum) with a maximum at 285 nm, minimum at 245 nm, and 260/280 ratio of .56, 285/245 rationof 2.7. Polysomes should have an A_{260}/A_{280} ratio of about 1.85 (Vessey and Keck, 1970).

Part II: Poly dT-Cellulose Chromatography

RNA Preparations

Four different Novikoff ascites cell RNA preparations were used in the experiments designed to characterise the poly dT - cellulose column behavior. The total RNA preparation defined as R_1 was made from cells taken from rats which had been injected with ascites cells 8 days previously. Its spectrum exhibited a 260/280 absorbancy ratio of 2.0. RNA preparations R_2 and R_5, made from rats injected 7 days previously, had 260/280 ratios of 2.2 and yields of roughly 20 A_{260} units per gram ascites fluid. Preparation R_4 from 7 day cells had a 260/280 ratio of 2.5 and a yield of around 10 A_{260} units per gram ascites fluid. The procedure used during preparation R_4 differed in that the initial phenol extraction mix was chilled 5 hours before centrifugation and in that the combined aqueous phases of the reextraction step were shaken with 1/3 volume phenol for 3 minutes at room temperature before precipitatingsthe RNA in acetone.

All RNA preparations exhibited absorbancy maxima at 258 nm with minima between 228 (R_4) and 233 (R_5) nm. None of the preparations showed significant degradation of ribosomal RNA on 2 % polyacrylamid e gels.

Artemia salina RNA prepared from dormant cysts had a 260/280 absorbancy ratio of 1.75 with a maximum at 254 nm and a minimum at 230 nm. Approximate yields were 960 A_{260} units from preparation I dormant cysts (83 A_{260} units/ gram damp cysts) and 810 units from preparation II, incubated cysts (74 A_{260} units/ gram hydrated cysts).

Fraction	Buffer A 0.01M tris 0.1M KCl, 4°	Buffer B room temp. 0.01M tris	$(NH_4)HCO_3$ 0.01M or 0.1M, 4° or room temp.
unused buffer	.001	.001	.001
running column, random sample	.004-.02	.001 - .015	.001-.015
first fraction	.014-.030	.010 - .033	.010-.035

Table 1. Range of A_{260} Background Observed During Blank Runs on Poly dT-Cellulose Columns

Blank runs involved standard chromatographic procedures as described only without prior application of any sample.
First fractions were taken after interruptions in column flow which varied from 1 minute to 40 minutes. There was direct correlation between length of interruption and level of absorbancy observed.
Ranges represent at least ten measurements under each condition

Characterigation of Poly dT-Cellulose

Since the poly dT-cellulose technique was intended to be an assay for extremely small quantities of mRNA, it was first necessary to define the cellulose's limits of adsorption. Background absorbancy characteristics of the columns were monitored during blank runs (no samples). Ranges for at least 10 measurements under each condition are reported in Table 1.

The capacity of each poly dT-cellulose preparation to adsorb and release poly A was determined by passing various amounts of poly A over several columns made from each batch. Column A with a 1.4 cc bed volume of cellulose contained 0.65 g cellulose after drying 24 h at 100°. A_{260} units/g dry cellulose were calculated using this factor. The data for A_{260} units of poly A reversibly adsorbed demonstrated that poly dT-cellulose columns could be expected to retain up to 6.5 A_{260} units of poly A per g dry cellulose (batch I) or up to 11 units (batch II), regardless of buffer system used. This is roughly 0.3 mg poly A/g dry cellulose and 0.4 mg/g respectively, using a specific absorbancy coefficient of 25 A_{260} units per mg poly A. Since poly A covalently bound to native RNA would have, theoretically, up to 7 times as much A_{260} absorbancy per poly A sequence as synthetic poly A, the columns may be expected to retain up to 28 A_{260} units of poly A RNA per g dry cellulose. Aviv and Leder (1972) give a value of 0.5 g dry weight per 2 ml of their preparations of poly dT-cellulose, which is less dense than the preparation used in this work. One g of their preparation retained 160 - 200 ug poly A (using a 0.5 M KCl buffer for application) which corresponds to the retention capacity of these columns. Edmonds

Source of RNA	A_{260} Applied	A_{260} in Fractions A	A_w	B	Percent B Fraction	% of A_{260} Recovered
Mycoplasm	5.1	4.4	*	.02	0.4	86
	10.2	8.85	*	.04	0.4	87
	122.	121.0	0.1	.0	-	97
Novikoff Ascites Cells, Prep R_1	130.	121.5	1.60	1.56	1.3	96
	130.	123.5	1.63	1.64	1.3	97.4

Table 2. Adsorption Properties of Poly(dT)-Cellulose as a Function of RNA Source

Fraction A was collected in Buffer A(0.1 M KCl, 0.01 M Tris) at 4°; Fraction A_w in Buffer A at room temperature. Fraction B was collected in Buffer B(0.01 M Tris) at room temperature.

Mycoplasmic RNA was made by F.Rottman on September 29, 1970. Total RNA was extracted at room temperature with phenol at pH 5. The RNA was salt-washed and precipitated using the 2-methoxyethanol-cetyltrimethyl ammonium bromide (CTAB) method of Bellamy & Ralph(1968). 2% acrylamide gel analysis revealed little evidence of RNA degradation.

* - No mixing of cellulose and sample and no A_w fractions collected.

and Caramela (1969) however report the binding of 1 - 2 mg poly A (applied in 0.1 M NaCl) by 1 g dry weight poly dT-cellulose.

The total recovery of applied RNA ranged from 76 to 114 % with 94 - 100 % being most common. Edmonds et al.(1971) report 80 - 90 % recovery of poly A. Faust et al. (1973) report that 1.5 % of poly A applied was never eluted from poly dT-cellulose except upon washing with 0.1 M KOH, but that 92 % eluted in 0.01 M Tris, 25°. The extremes of recovery range observed in the present work may be due to the use of uncalibrated micropipets or to drop losses occurring during manual collection of fractions.

Values for percent RNA reversibly adsorbed (B fraction) as a function of RNA source are reported in Table 2. Values for percent RNA eluted in buffer B are not considered significant relative to experimental variation when the sum of absorbancy units in the total B fractions fell below 0.1 A_{260} unit. Exception was made when the fraction's volume was less than 1 ml and experimental A_{260} values in individual fractions was above 0.04 A_{260}/ml, the upper limit of background absorbancy (Table 1). By this criterion, no significant mycoplasmic RNA was found to bind to the poly dT-cellulose. Mycoplasm, as a procaryotic organism, was not expected to have poly A sequences in its RNA population (Green and Cartas, 1972). Novikoff ascites cell RNA prepared as stated, was found to have around 1.2 - 1.8 % of its total A_{260} units adsorbed to poly dT-cellulose. These experiments demonstrate that RNA is probably not being non-specifically and reversibly adsorbed to the poly dT-cellulose columns.

The efficiency of polymer adsorption by poly dT-cellulose was

evaluated by passing various fractions over the columns a second time. If adsorption is indeed specific for poly A sequences (or any other characteristic), one would expect to find no RNA from an A fraction adsorbed by the column in a second pass, and no RNA from a B fraction not adsorbed. Results show a definite trend in this direction. RNA preparation R_1 shows 1.3 % in the B fraction after one pass, while the fraction B obtained when the fraction A of the first pass was run through the column a second time, showed only 0.2 % retention. Total RNA preparation R_4 had 1.3 % in the B fraction, but preparation R_4's A fraction had only 0.1 % in the B fraction obtained by a second column pass. Fraction B from the original pass of preparation R_4 shows 111 % in the B fraction after a second pass. However, in the case of passing fraction B RNA over the column for a second time, 46 % of the total applied A_{260} units appeared in the new A fraction, giving a total recovery of 157 %, a phenomenon which is hard to explain. Faust et al. (1973) found that 98.6 % of rRNA (mouse myeloma tumor) was not adsorbed by poly dT-cellulose, while 1.6 % of 18 S RNA and 0.8 % of 28 S rRNA are reversibly adsorbed and there is 1 % contamination of the B fraction by rRNA. It is not known if this indicates the presence of poly A sequences in rRNA or rather non-specific adsorption by the cellulose.

Novikoff ascites B fractions (poly dT adsorbing RNA released at room temperature with low salt) was isolated for use in standardizing methods needed to characterize anticipated Artemia salina B fraction RNA. There was a high degree of reproducibility of % B fraction obtained from different columns using a given RNA

preparation when the calculation was based on the A_{260} units recovered in fraction A. The assumption was made that "A_{260} units applied" is the less accurate figure due to the effect of systematic errors involving highly concentrated samples. The ranges of percent B fraction observed were, for different RNA preparations: R1, 1.3 - 1.4 %; R_2, 0 - 0.25 %; R_4, 1.4 - 1.6 %; R_5, 1.8 - 1.9 %.

Characterization of poly dT-Cellulose B Fraction

Preparation for base analysis by collection of RNA on filters followed by enzyme digestion: It was necessary first to establish the effectiveness of the method; that is, the completeness of RNA digestion by the enzyme solutions. 1.16 A_{260} units of ^{14}C-labeled E. coli RNA (49,000 cpm in Bray's counting solution) were precipitated with trichloroacetic acid and counted directly or treated by enzyme solution or non-enzyme buffer as described. Enzyme treatment removed essentially all counts (20,500 cpm before digestion(control) to 111 cpm after digestion). Buffer washing was nearly as effective, averaging 347 cpm for duplicates. The first 2 ml of ethanol rinse contained: 201 cpm and it can be concluded that essentially all counts have gone into the buffer solution.

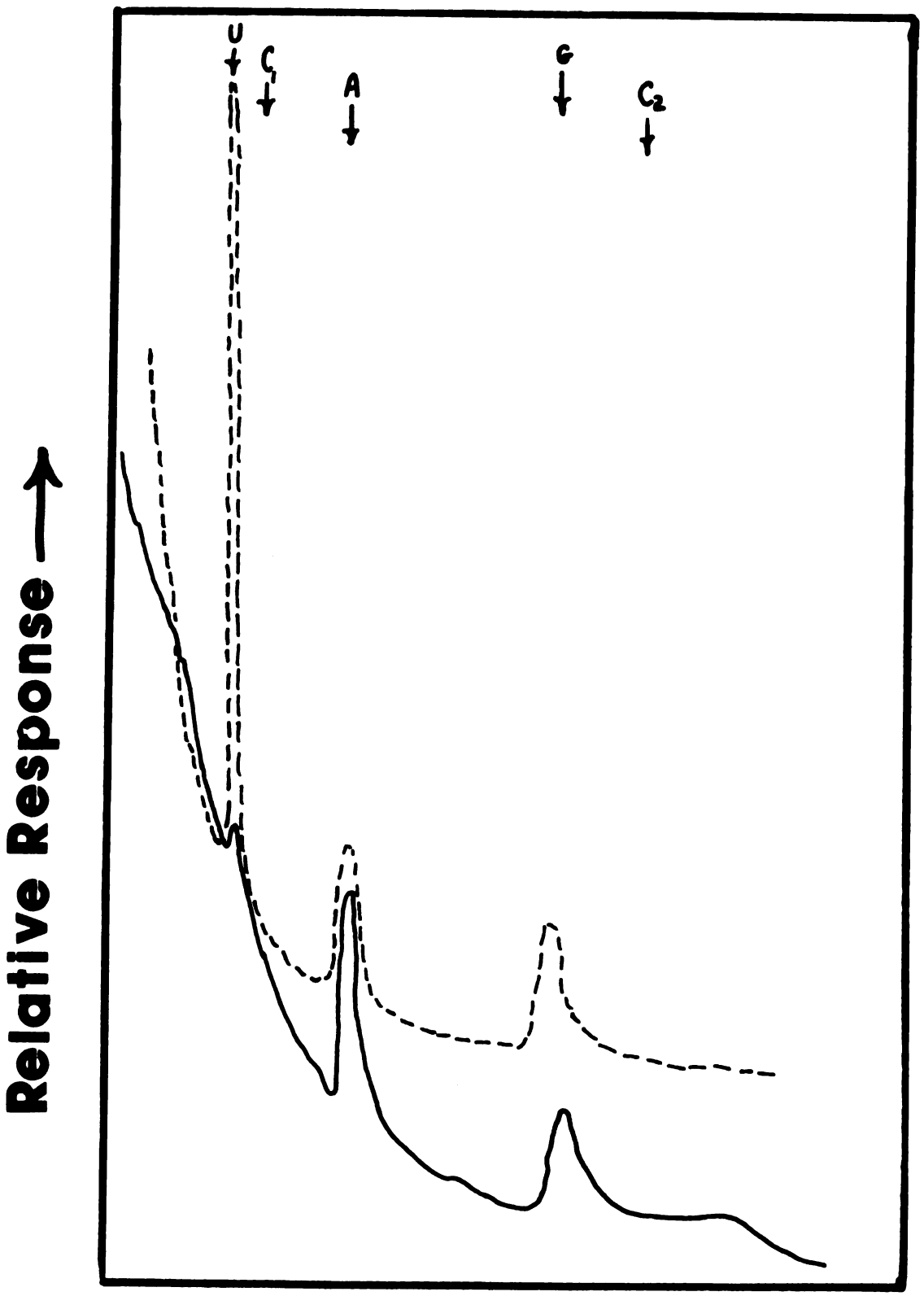
Since more buffer wash also removed the precipitated RNA from the filters, it was necessary to assure the effectiveness of the enzyme digestion. A test digestion of 112 A_{260} units of Pseudomonas putida rRNA was carried out according to the method described, using enzyme solution II. At various times samples of the mix were spotted on Whatman 3 mm paper and electrophoresed in 0.05 M Tris acetate pH 8.75, at 4 ma for 1.5 h. After 60 h, all visible A_{260} absorbing material was

Figure 6. [illegible] Analysis of [illegible] ascites cell total [illegible] free [illegible] and of Fraction B as obtained by poly[illegible]-cellulose chromatography of free [illegible]

[illegible] A[illegible] fraction B [illegible] (reversibly adsorbed to poly [illegible]-cellulose and presumed to contain poly A sequences) and [illegible] A$_{260}$ total [illegible] of free [illegible] were enzymatically digested and derivatized in preparation for base ratio analysis as described in the methods.

[illegible] of fraction B- the [illegible] derivative (approximately [illegible] A$_{260}$) ———— and [illegible] (approximately [illegible] A$_{260}$) of the total [illegible] derivative .... were injected as described by Pike ([illegible]).

Retention times of standards are indicated by arrows at the top of the figure.


U
C1
A
G
C2
Relative Response
Retention Time

Duplicate Samples	Values A:G / U:G		
	Total RNA	Fraction A	Fraction B
Day 1	0.4	0.4	1.8
Day 2	1.7	1.4	5.6

Table 3. Duplicate G.L.C. Response Ratios Obtained for Novikoff Ascites Cell RNA Previously Fractionated by Poly dT-Cellulose Chromatography

Total RNA used was preparation R_5.
Fraction A was the initial eluant in high ionic strength buffer, 4°.
Fraction B was the initial eluant in low ionic strength buffer, room temperature, and is presumed to contain poly A sequences.

found in the nucleotide and nucleoside regions.

Base analysis: Various known standard RNA's and polymers were prepared for base analysis as described, and injected into the G.L.C. in order to obtain values for response ratios in the G.L.C. system. There was no clear separation of U or G derivatives from those of C (Figure 6) and, in general, the system proved unreliable possibly due to incomplete derivatization of the enzyme digested samples which still had a relatively high concentration of salts after lyophilization.

The base ratios G:U and G:A varied independently from day to day for any given RNA type as can be seen comparing the difference between A/G and U/G ratios on any given day. For example, Novikoff total RNA on day 1 had an A/G value of 0.4 that of its U/G value, while on day 2 the A/G value was 1.74 times that of the U/G ratio(Table 3). The only consistent trend in comparison of ratios occurs with Novikoff B fraction where the A/G ratio is higher than the U/G value in all samples run. GLC chromatographs (Figure 6) did seem to indicate higher concentrations of adenosine in the RNA in fraction B.

T_1-RNAase digestion of poly dT-cellulose fractions: A or B_1 fractions were obtained by poly dT-cellulose chromatography of Novikoff total RNA, subjected to T_1-RNAase digestion as described and re-chromatographed on poly dT-cellulose. A_4 RNA (retained by the column in high salt at low temperature only) yields low or insignificant (less than 0.1 A_{260}) reversibly adsorbing A_{260} units after T_1-RNAase digestion as would be expected if material is primarily ribosomal RNA which is T_1-digestible. Gel analysis (Figure 7) does indicate considerable contamination by rRNA. Fraction B_1 (retained in high salt,

Poly dT-Cellulose Fraction Digested	Novikoff RNA Preparation	A_{260} Digested	Ratio of Peak I (oligers) to Peak II (mono-,dimers) of Sephadex G 50 Profile
B	R_1	1.93	0.16
B	R_4	6.10	0.15
B*	R_5	4.10	0.17
1	R_5	1.4	0.09
1	R_5	2.3	0.11
2	R_5	3.92	0.10

Table 4. Sephadex G-50 Analysis of T_1 Ribonuclease Digestion Products of RNA Fractions Obtained by Poly dT-Cellulose Chromatography

All chromatography was carried out in 0.1M $(NH_4)HCO_3$.
Fraction B was eluted from poly dT-cellulose column only at low ionic strength, room temperature and is presumed to contain poly A sequences.
Fractions 1 were B fractions from poly dT-cellulose chromatography which had been subsequently digested by T_1 RNase and rechromatographed on poly dT-cellulose.
The resulting A fractions (material not retained by poly dT-cellulose in high ionic strength buffer, 4°) were applied to Sephadex G-50 column.
Fraction 2 was the mono-,dimer peak (peak II) obtained when the fraction designated B* was rechromatographed on Sephadex G-50.

Figure 7. Representation of Results of Gel Electrophoresis Analysis of Selected RNA Samples

2% acrylamide-0.5% agarose gels were prepared and run as described.

1.) Total RNA, prep R_4 Novikoff ascites cells, 0.33 A_{260} 2.)Total RNA, prep R_5 Novikoff ascites cells, 0.46 A_{260}

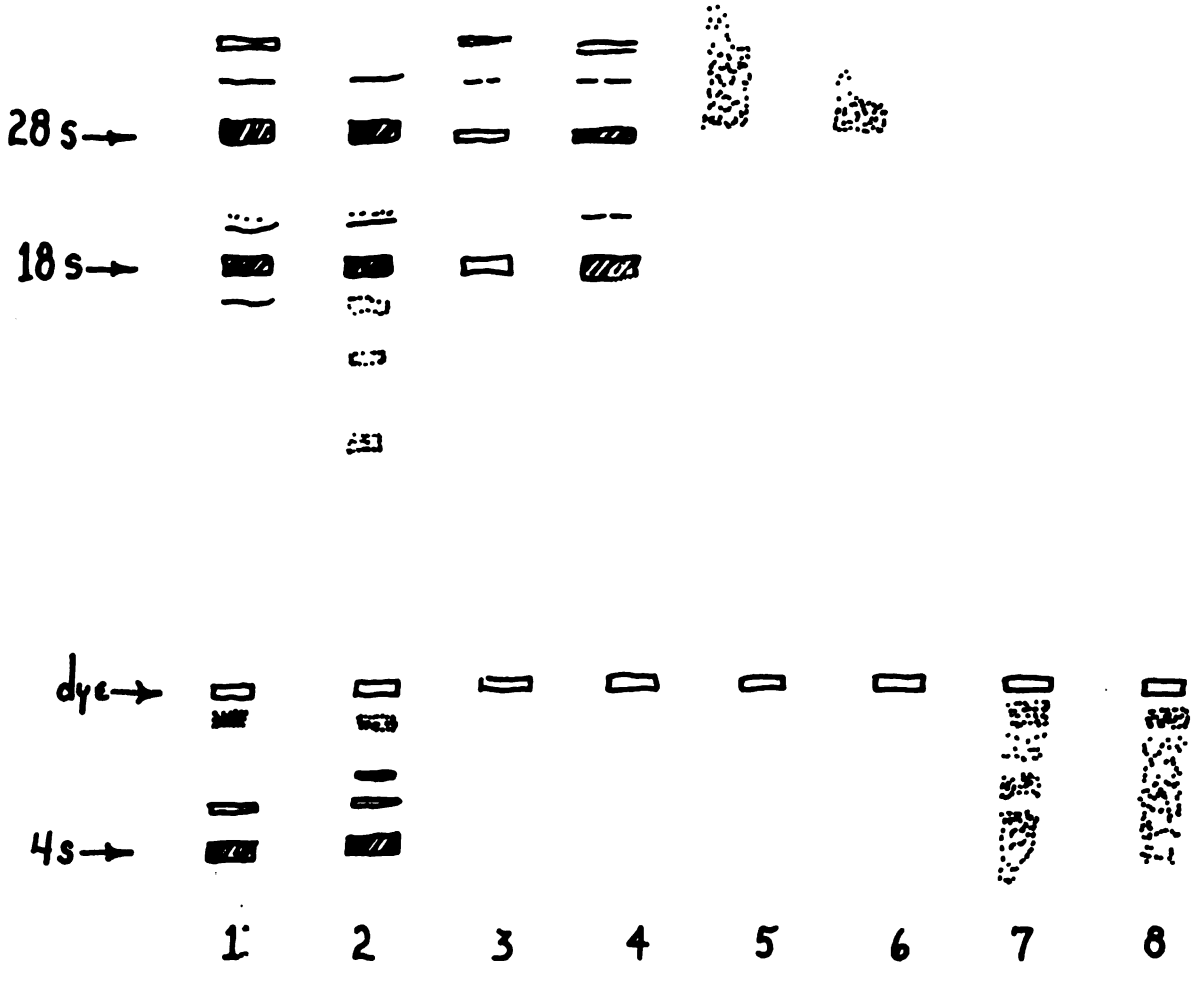
3.) Fraction A_w from poly dT-cellulose chromatography of Novikoff RNA prep R_4 , 0.24 A_{260}

4.) Fraction A_w of Novikoff RNA prep R_5, 0.47 A_{260}

5.) Fraction B from poly dT-cellulose chromatography of Novikoff RNA prep R_4 (Presumed poly A containing fraction) ,0.15 A_{260} 6.) Fraction B of prep R_5, 0.20 A_{260}

7.) Fraction A_w from poly dT-cellulose chromatography of Novikoff RNA prep R_1, treated with T_1 RNase and chromatographed on Sephades G-50. Resulting peak 1 (oligomers resistant to T_1 digestion) applied to gel, 0.2 A_{260}

8.) Fraction B from poly dT-cellulose chromatography of Novikoff RNA prep R_1 treated as in 7. above. 0.2 A_{260}


28 s
18 s
dye
4s
1
2
3
4
5
6
7
8

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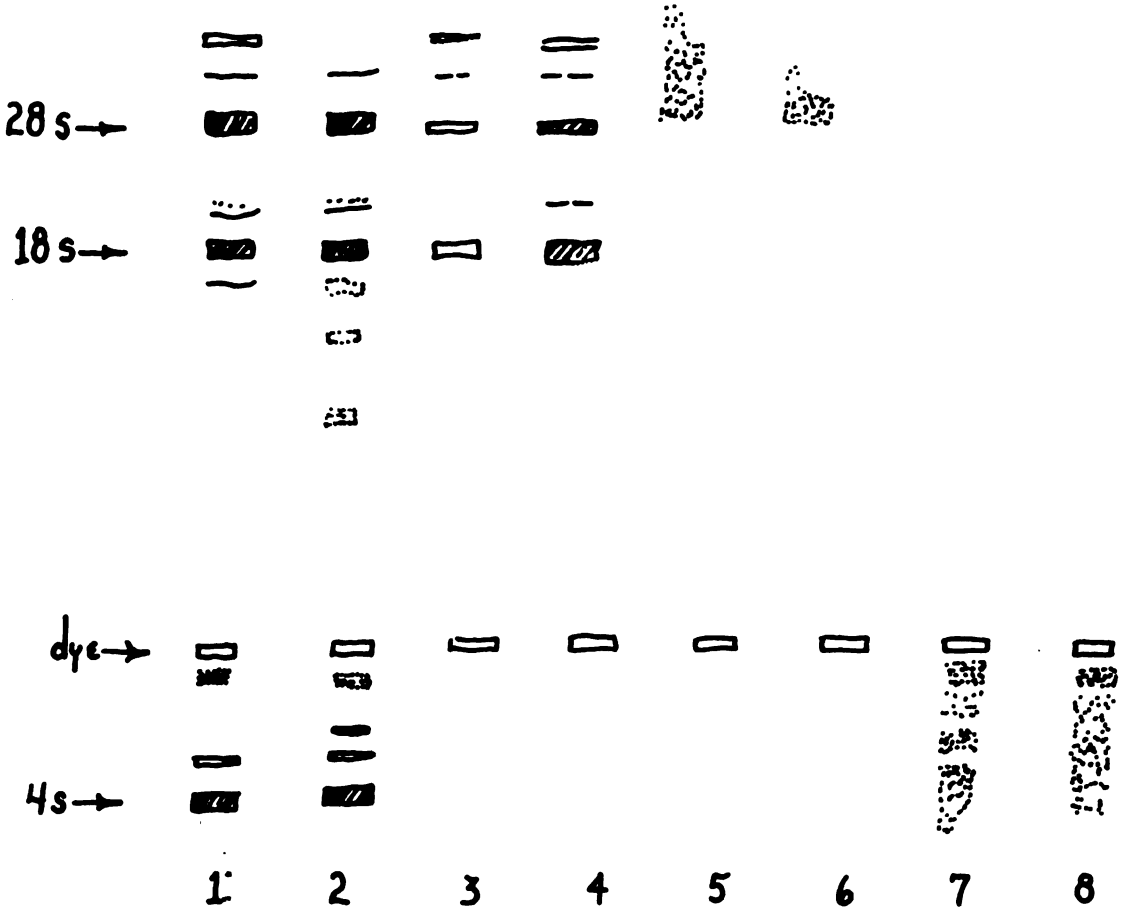
7.) Fraction A_w from poly dT-cellulose chromatography of Novikoff RNA prep R_1, treated with T_1 RNase and chromatographed on Sephades G-50. Resulting peak 1 (oligomers resistant to T_1 digestion) applied to gel, 0.2 A_{260}

8.) Fraction B from poly dT-cellulose chromatography of Novikoff RNA prep R_1 treated as in 7. above. 0.2 A_{260}


28 s
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released in low salt) however, gave 7.1 % and T_1 RNAase-stable, poly dT-reversibly bound A_{260} units.

Table 4 shows results obtained from Sephadex G-50 chromatography of T_1 RNAase digestion products. B fractions directly analysed after T_1 digestion gave ratios of .15 - .17 (15 %) for oligomer peak area(I) (RNAase-resistant material) to monomer peak areas(II).
The controls, poly dT-cellulose A fractions or peak II Sephadex fractions (monomers, dimers) passed over the columns for a second time gave much lower but not insignificant values for the resistant oligomer fraction (I). One would have expected no oligomers in these control fractions since in case 1 (Table 4) the T_1 resistantspoly A oligomers should have been retained by the poly dT cellulose and in case 2 monomer, dimers have already been selected for by using fraction II of the designated B run. The control values indicate that the margin of experimental error of the Sephadex G-50 system is at least 10 % and the true T_1 RNAase-resistant fraction of B_1 RNA may be closer to 5 - 7 % when analysed by G-50. This margin of error may be due to recorder noise at the high amplification used.

Gel analysis of B_1 and A_4 fractions from poly dT-cellulose chromatography and of T_1 RNAase digestion products of these fractions: Figure 7 is a representative figure based on polaroid picture of of a gel stained with Stains-All. Slots 8 and 5 (R_4 - A_4 fraction) show a high concentration of ribosomal RNA but no tRNA or other distinct smaller weight species. Slots 3 and 4, both B_1 fractions, display no ribosomal RNA bands but do have a quantity of heavy (greater than 20 S) heterodisperse staining material. Slots 1 and 2,

	Column	A_{260} in Sample	A_{260} in Fraction A	A_{260} in Fraction A_w	A_{260} in Fraction B	Percent Fraction B
I.	Dormant Cyst (rehydrated, 4°) RNA ... sample lost					
II.	Dormant Cyst RNA (30%) + 8 hr Incubated Cyst RNA (28°) (20%)					
	E	173.5	157.1	0.2	0.4	0.26
	F	260.3	191.8	0.4	0.4	0.20
	*E	260.3	214.0	0.0	0.0	0.0
III.	8 hr Incubated Cyst (28°) RNA					
	E	146.	126.5	o.1	0.6	0.47
	F	219	179.8	0.1	0.9	0.48
	*F	219	208.9	0.0	0.06	0.03

Table 5. RNA Prepared from Artemia salina: Characteristics of its Adsorbance on Poly dT-Cellulose as a Function of Stage of Development (length of time incubated at 28°).

* - Artemia RNA samples pre-incubated with poly U as descrioed in methods

the oligomer peak from Sephadex G-50 chromatography of T_1-digest of R_1 A_4 and the R_1 B_1 fraction, respectively, show only light (5 - 9 S) heterodisperse staining material as one would expect from T_1-stable poly A.

Poly dT-Cellulose Adsorbing RNA in Total RNA from Artemia salina

RNA preparations from dormant cysts and from incubated cysts (8 h) were passed over poly dT-cellulose columns to determine if there was a poly dT-adsorbing fraction in these RNA populations and whether or not such fractions would differ quantitatively as a function of developmental stage.

The dormant Artemia salina cyst RNA was lost due to error but both the 8 hour incubated cyst RNA and the mixed dormant plus 8 hour incubated RNA samples exhibited real, albeit small, amounts of poly dT-cellulose adsorbing RNA. The % B fraction value for 8 hour incubated cysts is 0.47 % and for the mixing experiment, 0.26 %, a value one would expect if the dormant cyst RNA (80 % of the total RNA of the sample) has a B fraction of about 0.1 %. Pre-incubation of the total RNA preparations with poly U results in essentially complete disappearance of the B fraction as one would expect if this fraction's adsorbanss to the poly dT-cellulose is dependent on free poly A sequences.

DISCUSSION

Part I: Polysome Isolationn

Yield

A maximum yield of RNAase-sensitive PAM of 40 - 50 % of total absorbancy could be isolated from incubated *Artemia* cysts, given a typical preparation from normally developing uncontaminated cysts. This means that a 1 X pellet yield of 7 A_{260} units per g of wet cysts would produce 40 A_{260} units of PAM from 15 g cysts. Assuming that PAM is entirely polysome material, free of contaminants and that mRNA does represent 1 - 2 % of polysomes' 260 nm absorbing material (Zomzely, et al., 1970; Evans and Lingrel, 1969), and that 100 % recovery of the RNA can be effected, 4 - 6 A_{260} units of mRNA could theoretically be obtained from standard preparations.

Yeast spheroplasts have usually 70 - 80 % of total cellular RNA in polysomes (Vessey and Keck, 1970; McLaughlin, et al., 1973). *E. coli* have 25 - 30 % of total ribosomes as polysomes, 40 % of the total lysate A_{260} as polysomes (Tai, et al., 1973). Collagen polysomes from 3T6 fibroblasts account for 66 - 77 % of the ribosomal A_{260} units. (Lazarides, et al., 1971).

The proportionally high percent of ribosomes in the *Artemia* ribosomal pellets may be due to mechanical disruption of polysomes during extraction or to cold-induced runoff (Fuhr, 1971; Friedman, et al., 1969), i.e. completion of already initiated protein molecules with resulting release of ribosomes while further initiation is prevented by temperature sensitive factors. In the *Artemia* polysome extraction procedure used, the intact cells were by necessity kept at 4° for at least 5 minutes prior to disruption during which period runoff could occur to a substantial degree. Mechanical disruption would

result in formation of monosomes-mRNA complexes still active in protein synthesis. Such complexes are stable at high ionic strength (Zybler, et al., 1970), whereas cold-induced runoff ribosomes or naturally occurring ribosome pools are dissociated. It may be possible to determine whether, in fact, a polysome population has been mechanically disrupted, creating the observed monoribosome population by examining the latter in high ionic strength buffer sucrose gradients.

Characteristics of PAM

Considering merely the optimal yield and the difficulty and nondependability of obtaining such a yield due to variations in hatching and to contamination, Artemia cysts do not seem to be a favorable system, per se, for extraction of mRNA from polysomes. This becomes even more obvious when one considers the nature of PAM isolated from Artemia cysts as defined by RNAase sensitivity, EDTA dissociation and spectral properties. Remarkably, although PAM appeared to be almost completely RNAase sensitive, it was never found to have a typical RNA absorption spectrum. With the exception of one experiment ($P^{25}H$) where it displayed a typical protein spectrum, it always gave the unusual end-absorption spectrum (type B, Figure 4), which was also often found for 1 X and 14,000 g supernatant fractions. This type of spectrum is assumed to be due to the effects of contaminants. There is excessive trehalose and glycogen in dormant cysts which may contribute to this absorption. The glycogen probably also accounts for non-RNAase sensitive material observed in sucrose gradient profiles. In some individual homogenates RNP particles and glycogen contamination may account for most of the 260 nm absorption(Finamore and Clegg, 1969).

In these cases a type B spectrum is found for 1 X pellet or even for 14,000 g supernatant, and RNAase digestion of the RNP particle contaminant yields only very light products that remain at the top of the sucrose gradients (Figure 5). The RNP particles may be largely protein resulting in the observed protein spectrum for PAM.

The RNAase-insensitive PAM present in EDTA gradients may also represent excessive amounts of glycogen, RNP particles protected from RNAase by effects of EDTA-induced aggregation (contaminants largely seem to be RNAase sensitive in Mg^{+2} buffers), or possibly aggregates of double stranded RNA.

Olsnes (1970) also observed a two phase ribosomal pellet similiar to that sometimes observed here. RNP contamination in this clear bottom pellet varied with centrifugation time but was always present in the upper layer. These contaminants were not affected by EDTA, were rapidly labeled and contained DNA-like RNA. He did not test for RNAase-sensitivity in EDTA.

Significance of PAM

Since this work was begun McClean and Warner (1971) have reported a large population of heavy polydisperse RNP particles in Artemia nauplii based on results from methylated albumin on Kieselguhr (MAK) column analysis of RNA, extracted at pH 7.5 with phenol at 60°. This fraction varied from 15 to 21 percent in prenauplii and in various stages of nauplius development, had DNA-like base ratio and was rapidly labeled.

The hypothetical RNP contamination in the present study may be related to the RNP particles reported in McClean and Warner's work and may be similar to the DNA-like RNA contaminants of Olsnes' system.

As such, these RNP particlescontaminants may be relevant to the question concerning presence of pre-formed mRNA in dormant Artemia cysts: there is some evidence that RNP particle "contaminants" in other systems may be directly related to mRNA metabolism. Aviv and Leder (1972) report template active 18 S RNA which directs amino acid products identical to those made by 9 S RNA of their system. This 18 S template RNA may well be an intermediate between HnRNA and mRNA as found in polysomes and may yield a smaller piece of protein-bound DNA-like RNA upon conversion. Greenberg and Perry (1972) propose that the RNA core of their RNP-contaminant has a more or less homogeneous size and that it is only non-specific binding of proteins that results in polydisperse sedimentation behavior in sucrose gradients. They found no evidence for poly A sequences on the RNA core of the RNP particles.

Part II: Poly dT-Cellulose Chromatography

Characteristics of poly dT-Cellulose

The retention capacity of the poly dT-cellulose preparations was judged to be sufficiently efficient to accommodate the levels of poly A RNA anticipated in natural RNA samples. In fact, essentially all poly dT-adsorbable RNA was adsorbed during the first pass over the column, and essentially none during the second pass (0.2 %). The general background adsorbance of the B fraction from poly dT-cellulose columns when non-poly A RNA samples are applied seems to be around 0.2 % of the material applied: RNA preparation R_2 : 0.25 %; mycoplasm RNA 0.4 %; fraction A RNA on a second pass: 0.2 %. The nature of this material is not presently known. Faust, et al. (1973) have found that their B fractions are contaminated by 1 % rRNA; 1. 6 % of applied 18 S_rRNA and 0.8 % of 28 S rRNA was found to be reversibly

adsorbed and eluted at low KCl concentration. McLaughlin et al. (1973) have found less that 0.04 % of yeast rRNA binds and that 0.5 % of poly A RNA doesn't bind. Whether this rRNA has long poly A sequences (McLaughlin et al. claim that poly dT retains poly A sequences longer than 20 nucleotides) or whether it is retained on the basis of secondary structure interactions with the cellulose, is not known. Other classes of RNA, such as those in RNP particles, may also be involved in this apparent adsorption to poly dT. The variance in total recovery (90 - 115 %) and literature reports of consistent loss of 3 - 19 % of the applied A_{260} units (Faust et al., 1973) indicates that there may be some non-specific binding phenomenon occurring on the cellulose, which may account for the binding of some rRNA.

Characteristics of B Fraction RNA

The poly A content of the reversibly adsorbing B fraction RNA was not definitely established due to difficulties with the GLC base analysis system. Only a weak indication for higher A:G ratios in B fractions was observed. One of the most serious difficulties was the small amount of material with which to work. Given a 3 A_{260} B fraction sample, at most 20 % (Faust et al., 1973), more likely 12 - 14 % (Raskas and Bhadari, 1973; McLaughlin et al., 1973) or 0.36 A_{260} could be expected to be RNAase-stable, i.e. poly A. Since radioactive labels could not be employed, carrier RNA methods could not be used to facilitate isolation of this fraction from contaminating nucleoside digestion products.

In this study, several attempts were made to determine the presencelof oligomers after T_1 RNAase digestion of B fraction RNA

under conditions where poly A is known to be stable. Around 15 - 17 % of the T_1 digested fraction was seen to run as oligomer through the G-50 Sephadex column, but considering the background of 10 % remaining after these digested samples were cleared of poly A by poly dT - cellulose chromatography, one concludes that only 5 - 7 % of this fraction is resistant to T_1 RNAase. Assayed directly by poly dT - cellulose, the T_1 digested B fractions show 7 % resistance. Literature reports commonly cite 12 - 15 % T_1-RNAase resistance for poly dT - cellulose adsorbed fractions (Raskas and Bhaduri, 1973; McLaughlin, 1973), but these values generally are based on ^{14}C-adenine labels and may not correspond to ratios determined on the basis of A_{260}. Faust et al.(1973) cites 5 - 6 % of the total ^{32}P counts or 2.5 % of the A_{260} units of the total applied RNA as the percent of mouse myeloma RNA retained after T_1-RNAase digestion. Values of percent RNAase resistance observed here might be affected by exonucleolytic digestion of poly A sequences at some stage of isolation, making them too short to bind, or by naturally occurring shorter poly A sequences in Novikoff ascites cells.

The gel patterns (Figure 7, slots 1 and 2) strongly indicate presence of low molecular weight resistant species in T_1 RNAase - digested fractions. The highly heterogeneous nature of this material may also be an indication of artificial degradation of the poly A RNA during isolation, as other investigators consistently find distinct banding in the 4 - 6 S regions of their gels. The gel patterns of B fraction RNA not treated with RNAase (slots 3 and 4) display heavy heterogeneous species which may indicate aggregation of poly A RNA

(usually 6 - 7 S) or of degradation products.

The poly dT-cellulose reversibly adsorbing material (B fraction) from Novikoff ascites cells had been shown, at this point, to include approximately 7 % of A_{260} units as T_1 RNAase resistant sequences. By commonly accepted convention, it can be said to contain 7 % in poly A sequences and is presumed to be mostly in mRNA (Faust, et al., 1973; Jelinek, et al., 1973).

Fraction A_w

The nature of the Novikoff ascites cell A_w fraction RNA (released from poly dT-cellulose in Buffer A, at room temperature) has not been investigated. Synthetic poly A also produces this fraction and it is possible that A_w represents specific poly A adsorption to the columns when natural RNA samples are involved. Table 4 results indicate that there is considerably less RNAase-resistant poly dT adsorbable RNA, percentage-wise, in A_w RNA than in B RNA. Since gel analysis (Figure 7) reveals considerable contamination by rRNA, the A_w RNA may represent a mixture of rRNA's retarded by the columns' structure and poly A RNA weakly adsorbed by the column. Faust et al. (1973) find up to 2 % contamination by rRNA even in B fractions; they also observed an A_w-like fraction which was too small to analyze. These authors theorized that the weak binding of poly A RNA in this fraction was due largely to shorter poly dTs sequences on the cellulose. However, Swan et al. (1972) also observed two fractions eluting at low salt and they propose that the difference in binding strength depends on the length of the poly A segment of the RNA. They find that the two fractions direct in vitro protein synthesis of the same product but that the B fraction with, theoretically, longer poly A sequences is more active (Swan et al., 1972).

One should be able to distinguish between these two theories by comparing S values of T_1 RNAase-digested samples by gel analysis or by rechromatographing fractions from each on the poly dT-cellulose column. According to the theory of Swan et al. (1972), the B fraction should elute as 100 % B fraction during the second pass. A B fraction should fractionate between A_w and B if it is dependent on poly dT length. The results reported here, although complicated by recovery of excess A_{260} units, indicate that fraction B RNA rechromatographed does not yield significant A_{260} units in the A_w fraction (0.2 %), but is quantitatively recovered in the new B fraction. Due to ambiguities about its nature, A_w fractions were in general ignored for the purpose of this study.

Novikoff Ascites Cell RNA

This RNA was shown to have between 1.3 and 1.8 % of the isolated total RNA in the reversibly adsorbed B fraction, depending on the particular RNA preparation. This is low compared to most other systems reported in the literature: 2.5 % for mouse myeloma (Faust, et al., 1973); 4.8 % of ^{14}C poly A in rabbit embryo RNA (Schutz et al., 1973); 2.4 - 4 % ^{14}C poly A in yeast RNA (McLaughlin et al., 1973). It is possible that the Novikoff ascites cells are naturally low in poly A *in vivo*, but the variation of 1.3 - 1.7 % among various preparations suggests that experimental artifacts such as endonucleolytic digestion or loss into the phenol layer, are the more likely cause of the lower percentages.

Artemia salina RNA

The former experiments sought to establish the reliability of the poly dT-cellulose system. Unfortunately there was little time at this point to devote to the application of the method to the central question: concerning Artemia salina mRNA. One poly dT-cellulose experiment was run with Artemia RNA samples to compare dormant cyst RNA with incubated cyst RNA. The results (Table 5) show that there are extremely low but real levels of poly A RNA in such samples. The levels of percent B fraction are not, at face value, significantly above the system's background of 0.2 % but prior complexation with poly U eliminates fraction B completely, indicating specificity for poly dT sites. The percent fraction B seems to increase with incubation time. Experimental conditions may have resulted in loss of poly A in the same manner that Novikoff poly A RNA seems to be lost, and the Artemia results may be spuriously low. Of course, there is no guarantee that all Artemia RNA's carry poly A (McLaughlin et al., 1973), or that the poly A RNA would be fully adenylated in dormant cysts. That is, the poly A sequences may not be long enough to effect retention by the poly dT-cellulose.

If preformed message is present in the dormant cysts, it could need processing or activating by adenylation, or its dormancy may be solely a question of non-availability of the ribosome binding site due to specific protein complexing as Lee et al. (1971) suggest for mouse sarcoma 180 ascites cells, that are translationally inactive due to amino acid starvation. If there is no stored message, one would expect to find rapid metabolism of nucleotides in the dormant cyst entering incubation since the cysts at this stage are impermeable to

nucleotides since they are virtually devoid of ATP and free guanine (90% of all nucleotides in the cyst contain guanine; Finamore and Clegg, 1969).

Conclusion

On the basis of the data reported here one may infer that there probably is a small amount of poly A RNA in dormant Artemia cysts and that the amount increases as metabolism is resumed. This could indicate either a small adenylated-template pool (mRNA plus HnRNA) in dormant cysts or simply a lack of adenylation. Contamination of the acetone-precipitated RNA preparations by guanosine dinucleotides and the naturally occuring high proportion of rRNA in all Artemia RNA preparations (Finamore and Clegg, 1969) may account for the low level of poly A RNA (0.4% of total RNA A_{260}applied) observed in total RNA preps from incubated Artemia cysts. Total RNA preparations were used in poly dT-cellulose chromatography rather than the more customary polysomal RNA preparations since intact polysomes could not be obtained with any consistant regularity or purity from Artemia cysts.

Further efforts should be directed at maximizing RNA extraction efficiency and minimizing nucleolytic artifacts. It would then be of interest to determine the variations in percent poly A RNA in Artemia as a function of metabolic state (dormant versus incubated); as a function of RNA localization (nuclear RNA versus cytoplasmic RNA); and as a function of RNA species, e.g. HnRNA. It would also be interesting to examine changes in poly A length with respect to these variables. Ultimately the cell free protein synthesis activity of poly A RNA, and possibly PAM-derived RNA, should be investigated to determine both the size of the mRNA pool in dormant cysts and the percentage of potentially template-active RNA which is adenylated.

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