CHARACTERIZATION OF TRANSPORT OF RIBONUCLEIC ACID FROM ISOLATED NUCLEI

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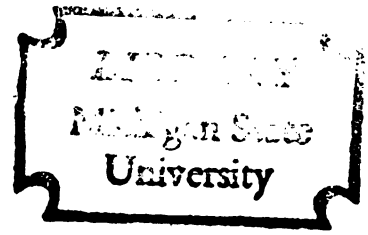

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Characterization of Transport of
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ABSTRACT

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By

Gary Alan Clawson

The question to be investigated in this thesis is "What factors are involved in the control of transport of genetic information to the cytoplasm?"

Nuclei were isolated via Dounce homogenization. Electron microscopy showed the ultrastructure of such nuclei was similar to in vivo controls. Pores and intranuclear structures were evident.

Transport of TCA-precipitable ribonucleic acid was monitored. From the rates of release of RNA at given temperatures, displayed on an Arrhenius graph, an activation energy of about 20 kcals/mole uridine was ascertained for facilitated transport. This activation energy was shown to be independent of nuclease action. A diffusion mediated release was also found for small RNA, with an activation energy of approximately 2.9 kcals/mole uridine.

Detergent treatment of nuclei was performed, and nuclei remained intact after such treatment. Chemical analysis revealed that the membranes had been removed. Close examination revealed that a fine fibrillar network existed in the remaining nuclear envelope which allowed the nuclei to retain their shape. It was hypothesized that the network is analogous to the actomyosin network found in muscle. Magnesium treatment of nuclei caused a reduction in size of about 50%,

and high concentrations (8mM) of magnesium inhibited all RNA transport. Magnesium causes conformational changes in this actomyosin-like network, which in turn cause the aforementioned phenomena.

Release of ribonucleic acids from detergent-treated nuclei was shown to be unaltered from the release obtained in Dounce homogenized nuclei. This information, in conjunction with the domain of temperatures used in the Arrhenius graph (which yielded a line of constant slope) clearly demonstrated that the facilitated transport of RNA is independent of the nuclear membranes.

Cell growth state was tested with regard to its effects on RNA transport. Basically, cytosol prepared from stationary phase cultures was much less effective in facilitating RNA transport than was cytosol obtained from logarithmically growing cultures. Similarly, stationary phase nuclei released a much smaller amount of RNA to both stationary phase cytosol and logarithmic phase cytosol than did logarithmic phase nuclei. It was demonstrated that the percentage of poly A containing RNA was lower in RNA released from stationary phase nuclei than in RNA released from logarithmic phase nuclei. Also, stationary phase cytosol facilitated transport of RNA with a slightly higher (2.7%) poly A+ content than did logarithmic phase cytosol, regardless of the type of nuclei used for release.

Through the various combinations of stationary and logarithmic phase nuclei and cytosol preparations tested, it was demonstrated that an inhibitor of the RNA transport process existed in stationary phase cultures (cytosol) with regard to facilitated RNA transport.

The RNA released was characterized in the logarithmic phase nuclei/logarithmic phase cytosol system. The RNP released was

heterogeneous with a prominent peak at 35S. The RNA extracted from the released RNP was again heterogeneous in the range 4S-28S, with no prominent peaks. The total poly A+ content of released RNP in this system was 35.6%. By comparison, logarithmic phase nuclei were found to release 41.7% poly A+ RNA to stationary phase cytosol.

An ATPase assay was attempted, and did not show localization of ATPase activity to the level of nuclear pores as previously demonstrated (Chardonnet & Dales, 1972), although control nuclei showed reaction product deposition. Further work is in progress.

In summation, the facilitated transport of RNA was shown to be independent of the nuclear membranes. The existence of a fine fibrillar network was demonstrated, which controls the transport of RNA, presumably through contraction and relaxation cycles, forcing RNA through the nuclear pores. The activation energy for facilitated transport of large RNA molecules was found to be approximately 20 kcals/mole uridine in TCA-precipitable RNA. A diffusion mediated transport for small RNA molecules was also found, with an activation energy of about 2.9 kcals/mole. Cell growth state was found to be important for release, in total amount of RNA transported, in RNA synthesized in nuclei from cultures at different cell densities, in the poly A content of the released RNA, and even in the gross size of the various nuclei.

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INTRODUCTION

The fundamental division of cell types is the eukaryotic-prokaryotic distinction. The basis for this distinction is the existence of a nuclear membrane (eukaryotic is Greek for true nucleus). However, besides being the basis for the eukaryotic-prokaryotic classification scheme, what function does the nuclear envelope perform?

Nuclear membranes have been found to be distinctly similar throughout a wide variety of organisms. In other realms of biology, invariant structures are invariant because the function they perform cannot be dispensed with or fulfilled by other structures. Extrapolation leads to the same conclusion with regard to the nuclear envelope: The structure of the nuclear envelope is invariant because such a structure performs a vital function(s) in the eukaryotic cell. But what is this vital function? It is control of the transport of genetic information to the cytoplasm, to the machinery of the cell. Information to be presented throughout the body of this text points to the control of the transport of ribonucleic acids, as the most vital function performed by the nuclear envelope.

Thus, the main thrust of this investigation is addressed to answering the question "How does the nuclear envelope control the transport of genetic information to the cytoplasm?", since the existence of the nuclear envelope seems designed to allow for such control; transport factors, poly A tracts, etc., probably function by aiding or

mediating physical transport of genetic information across the nuclear envelope, in conjunction with its structural aspects. To answer this question, a study of the characteristics of nuclear RNA transport was performed, delving into energy requirements, structural aspects, analysis of released materials, etc.

Another form of control investigated was the control exerted by the state of cell growth. Such control is presumably not directly involved with the nuclear envelope per se. Rather, it is mediated through transport factors and inhibitors, changes in HnRNA synthesis and processing, etc. However, structural changes were noted between logarithmic phase and stationary phase nuclei, as will be discussed later.

REVIEW OF THE LITERATURE

I. Nuclear Organization

A. Formation

The first study of the nuclear envelope using the electron microscope was undertaken in 1950 by Callin and Tomlin (Callin & Tomlin, 1950). This study showed that the nuclear envelope contained pores with a diameter of a few hundred Angstroms (10Å = 1nm), and that these pores had a raised annulus associated with them. Subsequent investigations (Gall, 1964) have shown that double-layered nuclear envelope with pores and annuli is a feature common to all eukaryotic organisms. The reported diameters of pores have ranged from 30-100nm. This wide range has been assumed to imply real differences among organisms.

The development of the nuclear envelope is an interesting process, and information can be gleaned from its study. It has been shown (Porter & Machada, 1960; Fawcett, 1965) that there is a direct continuity of the outer nuclear membrane with endoplasmic reticulum in a wide variety of organisms, so that the perinuclear space is in direct contact with the lumen of the endoplasmic reticulum. It is known that the nuclear envelope is developed from the endoplasmic reticulum at telophase (Feldherr, 1972B; Porter, 1961). The membrane on the inner surface of the nuclear envelope (next to the nucleoplasm) loses its ribosomes and the nuclear pores develop as the membranes become associated with chromosomes (Roberts & Northcote, 1971). The membrane portions are often seen

to fuse and become a complete nuclear envelope, which surrounds what is to become the nucleoplasm as the chromosomes disperse. In prophase, when the nuclear envelope breaks apart, strands of the nuclear envelope can be seen in the cytoplasm, still carrying pores (Northcote, 1974), although they are otherwise indistinguishable from the other membranes comprising the endoplasmic reticulum. After prophase, these nuclear envelope strands can no longer be distinguished, probably because the pore complexes are lost. Therefore, there are no apparent differences between the inner and outer nuclear membranes when the nuclear envelope is formed. Both of these membranes, as they trail away from the chromosomes in the early phase of nuclear envelope formation, carry ribosomes. When the envelope disperses both sides have the same appearance. Therefore, since chemical differences exist between the inner and outer membranes (Monneron, 1974; Chardonnet & Dales, 1972) changes presumably must be reversible and must take place when the membranes are in close proximity to the chromatin. For example, when the membranes are near the chromosomes the nuclear pores form (Roberts & Northcote, 1971). It seems likely that the presence of pores is "reversible" in the sense that they can be removed (degraded) from membranes. This probably occurs during prophase when the membranes of the nuclear envelope dissolve.

In some plant cells, particularly those grown in culture (which often become tetraploid or of higher ploidy), the inner membrane folds into the nucleoplasm (Wright & Northcote, 1973). These folds extend the perinuclear space into the nucleus and increase the surface area of the inner nuclear membrane. Since chromatin is associated with the nuclear membrane (Oppenheimer & Wahrman, 1973; Kay & Johnston, 1973), these indentations increase the number of sites for chromatin organization

within the nucleus while allowing an association with the membrane. Also, the indentations increase the surface contact between the nucleus and the cytoplasm.

The continuity of the perinuclear space with the endoplasmic reticulum may provide a major transport connection between the nucleus and the cytoplasm. This can be extended due to the association of the endoplasmic reticulum with other cellular organelles and by the passage of the endoplasmic reticulum through the plasmodesmata (in plant cells), so that the endoplasmic reticulum of one cell can be connected with that of adjacent cells (Northcote, 1974).

A fascinating observation concerning the behavior of nuclei is that the surface of the nucleus can be seen to be constantly undulating when viewed in the living cell by differential interference optics (Roberts & Northcote, 1970). In fixed tissue, large indentations of the nuclear envelope are often seen, and these indentations may be taken to represent such undulations caught in a particular position by the fixation. An interesting conjecture is that this indentation phenomenon may be associated with nuclear asymmetry. The indentations in the nuclear envelope are often large, and often contain cytoplasmic organelles, such as mitochondria. The indentations contain microtubules, which enter the indentations at their mouth and penetrate into them. Pores are found on the nuclear envelope which lines the indentations and cytoplasmic streaming along strands in the cytoplasm is often seen in plant cells (Northcote, 1974). In addition, the nucleus is moved about the cell and is not confined to any one position (Roberts & Northcote, 1970).

A common technique in biology has been to perturb a system, with the hope that the result of such a perturbation will help to elucidate

the structure and/or function of the system. Such a perturbation technique has recently been applied to amoebae (Flickinger, 1974). Amoebae were placed on the surface of an agar-coated slide. The nucleus was physically pushed from the amoeba with a pipette, damaged with this pipette, and reinserted into the amoeba. Beginning approximately fifteen minutes after the damage was inflicted, pieces of rough endoplasmic reticulum intruded into the holes in the nuclear membrane. The pieces of membrane were seen to become connected to the nuclear membranes at the edges of the holes. Patches of disorganized looking fibrous lamina were present for a time after injury, but this disorganized fibrous lamina showed evidence of returning to normal appearance. This process of repair is similar to the formation of the nuclear envelope at telophase. Often, after the endoplasmic reticulum had covered the holes in the nuclear envelope, ribosomes were round (temporarily) on the inner nuclear membrane. The ribosomes were subsequently removed by some unknown process and the endoplasmic reticulum leaflets flattened out and a normal configuration for the nuclear envelope was noted.

The repair mechanisms noted above are different from the repair mechanisms in various kinds of eggs and plasma membrane in the amoeba (Gingell, 1970). In the repair of these membranes, it is thought that bundles of cortical filaments may cause contraction of the margin of the holes in the membrane (Gingell, 1970). Alternatively, a new membrane may be formed external to the plasma membrane (Szubinska, 1971) or dense droplets of lipids which adhere to the cytoplasmic surface of the membrane may insert into it (Bluemink, 1972).

Following this technique of physically damaging amoeba nuclei, a few tears were observed in the fibrous lamina. In general, however, the

fibrous lamina seemed to be much more resistant to injury than were the nuclear membranes. The main change observed in the fibrous lamina occurred later, and consisted of replacement of the normal honeycomb-like organization by a thick, disorganized mass of filaments. This effect was shown not to be a direct effect of the injury. The change could have been a degenerative change which followed damage to the nuclear membranes, but this possibility was argued against, since in the cells that had survived to this point the nuclear membranes were intact. Another possibility mentioned was that the disorganization of the fibrous lamina might be a preliminary step in its reorganization in order to accommodate an altered nuclear size or contour. This possibility was supported by data which suggested that there appeared to be a progressive organization of the affected regions, which often were found to contain plates (Flickinger, 1974). Of interest in this study, however, was the fact that although some of the nuclear membranes showed repair, the nuclei were not fully returned to normal since none of the cells were observed to divide. Possible reasons for this non-dividing behavior were: 1. A difficulty in nuclear pore formation; 2. A difficulty in reconstruction of a normal fibrous lamina; 3. Normal relationships between the nuclear envelope and intranuclear structures not restored; or 4. Some contents of the nucleus were irretrievably lost.

The sequence of events seen in amoeba nuclear envelope repair (Flickinger, 1974) and the well known formation of the nuclear envelope from endoplasmic reticulum in telophase (Feldherr, 1972B; Porter, 1961) appear to be straightforward examples of the exchange of large, intact membrane fragments between two different classes of cellular membranes. In this context, however, it appears that the nuclear membrane at this

stage of development is indistinguishable from the endoplasmic reticulum. In this stage of development, then, a nuclear membrane class of membranes is nonexistent and will eventually evolve through a limited diffusion and differential incorporation of components. Such a transfer is not between two different classes of intracellular membrane systems. The extent to which patches of membranes or membrane components are transferred from one organelle to another poses additional questions which are important, for instance, in secretory processes. Transfer of portions of membranes and mixing of membrane components between the endoplasmic reticulum, Golgi apparatus, and plasma membrane during the normal secretory process continues to be debated (Meldolesi, Jamieson, & Palade, 1971; Meldolesi & Cova, 1972; Siekevirz, 1972; Meldolesi & Cova, 1971).

B. Nuclear Membrane Composition/Properties

The major phospholipids in the nuclear envelope in rat liver have been found to be phosphatidylcholine (PC), phosphatidylethanolamine (PE), with diphosphatidylglycerol (DPG) as a minor component (Stevens, 1967). However, two enzymes used for the synthesis of PE and three enzymes used in the biosynthetic pathway for DPG in rat liver are located primarily in the mitochondrial fraction (Dennis & Kennedy, 1972). Thus, this fraction supplies part of the machinery necessary for the synthesis of new nuclear membrane phospholipid units. The endoplasmic reticulum is also involved in the synthesis of nuclear membrane since PC is synthesized in the microsomal fraction. Dennis and Kennedy propose that in the synthesis of mitochondrial membranes, PC is transported from the microsomes to the mitochondria. An analogous transport may be needed to form the nuclear envelope. It is also theorized that ATP may be transported from the mitochondria to the nucleus.

Extensive examinations have been made of the nuclear membranes with regard to their composition and properties. Kashnig and Kasper (Kashnig & Kasper, 1969) isolated rat liver nuclei using the method of Blobel and Potter (Blobel & Potter, 1966). The nuclei were then disrupted by sonication. Solid potassium citrate was added, which resulted in solubilization of much of the nucleoplasm which otherwise was found to adhere to the membranes. The ruptured nuclei were pelleted and the pellet was resuspended in a solution of density 1.22g/cc. This membrane solution was overlayered with solutions of densities 1.20, 1.18, and 1.16g/cc. This discontinuous gradient was developed at 100,000g for 12 hours. Nuclear membrane was isolated at the interface densities of 1.18:1.20g/cc and 1.16:1.18g/cc. The composition of the membrane bands at these interfaces was analyzed. Cytoplasmic membrane was shown not to be a contaminant as it layered at the air:1.16g/cc interface. The compositions found at the respective interfaces were as follows:

1.16:1.18g/cc interface - protein 58.8%, carbohydrate 2.9%
(neutral sugars 2.6%, hexosamine 0.24%, sialic acid 0.06%),
lipid 35.2% (phospholipid 29.2%, cholesterol 6.0%),
RNA 3.1%, DNA 0%

1.18:1.20g/cc interface - protein 62.3%, carbohydrate 3.9%
(neutral sugars 3.6%, hexosamine 0.21%, sialic acid 0.09%),
lipid 27.7% (phospholipid 24.1%, cholesterol 3.6%),
RNA 6.1%, DNA 0%.

Purified nuclei were found to have a ratio of lipid phosphorus:DNA phosphorus of 0.11. This previously had been calculated to correspond to four monolayers of lipid around a single nucleus (Gurr, Finean, & Hawthorne, 1963). The nuclear membrane composition differed greatly

from the composition of erythrocyte membrane. In erythrocytes, the membrane composition was 49.2% protein, 43.6% lipid, and 7.2% carbohydrate. Other differences were of interest. The erythrocyte membrane contained glucosamine and galactosamine in a ratio of 2:1. However, only glucosamine was found in the nuclear preparations. Also, intact red blood cell membrane contained twelve times the sialic acid as its nuclear counterpart.

In the nuclear preparation, lipid phosphorus was found to represent approximately 75% of the total membrane-bound phosphorus. Since the procedure used (Kashnig & Kasper, 1969) employed RNAase digestion, a component which contained phosphorus, was resistant to ribonuclease digestion, and was not extracted with chloroform-methanol was present. It was suggested that such a component represented RNA which was integrally attached to the membrane (and was thus not accessible to ribonuclease). Plasma membrane isolated from the same tissue gave a density of 1.185-1.194g/cc, a value close to the density found in the nuclear preparation, although large differences existed in the compositions.

Important enzymatic data were also given by Kashnig and Kasper (Kashnig & Kasper, 1969). It was shown that a rotenone-insensitive DPNH-cytochrome c reductase and a glucose-6-phosphatase were present in the nuclear membrane at approximately 50% of the specific activity of these enzymes in the cytoplasmic membrane. This was taken as evidence that these activities were localized on only one of the leaflets of the nuclear envelope, most likely the outer. Following this questionable line of reasoning, it was suggested that the inner leaflet

was biochemically distinct from the outer leaflet. I view this as just a possibility.

Monneron (Monneron, 1974) also has done extensive research on nuclear membranes. She starts with purified fractions from rat liver and also from calf thymocytes. To obtain purified nuclear preparations, cells were homogenized. After lysis, the entire nuclear preparation was sedimented through layers of sucrose. Nuclei were the most dense structures so the contaminating cytoplasmic components did not reach the dense sucrose layer as in the previous technique (Kashnig & Kasper, 1969). Treatments of the purified fractions were one-step treatments and consisted of treatment either with 0.5M $MgCl_2$ or with an alkaline solution of pH 8.5. High concentrations of divalent cations separated the nuclear envelope from its bound chromatin.

The nuclear suspension was prepared with the high salt technique and was overlayered with a linear sucrose gradient. A milky band was obtained at a density of 1.17-1.18g/cc, similar to the results of Kashnig and Kasper. When the absorbance of the gradient was monitored at 440nm, only the sharp membranous peak adsorbed. At 260nm, the bottom of the tube showed strong absorption (containing the DNA), as did the membrane peak. If it is assumed that all the phospholipids are located in membranes, then recovery was 74%, with 55-60% of the phospholipids in the sharp milky band. The composition of this band was: protein 73 $\pm$ 4%, phospholipids 23 $\pm$ 2% (giving a phospholipid:protein ratio of 0.31), RNA 3 $\pm$ 1%, and DNA 0.6 $\pm$ 0.2%.

When the nuclear preparation which was isolated via the alkaline method was treated similarly, the density was 1.19-1.20g/cc. The composition of the band was: protein 65 $\pm$ 4%, phospholipids 21 $\pm$ 2%

(giving a phospholipid:protein ratio of 0.32), RNA 14 ± 2%, and DNA 0%. Thus, the alkaline treatment resulted in a preparation with a higher density and a higher percentage of RNA than the preparation which utilized the high concentration of divalent cations.

The major phospholipids of the nuclear membranes are phosphatidylcholine and phosphatidylethanolamine, with diphosphatidylglycerol as a minor component (Stevens, 1967). Thus, the nuclear envelope is consistently found to be richer in protein than the endoplasmic reticulum (Franke & Scheer, 1970; Kashnig & Kasper, 1969; Monneron, 1974). Similarly, nuclear membranes are richer in esterified cholesterol (Kleinig, 1970).

Studies on rat liver nuclei, rabbit liver nuclei, and hepatoma nuclei have been reported (Harris, 1974). Using the zonal centrifugation technique of Price et al. (Price, Harris, & Baldwin, 1970), nuclear ghost membranes were prepared. The density was 1.21 ± 0.01g/cc for rat liver and hepatoma nuclear membranes. A second technique was also used on rat liver and rabbit liver nuclei. Prior purification of nuclei was again required, after which the nuclei were burst, and placed in an isopycnic discontinuous sucrose gradient. This technique gave a density of 1.22g/cc. In comparing the results of these two techniques with results previously presented, it is possible that the purification here was not as complete, yielding higher densities.

A 5'-nucleotidase activity and an ATPase activity have been detected associated with nuclear membranes of calf thymocytes (Monneron, 1974), as well as a 3'-nucleotidase activity, although no marker enzymes have been found. The lead method of precipitation of phosphate (to be discussed) was used to monitor hydrolysis of 5'-TMP. The lead

precipitates were found to be much more abundant on nuclear envelopes than on whole cells, indicating an apparent localization of the 5'-nucleotidase activity. This 5'-nucleotidase activity was also demonstrated with 5'-AMP, although to a lesser extent. An ATPase activity was regularly found which was not dependent on potassium ions or sodium ions. Other investigations (Chardonnet & Dales, 1972) have demonstrated an ATPase activity at or near the pore complexes of the nuclear envelopes in HeLa cells.

The topology of sugar residues on nuclear membranes has also been studied by means of lectins (Monneron, 1974). Concanavalin A (Con A) binds in large amounts to both the inner and outer nuclear membranes, but does not bind to pores. Wheat-germ agglutinin was also used as it had been shown to induce aggregation of nuclei (Nicolson, Lacorbiere, & Delmonte, 1972). Binding of Con A implied that specific carbohydrates were located on the nuclear membrane. Both leaflets demonstrated Con A binding, although the outer membrane showed greater binding capacity. Binding was interrupted at pores. The inner side of the inner nuclear membrane was never labelled. Treatment of nuclei with detergents, such as Triton X-100 or deoxycholate (DOC) prevented binding of Con A to nuclear envelopes. These results implied that large amounts of specific sugars were present in nuclear envelopes and were localized mainly on nuclear membranes. As a test for fluidity (for comparison, see Frye & Edinin, 1970; Comoglio & Filogamo, 1973), isolated nuclei were submitted to treatment with labelled Con A at 37C for 1 hour. Control thymocytes showed obvious cap formation (Davis, Dulbecco, Eisen, Ginsberg, & Wood, 1973), but the distribution of Con A binding sites on the nuclear membranes was not modified. This contrasts with results on plasma

membranes. The lack of redistribution of Con A binding sites on nuclear membranes (Monneron, 1974) is explainable since the nuclear membrane is interrupted by pores, has chromatin tightly bound to its leaflets, and ribosomes bound to its outer leaflet, all of which could conceivably restrict any lateral motion.

C. Structure of the Nucleus

Besides the nuclear membranes, other integral structures seen in nuclear preparations are the chromatin layers. These chromatin layers were referred to as nuclear envelope limited monolayers of chromatin in previous studies (Haynes & Davies, 1973). Monolayers of chromatin, containing structural units of about 33nm width, were reported in Burkitt's lymphoma cells. They were enclosed on both sides by the nuclear membranes (envelope) and were located in the cytoplasm or the nucleus. Nuclear asymmetry was defined in terms of the increase in membranes over the amount needed to enclose the same area in a circular configuration. The percentage of cells (Ps) with nucleus-associated sheets was defined, averaged over all stages of the cell cycle. Ps was found to increase with cell viability and with nuclear asymmetry. During S phase of the cell cycle (DNA synthesis), a marked increase in Ps occurred with no change in nuclear asymmetry. The data were taken to suggest that nuclear asymmetry is a necessary but not sufficient condition for causing sheets of chromatin to form. It was also shown that condensed chromatin, in a variety of vertebrate cells, contained a structural unit about 17nm in diameter. This component was well defined because of its tendency to form an ordered layer (or layers) of definite geometry on the surface of the nucleus, in apparent contact with the

nuclear membranes. The outer layer seemed to give rise, through delamination and membrane flow, to monolayers seen to be confined between pairs of membranes. Since the sheets were nearly constant in width, this was taken as evidence for the general occurrence of a structural unit called a unit thread. This unit thread consisted of DNA and protein. The larger dimension of 37nm was reported due to projections which radiated from the central 17nm unit.

Microtubules have also been examined (Haynes & Davies, 1973). They were found to be present in the cytoplasm and their morphological arrangement suggested a role in determining nuclear asymmetry. Cells were treated with colcemid, a microtubule depolymerizing agent. Such treatment did not alter the existing nuclear asymmetry or Ps of the cells. However, cells treated early in S phase with colcemid had a considerably delayed reappearance of sheets in the G2 phase. Reappearance, when it did occur, took place with the microtubules still depolymerized. It was also suggested that the final nuclear configuration was dependent on which configuration was the most favorable energetically at the time.

An arrangement of microfibers in bundle form near chromatin in midprophase cells has been reported (Chai, Weinfeld, & Sandberg, 1974). The chromatin was located in the area where nuclear envelope had existed. The microfibers were oriented in one direction and had a diameter of approximately 7nm. They were occasionally noted immediately adjacent to the nuclear envelope that was in the process of dissolution (and similarly for regions where new nuclear envelope was being formed).

A platinum compound which causes cross-linking of complementary strands of DNA (Roberts & Pascoe, 1972) has been used to treat Sarcoma-180 ascites cells (Aggarwal & Sodhi, 1973). Similarly to

previous results, it was reported that 6-7nm thick, elongated cytoplasmic filaments were distributed throughout the cell, but were more concentrated in the perinuclear areas and seemed to be associated with the outer nuclear membrane. After short treatment with the platinum compound, a concentration of all the microfilaments into a discrete band around the nucleus was induced. Following longer platinum treatment, this filamentous band disappeared and the mitochondria, which still appeared normal in their morphology, concentrated around the nucleus in the form of a distinct band. This suggests some connection between the mitochondria and the nucleus, as was conjectured on the basis of chemical evidence (Dennis & Kennedy, 1972). The appearance of microtubules near the nucleus is interesting with regard to my results to be presented.

Autoradiographical and biochemical data have led to the hypothesis that DNA polymerase could be associated, at least temporarily, with the nuclear membrane and functions there, although this is much debated (Comings & Kakefuda, 1968; Infante, Nauta, Gilbert, Hobart, & Firshein, 1973; Freienstein, Freitag, & Suss, 1973). This possibility was investigated by Monneron (Monneron, 1974). Labelled isolated rat liver nuclei were labelled in vitro with ^{3}H-dTTP. The nuclei actively incorporated this precursor. Autoradiography of labelled nuclei did not show any predominant labelling of the nuclear envelope region. If isolated nuclear membranes were incubated in the same mixture, there was no incorporation of radioactivity, with or without added DNA primer. However, these experiments cannot be taken as conclusive proof that the DNA polymerase is not attached to the nuclear membrane, since it can be argued that the polymerase was inactivated by treatments, essential ligands were lost, etc.

Fawcett considered the chromatin layers, or the fibrous lamina, to provide structural reinforcement to the nuclear membrane (Fawcett, 1966). An elaborate honeycomb-like organization has been found in only a few cases, however. Results presented by Aaronson and Blobel (Aaronson & Blobel, 1975) showed that the fibrous lamina's organization was much more widespread than had been demonstrated previously. Aaronson and Blobel noted that liver parenchymal cell nuclei in situ did not show an amorphous layer separating the inner nuclear membrane from the chromatin. However, isolated rat liver nuclei that had been treated with detergents exhibited a densely staining amorphous layer approximately 15nm thick. This layer was often mistaken for a membrane. The results of Chai et al. (Chai, Weinfeld, & Sandberg, 1974) support Aaronson and Blobel's suggestion and will be discussed. Aaronson and Blobel suggest that the presence of a rigid lamina at the nuclear periphery may explain the fact that rat liver nuclei and many other types of nuclei retain their shape in the absence of a nuclear membrane. Furthermore, they speculate that the fibrous lamina might organize the nuclear pore complexes and might be responsible for the non-random distribution of nuclear pores in the nuclear surface.

D. Intranuclear Structures

Intranuclear structures have often been hard to visualize by electron microscopy and different treatments have been employed to obtain a better resolution of these structures.

For instance, Chai et al. (Chai, Weinfeld, & Sandberg, 1974) used direct fixation with chrome-osmium and compared this treatment with the double-fixation procedure utilizing glutaraldehyde-osmium. Double fixation showed the heterochromatin well, whereas it was only faintly

visible with direct fixation. However, nucleolar structure and detail were revealed more clearly with direct fixation. Direct fixation was also shown to remove the visual interference of the amorphous lamina caused by the densely-staining heterochromatin in double fixation. This result could explain why the amorphous lamina has not been noted in all types of nuclei. Direct fixation was also reported to allow good visualization of the non-heterochromatin nucleolar materials. In early prophase, the nucleoli were seen to disperse, and highly electron dense material was located in a few perichromosomal areas (Chai, Weinfeld, & Sandberg, 1974). This highly electron dense material was considered to be of nucleolar origin, and some RNA was present in these areas (Phillips, 1972).

The partially characterized regions of the nucleus include the chromatin, the nucleolus, and nuclear helices.

The helices have been shown not to contain DNA (Wise & Goldstein, 1972) but they do contain RNA (Wise, Stevens, & Prescott, 1972) and protein (Stevens, 1967). The most reasonable function attributed to these nuclear helices is that they package (or contain) the mRNP particles destined for transport to the cytoplasm (Wise, Stevens, & Prescott, 1972).

Some of the proposed functions attributed to the chromatin have been previously discussed, and include spatial organization of the pore complexes (Aaronson & Blobel, 1975) and structural reinforcement for the nuclear membranes (Fawcett, 1966) by the peripheral chromatin. Other proposed functions are the regulation of genetic transcription (Britten & Davidson, 1969; Jacob, Gillies, MacLeod, & Jones, 1974; Wise & Goldstein, 1973) or replication (Jacob, Gillies, MacLeod, & Jones, 1974).

The proposed functions of nucleoli include the transcription of ribosomal RNA (Schildkraut & Maio, 1968; Mattoccia & Comings, 1970) and recent work has indicated that the nucleolus may be involved in controlling the transfer of informational RNA from the nucleus to the cytoplasm (Deak, 1973; Harris, Sidebottom, Grace, & Bramwell, 1969; Deak, Sidebottom, & Harris, 1972; Sidebottom & Harris, 1969). Some of the studies and techniques used to delineate the function of these various structures will be detailed, although most of them do not allow ultrastructural detail for separation of the components involved.

An interesting perturbation technique was used to investigate intranuclear structure and function (Deak, 1973), in BSC-1 cells derived from monkey kidney, grown in culture. The nucleolus (or other parts of the nucleus) was inactivated by a microbeam of light (of diameter 4 micrometers for irradiation of nucleoli and 25 micrometers for irradiation of whole nuclei). These experiments showed, via labelling and autoradiography, that the inactivation of the nucleolus inhibited the appearance in the cytoplasm of ribosomal RNA and other types of RNA, most notably tRNA. In this study, the cells were incubated at 37C for 1 hour after irradiation and this could be a crucial factor in these results (see Rizzo & Webb, 1972). Observation of cells that were not irradiated indicated that ribosomal 18S and 28S RNA represented 55% of the radioactivity released, transfer RNA 40%, and other types 5% (Deak, 1973). In irradiated cells, Deak found that nucleoplasmic labelling occurred even though complete inhibition of cytoplasmic labelling occurred. These results indicated that approximately 50% of the nucleoplasmic labelling was dependent on nucleolar activity. It seems that these experiments permit the conclusion that the nucleolus is involved in

the transfer to the cytoplasm not only of ribosomal RNA but also other types of RNA, since "nucleolar irradiation clearly inhibits the flow of transfer RNA to the cytoplasm" (Deak, 1973).

Sidebottom and Harris (Sidebottom & Harris, 1969) reactivated erythrocyte nuclei in heterokaryons and found that they did not contribute detectable amounts of labelled RNA to the cytoplasm of the host cell until the transferred nuclei had first developed nucleoli. In addition, intensive inactivation of the nucleolus in nuclei that contained only one such structure resulted in a reduction of cytoplasmic labelling to low levels. A possible argument against nucleolar control of tRNA transport based on this study would be the assumption that an abolition of nucleolar ribosomal RNA synthesis had occurred. However, Deak's results (Deak, 1973) seem to invalidate this argument.

Wise and Goldstein (Wise & Goldstein, 1973) also used nuclear transplantations in conjunction with electron microscopic autoradiography. To enrich the nuclei for non-migrating labelled RNA the labelled nuclei were placed in enucleate amoeba for a period of time, then transferred into another unlabelled enucleate amoeba. Thus migrating RNA would be diluted. Similarly, to localize the migrating nuclear RNA, labelled nuclei were transplanted into unlabelled amoeba containing a nucleus, thus forming binucleate cells. Autoradiography was then performed to determine the localization of RNA which had migrated into the unlabelled host nucleus.

A class of non-migrating low molecular weight RNA (4-6S) was found to be associated with the structureless region of the nucleoplasm, but definitely not with the nucleoli, nuclear helices, or chromatin. Therefore, it appears that this class of RNA was not involved in genetic

regulation. Similarly, Hodnett and Busch (Hodnett & Busch, 1968), due to the lack of association with chromatin, concluded that non-migrating RNA was not involved in the regulation of genetic transcription.

Another class of low molecular weight migrating RNA was found to "shuttle" between the nucleus and the cytoplasm. This class of RNA was not associated with the nucleoli or with the nuclear helices, although it was apparently involved with the chromatin since some radioactivity was found there. This shuttling RNA might therefore be involved in genetic regulation. It was theorized that a small proportion of the shuttling RNA may be associated with the chromatin at any given moment during interphase, somehow regulating genetic transcription (Wise & Goldstein, 1973).

Models exist which have hypothesized roles for particular classes of RNA in the regulation of specific gene transcriptions (Britten & Davidson, 1969). Wise and Goldstein have theorized that RNA shuttling between the nucleus and cytoplasm do so for the purpose of detecting changes in the cytoplasmic environment, much like repressor proteins in bacteria.

The finding that the shuttling RNA was found associated with chromatin is important since it provides a possible tie-in for regulation of transcription. It is contended that although the amount of shuttling RNA associated with the chromatin is small at any given instant, the significant fact is that shuttling RNA is proportionally more concentrated than is non-migrating RNA. The significance of this remains to be seen.

The nuclear helices were shown to contain no radioactivity via autoradiography, implying that the helices contained no low molecular weight RNA (Wise & Goldstein, 1973).

Another technique which has been used to study the distribution of intranuclear sites is the technique employed by Jacob et al. (Jacob, Gillies, MacLeod, & Jones, 1974), using mouse L-cells. This technique makes use of in situ hybridization of nucleic acids in cytological preparations (John, Birnstiel, & Jones, 1969). Autoradiography was used to detect labelled components. Molecular hybridization in ultra-thin sections was demonstrated once prior to this experiment (Jacob, Todd, Birnstiel, & Bird, 1971).

Satellite DNA was isolated, checked for purity, and then used as a template for the synthesis of complementary RNA in vitro. Upon hybridization, satellite DNA was found (via autoradiography) in fairly large patches associated with the nuclear envelope, associated with the chromatin around the nucleolus, and often dispersed. Therefore, these areas were assumed to be rich in satellite DNA sequences. Stretches of hybridized chromatin could also be seen extending into the interior of the nucleoli (not pernucleolar chromatin). It has previously been demonstrated that the DNA sedimenting with the nucleoli was considerably enriched in satellite sequences when compared with DNA from the whole nucleus (Rae & Franke, 1972).

For localization of ribosomal DNA sequences, a different method was used to obtain complementary RNA. It has been demonstrated that ribosomal RNA from one eukaryote can hybridize with ribosomal DNA from another eukaryote (Borwn, Webber, & Sinclair, 1967; Pardue, Gerbi, Ehardt, & Gall, 1970). Therefore, labelled 28S ribosomal RNA from Xenopus kidney cells was used to hybridize with mouse L-cell DNA. A low background was obtained indicating little non-specific binding. When autoradiography was performed, the distribution of the silver

grains on the nucleoli was similar to that obtained after hybridizing satellite-complementary RNA to sections of mouse cells. That is, practically all the silver grains were found over nucleolar chromatin (Jacob, Gillies, MacLeod, & Jones, 1974). These results suggest that the satellite sequences and ribosomal sequences are present in the intranucleolar and perinucleolar chromatin. This technique thus allowed localization of a particular molecular species in the nucleus. From this information, it was argued that the centromeric regions of the chromosomes (the location of the satellite DNA regions) get positioned predominantly along the nuclear envelope. This finding may be related to the phenomenon of chromosome condensation to the nuclear envelope during prophase of mitosis, or to replication or initiation of DNA replication on the nuclear membrane (Comings & Kakefuda, 1968; Freienstein, Freitag, & Suss, 1973; Infante, Nauta, Gilbert, Hobart, & Firshein, 1973). The precise location of the nucleolar organizer in the interphase cell nucleoli remains unknown, although these organizers have been shown to be situated near the centromeric heterochromatin which contains the satellite DNA (Pardue & Gall, 1970). Such detailed ultrastructural studies are needed to pinpoint the precise locations of various components inside the nucleus.

E. Pore Structure/Properties

Nuclear pores and pore complexes are the most singular component in the nuclear envelope. Many studies have been done on the nuclear pore complexes and such studies have defined components of the pore complexes such as annuli with eight subunits, central granules, inner rings, and fibrillar materials (Franke & Scheer, 1970; Harris & Agutter, 1970). Molecular conformations are unknown.

Nuclear pores vary in their number and distribution pattern in different nuclei from the same organism and possibly at different stages of development in the same nucleus (Northcote, 1974). It seems likely that pores can be formed and reformed so that their organization and numbers can vary on the nuclear surface. For an idea of the number of pore complexes present on a nucleus, in a typical cell nucleus from a sycamore plant there are approximately 12-15 pores per square micrometer (approximately 10% of the surface area). An average cell nucleus with a diameter of 15 micrometers carries about $9x10^3$ pores. This figure agrees basically with previous studies (Franke & Scheer, 1970).

Reported pore diameters have ranged from 30-100nm, and this range has been taken to imply definite differences between species and cell types (Gall, 1967). However, Gall (Gall, 1967) has shown that the pore diameters are almost identical in three different species. He suggested that the discrepancies in the literature are due to difficulties in defining the relationship between the pore per se and its accompanying annulus. Gall coated electron microscope grids with colloidion and placed a drop of solution containing the isolated nuclei on the grid. Most of the solution was drawn off and another drop of liquid was added, breaking the nucleus. The portion of the nuclear envelope in contact with the colloidion coat adhered tightly. Fixation of the nuclear envelope was performed with OsO_4, the grids were washed, and a drop of phosphotungstate solution (a negative stain) was added. The results showed nuclear pores and pore complexes. The pores were octagonal rather than circular and the width of the pores was 66.3 $\pm$ 0.5nm. The inner and outer diameters of the annulus associated with each pore were respectively greater and less than the width of the pore itself. It was therefore argued that surface views of the nuclear envelope, unless they are negatively

stained, fail to show the true dimensions of the pores. The pore annulus was reported by Gall to consist of three basic regions. First, there was a more or less uniformly dense central area. Second, there was a much less densely staining line of about 6nm thickness which surrounded the central area and defined an octagon. Third, there was a region outside of the octagonal line which was densely staining (depending on the intensity of the stain). A photographic rotation technique of Markham et al. (Markham, Frey, & Hills, 1963) was employed by Gall (Gall, 1967) to demonstrate that the pore complex was octagonal. In this technique, a micrograph is printed n times, with the enlarging paper being rotated (360/n) degrees between exposures. Structures with n-fold radial symmetry should show reinforcement of detail since random details will be averaged out. Also, the same structures should not show n $\pm$ 1 symmetry.

Harris (Harris, 1974) examined nuclear membrane ghosts. In the preparations with intact nuclear ghosts, he showed that four layers of membrane must be penetrated in micrographs, and that much detail will therefore be lost. Harris also employed a negative stain, and also found that intact nuclear ghosts were disrupted by surface tension and spreading forces when a drop of stain was placed on a grid. Again, the patches of membrane in contact with the coated grid furnished much greater detail.

Around the central pore, Harris found microcylindrical or hollow disk structures with electron-opaque centers which represented the annular granules of the nuclear pore complexes. These cylinders appeared to underlie each of the eight annular granules. Often partially broken pore complexes were seen. Some of the diffuse materials composing the annulus were seen splitting away in these complexes, and the microcylindrical molecules could be seen more clearly. The external diameter

of each was approximately 20nm and the internal diameter was about 5nm. These structures were taken to be representative of a hollow disk lying on its side, with the central hole filled with stain. The suggestion that the eight annular granules were actually cylinders had been made previously (Wischnitzer, 1958; Vivier, 1967; Abelson & Smith, 1970), but this was the first good demonstration of this characteristic. Information regarding the height of the annular granules was not obtained by Gall or Harris. However, Aaronson and Blobel (Aaronson & Blobel, 1975) reported many goblet-shaped structures of approximately 65nm at the stem. These structures were assumed to be pore complexes in lateral view.

Harris (Harris, 1974) also employed the rotation procedure of Markham et al. (Markham, Frey, & Hills, 1963). This time, with more detail visible in some of the complexes, a 16-fold radial symmetry was demonstrated. It therefore appears that there is a very complex organization of subunits within the pore complex. The hollow disk macromolecules were within each of the eight annular granules and were shown to have diffuse material surrounding them. It was not known whether more than one disk was stacked on top of another, but the suggestion that the pore complex is 65nm in length (Aaronson & Blobel, 1975) makes this appear likely. Additional features of this pore complex model are the inner ring of material, the central microcylinder, and the radial fibers. It was suggested that the more detailed pore complex structure presented was visualizable due to the milder, low ionic strength treatment used.

Aaronson and Blobel (Aaronson & Blobel, 1975) characterized the components of the pore complex. Rat liver nuclei were incubated in a solution at pH 8.5 which contained pancreatic DNAase I, for 20 minutes. Detergent solubilization of the phospholipid and magnesium solubilization

of the remaining chromatin were then performed. The composition of each of the fractions was analyzed after precipitation with cold trichloroacetic acid for DNA, RNA, protein, and phospholipid. Electrophoretic analysis of the reduced and alkylated proteins was performed in sodium dodecyl sulfate thin-slab gels containing a linear gradient of polyacrylamide. In agreement with many other studies, nuclear ghosts contained a ribosome-lined outer membrane. The inner nuclear membrane had a clearly defined amorphous fibrous lamina associated with its inner aspect and there were occasional small aggregates of chromatin reported. The nuclear ghosts were found to remain intact even after treatment with detergents at concentrations as high as 5% Triton X-100. The ghosts were thought to consist of highly condensed peripheral chromatin. The reported 70-90nm diameters of the annuli are in good agreement with previously reported dimensions.

An amorphous lamina of about 15nm thickness was observed, which correlates well with the 17nm central unit dimension reported for chromatin threads. The contour length of the lamina could be followed for several micrometers, indicative of sheets. Without the DNAase treatments, it might be expected that the radial fibers previously reported to extend from the threads would be present, making the amorphous lamina appear larger.

It was demonstrated that Triton X-100 treatment solubilized 95% of the original phospholipid (Aaronson & Blobel, 1975), and about 30% of the protein. About 90% of the DNA and 75% of the RNA remaining after detergent treatment was removed by the salt treatment. Nuclear envelopes (as compared to whole nuclei) showed a marked enrichment in 4 bands in the gels with molecular weights of 69,000, 68,000, 66,000, and 50,000

daltons. There were also some histones and one slowly migrating band which entered the gel. Previous work showed that the 50,000 dalton band was a major constituent of the nuclear membrane (Aaronson & Blobel, 1973). In agreement with this finding, detergent treatment removed this band. The histones still remained after detergent treatment, probably still complexed to DNA. Salt treatment removed almost all of the histones. The 66,000, 68,000, and 69,000 dalton bands remained after salt treatment, as did the slowly migrating band.

The 65nm lateral dimension for the pore complex correlates well with the contention that the nuclear pore complex annuli extend outside of the outer nuclear membrane, progress through the outer and inner nuclear membranes, and on through the fibrous lamina.

Fawcett (Fawcett, 1966) demonstrated that RNA accounts for only 20% of the total weight of the pore complex lamina. However, Scheer (Scheer, 1972), using amphibian oocyte nuclear enbelopes, estimated that RNA should account for a large fraction by weight of the pore complex. It seems much more likely to me that it hinges on the isolation procedures used.

Some of the functions for the pore complexes which have been proposed include nucleocytoplasmic exchange of macromolecules (Stevens & Swift, 1966; Harris, Sidebottom, Grace, & Bramwell, 1969; Deak, Sidebottom, & Harris, 1972), and chromatin organization (Comings & Okada, 1970).

Besides the structural electron microscopic studies given above, another tack has been taken to discern pore structure. Information regarding the structure of the pore complex has been obtained based on correlations with the nuclear envelope permeability for small substances

(Paine, Moore, & Horowitz, 1975). The method employed was ultra-low temperature autoradiography (Horowitz, 1974), a technique which can be used to locate tracer substances, regardless of their diffusability.

It was shown previously that colloidal gold particles greater than 12.5-14.5nm in diameter did not penetrate the nuclear envelope in amoebae (Feldherr, 1965). Similarly, ferritin of diameter 9.5nm did not enter the nuclei of amphibian and cockroach oocytes (Gurdon, 1970; Paine & Feldherr, 1972). Below this 9.5nm size, penetration has been found to be inversely proportional to tracer size. Passage of proteins (12,000-67,000 daltons) 1.5-3.5nm in diameter was slowed by the nuclear envelope in cockroach oocytes (Paine & Feldherr, 1972). The degree of slowing increased with increasing molecular size and no slowing was seen for tracers of less than about 4000 daltons.

For the ultra-low temperature autoradiography studies (Paine, Moore, & Horowitz, 1975), dextran polymers were used to obtain flux and equilibrium data. Dextrans were used because they were of known size, hydrated, and because they behaved as spheres in aqueous solutions (Grotte, 1956). Two percent of the cell volume of the dextran solution was microinjected into the vegetal cytoplasm in mature frog oocytes (to avoid nuclear damage). Solute diffusion was permitted for given periods of time, then diffusion was stopped at -190C. Sectioning was done at low temperature and intracellular locations of tracers were determined using ULTA, with the autoradiographic exposure done at -96C. This procedure does not permit solute redistribution.

The kinetics obtained for the entry of labelled dextrans into the nuclei were used in equations to yield a functional pore radius of approximately 4.5nm. The equations included entries for steric hindrance

to pore entry, and pore wall correction factors, which described the frictional resistance to diffusion within a cylindrical pore relative to that in a free solution. Thus the labelled dextran nuclear entry kinetics were explained quantitatively as restricted diffusion through nuclear pores with a patent radius of about 4.5nm each. This finding is in basic agreement with the calculated patent pore radius found in insect oocytes (Gurdon, 1970), amoebae (Paine & Feldherr, 1972), and HeLa cells (Wu & Warner, 1971).

It was suggested that the pore complex central channels were the actual sites of nucleocytoplasmic macromolecular exchange. For instance, colloidal gold particles have been reported to be limited by the annular material of pores to the central channels of the pore complexes, as they pass from the cytoplasm to the nucleus (Feldherr, 1965). Ribonucleoprotein particles, presumably in nucleocytoplasmic transit, have been reported to "narrow down" to 10-20nm in diameter as they "squeeze" through the central region of the pore complex (Paine & Feldherr, 1972). This evidence is very sketchy at best and amounts to little more than guesswork, even accepting the large discrepancy between the calculated pore diameter of 4.5nm and the 10-20nm diameter of RNP molecules. As further evidence of the pore central channel functioning as the site of nucleocytoplastic transport, Paine et al. (Paine, Moore, & Horowitz, 1975) state that their data can account for large differences in passage of similar solutes based on small differences in their sizes. A much more plausible explanation will be given later. This concept of passive transport is in conflict with shuttling RNA studies by Wise and Goldstein, with the passage of informofers whose size is larger than the patent pore radius (for instance, Lukanidin, Kyl'Guskii, Aitkhozhina,

Komaromi, Tikhonenko, & Georgiev, 1973), and with the data of Roth et al. (Roth, Bolla, & Cox, 1976) regarding selective uptake of ribosomal proteins compared with cytosol proteins.

As an additional factor affecting transport of solutes, it has been demonstrated that frog annular materials have a net positive charge (Goldstein & Trescott, 1970).

An ATPase activity has been found in association with the pore complex (Cutler, Chaudhry, & Montes, 1974; Chardonnet & Dales, 1972). The effects of inhibitors of ATPase's have been examined (Schumm & Webb, 1975; Chardonnet & Dales, 1972) in many systems, and will be discussed regarding their effects on transport. However, the technique used to demonstrate ATPase activity is a complex technique, which bears discussion.

The lead method for precipitation of phosphate, based on a Gomori metal precipitation reaction (Gomori, 1952), was originally proposed in 1957 (Wachstein & Meisel, 1957) for demonstrating ATPase activity. Following this basic scheme, a great deal of research has gone into discovering what happens in such ATPase media (for instance, Novikoff, Drucker, Shin, Goldfischer, 1961; Marchesi & Palade, 1966; Toose, 1965; Rosenthal, Moses, Beaver, Schuffman, 1966; Rosenthal, Moses, Tice, & Ganote, 1969; Gilder & Cronshaw, 1973; Novikoff, 1967; Cronshaw & Esau, 1967; Gardner & Peel, 1969; Kluge & Ziegler, 1964; Pratt, 1954).

Following the basic Wachstein-Meisel procedure, lead ions for precipitation and ATP for substrate are added to the medium, along with other divalent cations, etc. ATP has been shown to chelate lead ions (Tormey, 1966; Berg, 1964; Tice, 1969; etc.), and other divalent cations compete with lead for participation in these complexes. Lead

has been shown to catalyze nonenzymatic hydrolysis of ATP in tissues (Tice, 1969; Moses & Rosenthal, 1968; Moses, Rosenthal, Beaver, & Schuffman, 1966), although little nonenzymatic hydrolysis occurred at lower lead concentrations. Also, lead has been shown to bind to tissue, as does ATP. It has been shown that the phosphate and lead do combine to form precipitates at a ratio of 1:1 (Tice, 1969). However, it was subsequently shown, using electron microscopic autoradiography, that ATP was also found in the precipitates (Rosenthal, Moses, Ganote, & Tice, 1969). Inhibition of enzyme activity has been demonstrated with lead (Jacobsen & Jorgensen, 1969). Tissue has been shown to inhibit lead catalyzed hydrolysis (Ganote, Rosenthal, Moses, & Tice, 1969). In short, the reaction is very much more complex than just having lead ions in solution to trap released phosphate as it becomes available.

Fixation of tissue prior to incubation in ATPase media has been shown to inhibit most of the ATPase enzymatic activity (Chardonnet & Dales, 1972; Novikoff, Hausman, Podber, 1958; Tormey, 1966), so care must be taken when choosing the procedure for fixation and the fixative itself.

As the technique is most often used today, low concentrations of lead ions are used. This concentration (0.1-1.0 mM) is chosen to be low enough so as not to inhibit enzyme activity and so that little non-enzymatic hydolysis occurs. This concentration range has also been shown to be efficient for trapping released phosphate before much diffusion occurs (Marchesi & Palade, 1967; Moses, Rosenthal, Beaver, Schuffman, 1966; etc.). In general, ATP is added in higher concentrations than in the original Wachstein-Meisel media (3.6mM as opposed to 0.7mM), although the typical experimental approach is to add ATP to the

experimental mixture and omit it from the control mixture. Due to the aforementioned interactions of the medium components, the significance of experimental vs. control in the strict sense is questionable.

Fixation is usually chosen so that ultrastructure as well as enzyme activity will remain (Chardonnet & Dales, 1972; Novikoff, Hausman, Podber, 1958; Tormey, 1966). Calcium formol (3%) has been employed as a fixative successfully, with fixation allowed for 5 minutes on ice.

In summation, with these precautions as to concentrations and procedures, the lead method for detection of ATPase activity is a valuable tool for probing into the enzymatic locations, and does permit localization of ATPase activity.

II. Nuclear Release of Ribonucleic Acid

A. Systems Designed to Measure Release of Ribonucleic Acid

The first system designed to examine the release of RNA from the nucleus was that of Schneider (Schneider, 1959; Schneider, 1961). Rats were injected with ^{14}C-adenine for 2 hours prior to sacrifice and the nuclei were isolated from a cell homogenate. Nuclei were then pelleted, resuspended in 0.25M sucrose, and recentrifuged.

Schneider found that part of the labelled RNA was released from the nuclei and the remaining nuclear RNA had a much lower specific activity than before release. This was shown not to be due to hydrolysis of nuclear RNA to the level of nucleotides that could be incorporated into cytoplasmic RNA or to a dilution of the nuclear RNA from other cell fractions.

Release was found to occur only at temperatures above 20C and magnesium was found to inhibit release. High concentrations of fluoride,

malonate, cyanide, and iodoacetate did not significantly inhibit or stimulate RNA release, suggesting release was energy independent. Adenosine triphosphate, KCN, and citrate stimulated release, as did the removal of magnesium from the incubation mixture. An interesting idea expressed by Schneider was that "if some complex does exist between magnesium and ribonucleoprotein, then the strength of that complex will determine whether it can be broken by the formation of a second complex forming agent or not." Therefore, the effect or lack of effect of a chelating agent may be due to differences in the association constants of the magnesium complexes. Schneider expressed the belief that the effect of temperature on release might be due to some enzymatic action or other physiological phenomenon or to a change of some lipoidal material forming complexes with the ribonucleoprotein from the solid state to the liquid state at about 20C. Schneider concluded that the ATP effect was a physical reaction and not a biological reaction.

Chatterjee and Weissbach (Chatterjee & Weissbach, 1973) have used a system which consists of HeLa cells or mouse L-cells. Nuclei were prepared by cell lysis in a Dounce homogenizer. Detergent treatment was also mentioned, although it was not clear whether this was part of their standard isolation procedure. When DNA was to be labelled, precursor was added at 37C for 50 minutes prior to isolation. The incubations for release were carried out at 30C.

Using this system, RNA and protein were released from nuclei in the presence of ATP. No release was observed at 0C, but good release was obtained at 30C with various concentrations of ATP, with release maximized at 6mM ATP. Protein was also released, but ATP was found to

stimulate its release only slightly; RNA release was increased 300% with ATP, whereas protein release increased only about 25%. As would be expected, it was found that some of the released TCA-insoluble (Trichloroacetic acid insoluble) counts were degraded to TCA-soluble counts. Degradation of TCA-insoluble counts could be prevented by the addition of disodium ethylenediaminetetraacetate (EDTA).

The kinetics of release were also monitored and linear release was reported for 30 minutes at 30C with 25-30% of the RNA released. Nine percent of the RNA was released in the absence of ATP and a magnesium inhibition of release was also noted.

Other additions to the incubation medium were examined, including ADP, CTP, GTP, UTP, the non-hydrolyzable analogues AMPPCP and GMPPCP, and inorganic phosphate. Inorganic phosphate and AMP did not stimulate release although all of the other abovementioned additives did. This result contrasts with the results of Ishikawa et al. (Ishikawa, Kuroda, & Ogata, 1969), in which ADP was unable to stimulate release.

The results suggested that hydrolysis of nucleoside triphosphates was not required (Chatterjee & Weissbach, 1973), and Schneider's suggestion (Schneider, 1959) that nucleotides functioned by chelating magnesium was discussed. Chatterjee and Weissbach drew interesting conclusions from their results. With no added magnesium, more TCA-insoluble and TCA-soluble label was released in the presence of ATP than in the absence of ATP. It was therefore suggested that the endogenous magnesium in the nucleus might be inhibitory for RNA release from the nucleus and that this endogenous magnesium was being removed by the addition of nucleotide. In addition, essentially all of the label released without magnesium was TCA-insoluble. EDTA caused a

two-fold increase in release of RNA. This also supports the view that magnesium inhibits release and also stimulates degradation of released RNA. An observation reported was that the size of the nuclei was considerably reduced in 8mM $MgCl_2$, in good agreement with my results. When DNA was labelled, no release of DNA was observed and no DNA-dependent RNA polymerase activity was found. Preliminary results by the same investigators (Chatterjee & Weissbach, 1973) suggested that the same conditions hold for adenovirus-2 infected KB cell nuclei. The conclusion drawn by Chatterjee and Weissbach was the same conclusion originally suggested by Schneider. ATP dependence in many reported systems may be due to chelation of magnesium ions.

Chatterjee and Weissbach (Chatterjee & Weissbach, 1973) have carried out polyacrylamide gel electrophoresis analysis on released RNA or RNA taken from whole nuclei. When the material released after 15 minutes of incubation at 30C was examined, the RNA was heterogeneous and contained RNA species which ranged from 4-6S to 28S or more. The RNA taken from whole nuclei showed species much larger than 28S. The predominant released species appeared to be in the 18-28S size range, with many species that were much larger also present.

Webb and co-workers have investigated the release of RNA from nuclei of cells from regenerating rat liver, moderately differentiated hepatoma 5123D, and poorly differentiated Novikoff hepatoma. The transport system consisted of the following constituents: 50mM Tris-HCl (pH 7.5), 25mM KCl, 2.5mM (or 250mM?) $MgCl_2$, 0.5mM $CaCl_2$, 0.3mM $MnCl_2$, 5.0mM NaCl, 2.5mM Na_2HPO_4, 5.0mM spermidine, 2.0 (or 2.5)mM dithiothreitol, 2.0mM ATP, 2.5mM phosphoenolpyruvate, 35 units (or 6.4 units) of pyruvate kinase, 500 micrograms of yeast RNA per ml, and dialyzed cytosol which

had a protein concentration of 10.3 ± 2.3 mg/ml (or 19 mg/ml), with $5x10^6$ nuclei/ml. The normal mixture contained 0.6 volume of cytosol. The entire mixture was incubated at 30C, with additional phosphoenol-pyruvate added (1.0mM) at each 10-minute interval. Labelled RNA was obtained by intraperitoneal injection of labelled orotic acid into rats 30 minutes prior to nuclear isolation.

The time course of release in this system was followed (Schumm, Morris, & Webb, 1973). Approximately 4% release from normal rat liver nuclei to normal liver cytosol was obtained at 30C after 30 minutes. After 60 minutes, approximately 8% was released. A biphasic release was noted, with a marked two-part response in normal liver. However, the regenerating liver nuclei release was not biphasic and the hepatoma 5123D release might have been biphasic, although no standard deviations were presented to support their claim that the release was biphasic. It was claimed (Rizzo & Webb, 1972; Schumm, Morris, & Webb, 1973) that a biphasic time course of release was predictable from facts known about processing and turnover time of mRNA and rRNA in intact cells. In other studies by these same investigators, this type of response curve was explained as utilization of exogenous energy source (see Yu, Racevskis, & Webb, 1972; Racevskis & Webb, 1974). These explanations are not necessarily compatible or consistent.

The effect of cytosol was examined (Schumm, Morris, & Webb, 1973) by incubating nuclei in cytosol fractions obtained from different cells. Maximal release was obtained with nuclei from normal liver incubated with normal liver cytosol. Transport from these nuclei was significantly reduced when cytosol was derived from hepatoma 5123D cells and was very much reduced when the cytosol was derived from regenerating

liver cells. As was mentioned, the protein concentrations of the cytosol preparations were very similar and it was shown that a 10-fold difference in protein concentration was required to cause a 50% reduction in transport.

Fractionation of the cytosol was attempted (Racevskis & Webb, 1974). Ammonium sulfate precipitation in the ranges of 0-20%, 20-60%, and 60-95% saturation was attempted. The insoluble proteins were removed by centrifugation after each addition, resuspended, and dialyzed. This fractionation resulted in an increase of 2.5 times the specific activity for RNA release of the unfractionated cytosol when the 20-60% fraction was used. This was the best enhancement obtained. Cytosol was also fractionated in a step-wise manner on DEAE-cellulose columns. Non-absorbing proteins were eluted with buffer at pH 8.5. Absorbed proteins were eluted with 0.6M KCl in buffer, pH 7.5. Most of the proteins which stimulated mRNA release as informosomes were found in the fraction which did not absorb to DEAE-cellulose at pH 8.5.

It was also shown (Yu, Racevskis, & Webb, 1972) that released RNA did not originate from perinuclear ribosomes, but was released as 40S and 60S ribosomal subunits. The investigators claim that the transport factors were not ribosomal proteins since supplementation of the medium with proteins isolated from purified ribosomes either had no effect on release or slightly inhibited release.

An earlier study of this system (Yu, Racevskis, & Webb, 1972; Racevskis & Webb, 1974) showed that spermidine and cytosol both were necessary to maintain the structural integrity of liver nuclei. The rate of release was maximal at a protein concentration of normal liver cytosol equal to or in excess of 12 mg/ml. A lower release was obtained

with 2.45 mg/ml than with buffer alone for up to 10 minutes, after which lysis occurred in buffer. Freezing at -15C reduced transport activity. In other studies (Racevskis & Webb, 1974) transport factors were in considerable excess at 19.6 mg/ml cytosol concentration and when the cytosol was frozen at -15C, macromolecular factors which facilitated rRNA transport were limiting in the assay at lower protein concentrations. If this interpretation is correct, then the initial rate of release should be the same, since rRNA is released in the second portion of their biphasic response curve; it was not.

In other studies (Yu, Racevskis, & Webb, 1972) cytosol was reported to contain other factors which were necessary for the preservation of nuclei. Complete omission of cytosol from the assay mixture resulted in rapid release of RNA due solely to nuclear lysis, which after 10 minutes became exponential.

Other studies (Schumm, McNamara, & Webb, 1973) have shown that the transport activity could be dissociated from the stabilizing activity of the cytosol, since the former but not the latter was inactivated by prolonged storage at -15C. It was shown that with the higher concentrations of cytosol 3.5% release of RNA occurred in 6 minutes at 30C. A serious discrepancy in the data from this system was the finding (Racevskis & Webb, 1974) that the concentration curve for cytosol concentration vs. release was sigmoidal, whereas in another study (Schumm, McNamara, & Webb, 1973) this same curve was linear.

Attempts were made to investigate whether ATP or cytosol factors affected the degree of processing of precursor ribosomal RNA within the nucleus (Racevskis & Webb, 1974). In one experiment, an aliquot of nuclei was incubated at 30C for 10 minutes in an optimal system which

contained neither ATP nor an energy generating system. Another aliquot was incubated at 4C. Then both aliquots were incubated at 30C for 10 minutes with ATP present and the initial rate of release in the two aliquots was compared. The rates of release were found to be identical. It was concluded that either the intranuclear processing of the ribosomal RNA was energy dependent, or that transport (release) was rate limiting, or both. In another experiment, nuclear RNA species in regenerating liver nuclei were examined after incubation at 0C, at 30C in a complete system without ATP, or in a complete system at 30C (after 10-minute incubations). The processing of 45S ribosomal precursor RNA occurred at 30C in the absence of ATP, although conversion was markedly greater in the presence of ATP. A 60% conversion was obtained with ATP. From these experiments, it was not clear whether this enhancement by ATP was due to the involvement of ATP as an energy source at the level of processing, or was a secondary result of a coupling between processing and transport. Another effect which was reported was a 40% greater processing of 45S ribosomal precursor in the presence of 12 mg/ml cytosol as opposed to 2.45 mg/ml cytosol (stored frozen, whereas the 12 mg/ml cytosol was fresh).

As different aspects of release with different types of nuclei were examined, a differential effect of ATP on release of RNA from normal or neoplastic cell nuclei was discovered (Schumm & Webb, 1975). In particular, the release from isolated rat liver nuclei, 5123D hepatoma nuclei, and Novikoff hepatoma cell nuclei was monitored. In contrast to earlier studies (Schumm, Morris, & Webb, 1973) a differential energy dependence was noted. RNA transport from liver nuclei was shown to have an absolute requirement for an exogenous energy source. RNA transport

from 5123D hepatoma nuclei had a 25% energy dependence while RNA release from Novikoff hepatoma nuclei was totally energy independent.

The effect of beryllium nitrate on transport from nuclei of normal liver and hepatoma cells was examined (Schumm & Webb, 1975). Beryllium nitrate has been shown to have a specific inhibitory effect on a nuclear pore phosphatase (Cutler, Chaudhry, & Montes, 1974). Therefore, this salt was tested as a possible inhibitor of RNA transport. Beryllium ions were found to drastically inhibit the ATP-dependent release of RNA from normal liver nuclei, had a partial inhibitory effect on RNA release from 5123D hepatoma nuclei, but had no inhibitory effect on RNA release from Novikoff hepatoma nuclei (ATP-independent).

Another effect reported in this system (Schumm & Webb, 1975) was the differential stability of cell nuclei from the different cell types. Previous studies had shown that spermidine and calcium ions were necessary for nuclear stability. It was also reported (Schumm, McNamara, & Webb, 1973; Racevskis & Webb, 1974) that a reduction in the concentration of cytosol protein below 3 mg/ml resulted in nuclear lysis, and that this lysis was enhanced with ATP present. In contrast to the lysis observed at 3 mg/ml with normal liver nuclei, Novikoff hepatoma nuclei appeared more resistant to lysis, even at 1 mg/ml. Less release at 1 mg/ml cytosol protein content was obtained than at 10 mg/ml, with the Novikoff hepatoma nuclei, and the investigators argued that this was due to limiting cytosol transport proteins. However, similar kinetics were shown in both the presence and absence of ATP. With Novikoff hepatoma cells there was no evidence of nuclear lysis even in the absence of cytosol.

A great deal of work has also been done on characterization of the released RNA using the above system. Most of the released particles were found to sediment at approximately 40S, which was comparable to that of informosomes isolated directly from intact rat liver cells (Henshaw, 1972). The identity of the particles as informosomes rather than ribosomal subunits was established by the use of buoyant density centrifugation (Schumm & Webb, 1972).

The size distribution of the labelled RNA released from normal liver nuclei, hepatoma 5123D nuclei, and Novikoff hepatoma nuclei to autologous and heterologous cytosol was studied (Schumm, Morris, & Webb, 1973). It was found that normal nuclei in normal liver cytosol released RNA that sedimented primarily in the 10S region of the gradient with minor species up to 28S also present. Very similar size distributions were obtained when normal nuclei were incubated in regenerating liver cytosol. However, when normal nuclei were incubated in hepatoma cytosol a much larger fraction sedimented in the 10S-28S region of the gradient. In contrast to this, when hepatoma nuclei were incubated in autologous cytosol, the released RNA was primarily 10S, with none at or above 18S.

Labelled regenerating rat liver nuclei incubated in normal liver cytosol released predominantly 10S RNA, with very little RNA at or above 18S. However, in regenerating liver cytosol or hepatoma 5123D cytosol regenerating liver nuclei released 10S, 18S, and 28S RNA primarily.

Using the technique of Gillespie and Spiegelman (Gillespie & Spiegelman, 1965), as modified (Birnboim, Pene, & Darnell, 1967), DNA-RNA hybridization experiments were performed (Schumm, Morris, & Webb, 1973). It was found that 50% of the 10S material released in

the regenerating system was also released in the hepatoma system. Furthermore, of the total RNA sequences released from regnerating nuclei only 20% were present in the RNA transported by normal nuclei and 45% were present in the RNA transported by hepatoma nuclei. It was also reported that only 27% and 41% of the RNA released by hepatoma 5123D nuclei have sequences in common with the RNA released by regenerating and resting rat liver nuclei, respectively.

The significance of this hybridization data is obscure. The two classes of competing RNA do not have to be identical, since they may be competing against many different species of labelled RNA. Also, under the conditions used for this hybridization, most of the RNA binding measured by this competition hybridization technique is due to RNA transcribed from reiterated genes (Melli & Bishop, 1969). Unique sequences are not detected by this method.

Cytosol factors affected transport, as has been noted. RNA species were examined in material released from regenerating liver nuclei to regenerating liver cell cytosol (Schumm, Morris, & Webb, 1973). It was found that the RNA released from regenerating liver nuclei gave the expected 100% competition when competed against the released material in the same system. However, this competition dropped to 75% and 60% when the competing RNA was obtained from nuclei incubated in cytosol derived from hepatoma 5123D cells or from normal liver cells, respectively. This was taken as evidence that there was a significant fraction of RNA sequences transported from regenerating liver nuclei when they were incubated in autologous cytosol, that were not transported in the presence of heterologous cytosol.

The proportion of messenger-like RNA (those containing polyadenylic acid tracts) was estimated by measuring the proportion of the labelled released RNA that bound to nitrocellulose filters at high ionic strength (Brawerman, Mendecki, & Lee, 1972). The proportion of the 20S and 26S RNA released from regenerating liver nuclei that contained poly A tracts was 47%, which the authors concluded "attested to the mRNA nature of this fraction". The proportions of the total presumptive mRNA transported by normal liver nuclei and regenerating liver nuclei were 44% and 38% respectively (Schumm, Morris, & Webb, 1973). These measurements were taken using a phenol:chloroform (1:1) extraction procedure, a technique which has been shown to preserve poly A tracts found in mRNA and heterogeneous nuclear RNA (Perry, LaTorre, Kelley, & Greenberg, 1972). These percentages do not correspond with previous results of these investigators (Schumm & Webb, 1972). Estimates based on a 30-minute in vivo labelling of RNA with labelled phosphate have shown that 60% of the RNA released from tumor cell nuclei to tumor cell cytosol contained poly A tracts. The hepatoma 5123D system had too low an incorporation of labelled orotic acid to allow use of the same technique mentioned above.

As was mentioned, it was reported (Racevskis & Webb, 1974) that a separation of cytosol transport functions was obtained by DEAE-cellulose column fractionation. The activity of absorbent and non-absorbent fractions was monitored in concentrations equivalent to 12 mg/ml of the original unfractionated cytosol. It was found that the fraction which did not absorb to the DEAE-cellulose columns appeared to be specific for the transport of 4-10S species, whereas the fraction which did absorb seemed to possess most of the stimulatory activity for transport of 18S

and 28S ribosomal RNA, although some 4-10S species were also released by this fraction.

Heat lability of transport factors in the cytosol has also been studied (Racevskis & Webb, 1974). If the cytosol fraction was heated for 10 minutes at 50C prior to use, the resultant release was reduced 25-30% compared to control cytosol release. It was stated that heating beyond 10 minutes was impractical, due to a differential denaturing of the natural ribonuclease inhibitor.

The effects of 3'-deoxyadenosine (cordycepin) on release have been examined (Schumm & Webb, 1974). Cordycepin has been shown to be an inhibitor of nuclear poly A synthesis (Penman, Rosbach, & Penman, 1970). The suppression of labelling of cytoplasmic mRNA in the presence of cordycepin is thought to be caused by an inhibition of mRNA processing and transport as a result of an inhibition of poly A synthesis (Darnell, Philipson, Wall, & Adeswick, 1971). A 40% decrease in TCA-precipitable release of 10S, 20S, and 26S species from cordycepin-treated nuclei was noted.

The results of using different techniques to quantitate poly A containing RNA were examined (Schumm & Webb, 1974). Release from untreated regenerating rat liver nuclei showed a 40% poly A content (in presumptive mRNA) when measured with nitrocellulose filters, cellulose columns, and poly U columns. When nitrocellulose filters were used to analyze the poly A content of RNA released from cordycepin-treated cells, 6% poly A containing RNA was found. When cellulose columns were used, 22% poly A+ RNA was found. When poly U columns were used, 45% poly A+ RNA was obtained. These results suggested that cordycepin decreased the size of the poly A tracts in the transported mRNA in addition to inhibiting

the release of a fraction of the RNA transported from normal nuclei. This was based on previous studies showing that poly A tracts of greater than 200 nucleotides will bind to Millipore filters, but tracts containing less than 70 nucleotides do not bind (Eaton & Faulkner, 1972). Other studies showed that poly A tracts of less than 50 nucleotides will bind to poly U columns (Sheiness & Darnell, 1973), and it has been proposed that as few as 12 nucleotides may cause binding (Niyogi, 1969).

To verify this suggestion that the size of poly A tracts on mRNA transported from cordycepin-treated rats was reduced, the poly A tracts from control and cordycepin-treated systems were isolated and separated on sucrose density gradients. It was found that the poly A tracts were shorter in RNA transported from cordycepin-treated nuclei, with a probable length between 15-70 nucleotides.

An attempt has been made to correlate this in vitro release with the analogous in vivo system, with the use of DNA-RNA competition hybridization (Schumm & Webb, 1974B). In this study, a comparison was made between the RNA released to the incubation medium described previously in detail (A), an incubation medium containing 1mM ATP and 0.88M sucrose (among other things) labelled (B), and an incubation medium containing 6mM ATP and 250mM sucrose (among other things) here labelled (C). Unlabelled competitor RNA was prepared by incubating unlabelled nuclei in media A, B, or C, and then purifying the released RNA from the nuclei-freed supernatant with phenol:chloroform. Alternatively, a 60-minute in vivo labelled or unlabelled cytoplasmic RNA fraction was purified directly from rat liver cells. Liquid hybridization was performed and percent hybridization was assayed on hydroxylapatite columns (Goodman, 1973).

In this study (Schumm & Webb, 1974B), competition assays of labelled RNA from normal liver cytoplasm in the presence of increasing amounts of unlabelled competitor RNA released from normal liver nuclei to incubation media A, B, and C were studied. The similarity of the saturation curves for these three comparisons indicated that essentially all of the families transcribed from reiterated gene sequences which were released to the cytoplasm are released by liver nuclei to the three different incubation media.

As a measure of the in vivo equivalence of the RNA released in the cell-free systems, the reverse situation was tested. Labelled RNA released from isolated nuclei to different media was hybridized to DNA in the presence of increasing amounts of unlabelled cytoplasmic RNA. The findings of these experiments were interesting. It was found that 78% of the labelled RNA released to medium A (containing cytosol, etc.) was competed by cytoplasmic RNA. However, only 50% and 32% of the RNA released to media B and C were competed under the same conditions, respectively. The authors applied a correction factor, based on the presence of RNA released to all media at OC, which amounted to about 1% of the total nuclear counts. With this correction, the disparity between medium A and the in vivo release was only 10%.

A further correlation of the medium A system and the in vivo system was found when the effects of colchicine were examined. The effects of colchicine were examined since structures resembling microtubules reside in the vicinity of nuclear pores (Schjeide, 1970) and colchicine has been shown to disrupt microtubules (Wilson, Bamburg, Mizel, Grisham, & Creswell, 1974). The results showed that colchicine reduced by 64% the accumulation of labelled RNA in the cytoplasm of liver cells.

Pretreatment of isolated nuclei with colchicine for 15 minutes at 0C in medium A, before incubation at 30C, reduced labelled transport by 77%. This same treatment was found to reduce transport of labelled RNA 22% and 5% in media B and C, respectively. The meaning of this study is obscure. Colchicine has been shown not to inhibit RNA synthesis in HeLa cells (Mizel & Wilson, 1973), but it appears to affect RNA processing and transport. However, it has been shown to inhibit nucleoside transport in HeLa cells independent of its effect on microtubules (Mizel & Wilson, 1973).

Another interesting system for studying release of RNA from isolated nuclei was that of Sauerman (Sauerman, 1974). In this system, rats were injected with labelled orotic acid 40 minutes prior to sacrifice. The nuclei from liver cells were then isolated. The isolated nuclei were absorbed onto the surface of membrane filter pieces which were then transferred into microcolumns. The nuclear columns were kept in a thermostatically controlled water bath and medium containing sucrose, buffer, and a ribonuclease inhibitor was pumped through the columns. The effluent fractions were collected by a drop counter into liquid scintillation vials and the total radioactivity of the released material was determined in a liquid scintillation spectrometer. As a control to determine if radioactive material was being released as acid-soluble counts, fractions were collected on filter paper disks, and the disks were repeatedly washed with TCA, ethanol, ether, and counted.

The labelled material found in the effluent fractions was shown to consist of ribonucleoproteins which sedimented in a broad zone with a peak at about 22S in a sucrose gradient. Most of the material was found to have a buoyant density of 1.45g/cc is CsCl. There was a small fraction with a buoyant density of 1.54g/cc. When the RNA was isolated from the ribonucleoproteins, it sedimented with a peak at about 12S.

When 3mM $MgCl_2$ (or $MnCl_2$, another potent inhibitor of RNA release - see Ishikawa, Kuroda, & Ogata, 1969) was added at 18C, the same percentage of RNA was released as was released at OC without them. With the addition of 0.5mM ATP, only a slight effect was seen in release. When the column was perfused with 2mM ATP, an increased release of 1.7% was obtained (corrected total). When 6mM ATP was perfused through the column, a 3.0% release was obtained. Thus, the release could be modulated by additions to the effluent volume. In similar experiments, 9mM $MnCl_2$ was used. As before, the release became significant only when the concentration of ATP approached the divalent cation concentration. When the concentration of $MnCl_2$ was 1mM, significant RNA release occurred above a concentration of 0.5mM ATP.

Sauerman concluded that the ratio of nucleoside triphosphate to divalent cation was the decisive factor for regulation of RNA release and that significant RNA release occurred when the nucleoside triphosphate concentration was greater than about two-thirds of the divalent cation concentration.

It was also found that 2'-deoxyadenosine-5'-triphosphate could substitute for ATP, as could the 2'-deoxyguanosine analogue (dATP, dGTP). Also, the release of RNA could be induced by the chelating agents citrate and pyrophosphate.

It was then concluded that the transport of ribonucleoprotein particles from nuclei was not necessarily coupled to an ATP-dependent energy requiring process, as has been suggested (Raskas, 1971; Schumm & Webb, 1972). As suggested by Schneider (Schneider, 1959), the release process may be primarily affected by divalent cations, which is what Sauerman also concluded.

Another system used to investigate RNA release was reported by Raskas and co-workers (Raskas, 1971; Raskas & Rho, 1973; etc.). In this system cultured human KB cells, infected with adenovirus2, were used. Previous results showed that the infecting adenoviruses are nuclear DNA viruses, and that viral mRNA is transcribed in the nucleus and translated on cytoplasmic ribosomes (Raskas & Okubo, 1971; Velicer & Ginsberg, 1968; Thomas & Green, 1966). Viral RNA is synthesized as high molecular weight precursors, then cleaved to yield small viral RNA species associated with the polysomes (Parsons, Gardner, & Green, 1971). Eighteen hours after infection, most or all of the polysomal mRNA is virus specified (Thomas & Green, 1966).

No cytosol was used in this system. The nuclei were isolated by Dounce homogenization, and incubated in buffer. When appropriate, ATP was added to the incubation mixture, along with 5mM creatine phosphate and 20 micrograms/ml of creatine kinase (an energy generating system), or other additions as noted. Incubations were carried out for 20 minutes at 37C.

The adenovirus infected cells were found to release RNA as ribonucleoprotein. The release was shown to be temperature dependent (Raskas, 1971). When the nuclei were incubated for 20 minutes at 37C, they were found to release 10% of their RNA. However, when ATP and an energy generating system was included in the incubation mixture, the nuclei release 50-60% of their RNA. This amount of release seems far out of proportion to other results obtained, although it should be noted that this is viral RNA. Transport of RNA in this system occurred without concurrent RNA synthesis, for release was not inhibited by actinomycin D. During the

incubations, 80-90% of the label remained TCA-precipitable and the number of nuclei remained constant (Raskas, 1971).

Incubations were carried out with AMP, ADP, GTP, CTP, and other ingredients. It was found that adenosine did not stimulate release greater than that obtained in buffer alone. AMP was found to cause 5-10% of the release which occurred with ATP present. ADP caused a 10-20% release. The other three ribose triphosphates were found to stimulate release as effectively as ATP. With magnesium concentrations of 5mM or higher, release was drastically inhibited. Concentrations of 3mM Mg^+ were commonly used. It was suggested that divalent cations have a role in maintaining nuclear structure. Incubations were also performed with EDTA and pyrophosphate, two other chelating agents. EDTA showed no activity, whereas pyrophosphate stimulated release to the same extent as did ADP, when rapidly growing cultures were used. The metabolic state of the cultures affected the response of nuclei to added pyrophosphate. If slowly growing cells were used to obtain nuclei, then pyrophosphate was found to be nearly as effective as ATP in stimulating release (Raskas, 1971).

The possibility that in vitro mRNA transport might require hydrolysis of ATP (NTP's) was investigated using AMPCPP and AMPPCP, non-hydrolyzable analogues of ATP. These stimulated release to the same extent as ATP.

To achieve maximal activity of the in vitro release, both ATP and an ATP-generating system were required. Omission of creatine phosphate and creatine phosphokinase from the incubation mixture reduced the release by approximately 75%, whereas absence of ATP reduced the RNA release by only 30% (Raskas, 1971). It was suggested that the nuclear preparations contained sufficient adenosine to serve as substrate for phosphorylation.

When nuclei were isolated from pulse-labelled cultures, most labelled viral RNA was found in high molecular weight precursor forms. However, the effect of ATP was shown, via polyacrylamide gel electrophoresis, to be selective for molecules the size of mRNA (Raskas, 1971). It was shown that during in vitro incubation, intranuclear viral RNA was first cleaved to yield molecules the size of polysomal viral RNA which were then released from the nuclei (Brunner & Raskas, 1972). The ATP seemed to stimulate release of molecules only after they underwent cleavage, suggesting that this feature of RNA metabolism in vivo was maintained. It is not clear whether ATP was needed for processing or for transport, or for both.

The nuclei, after isolation, contained newly synthesized low molecular weight RNA molecules, including newly made tRNA and also a viral RNA species synthesized in large quantities after infection (Ohe, 1972). The transport of these molecules was found not to be dependent on the presence of ATP, since they were released in buffer alone. Exogenous ATP appeared not to be needed for transport of these molecules. It has been shown that ATP stimulated release of RNA from various types of nuclei as well as from cores of the cytoplasmic vacinia virus (Kates & Beeson, 1970A). It is not clear whether ATP added to suspensions of isolated nuclei acts in the same manner that it does in vivo, but these results suggest a common effect in all cellular components which release RNA.

Raskas and Rho (Raskas & Rho, 1973) concluded that it would be difficult to propose a mechanism for mRNA transport which was controlled "exclusively by localized ATP concentrations and was at the same time selective for the mRNA sequences transported". However, selective binding of transport proteins could select for given sequences and

localized ATPase activity (Chardonnet & Dales, 1972) could indicate selective use of ATP in specific locations. It was concluded by Raskas and Rho that the selection of mRNA sequences occurred by controlled degradation of precursor RNA molecules.

The RNA released from adenovirus-infected KB cell nuclei has been characterized (Raskas, 1971; Raskas & Rho, 1973). The released RNA showed two broad peaks upon sucrose gradient centrifugation, one sedimenting more slowly than the small ribosomal subunit (35S) and second low molecular weight component at approximately 10S.

A replicate sample of this released RNA was adjusted to high salt (0.5M NaCl, 0.05M $MgCl_2$) and centrifuged through gradients which contained high salt. The released RNA of the 35S component could no longer be detected, and it was concluded that it sedimented at a lower rate and was not resolvable from the 10S component. This behavior suggested that the 35S component contained protein as well as RNA (Girard & Baltimore, 1966).

The 35S and 10S released components were combined, treated with glutaraldehyde, and analyzed on preformed caesium chloride gradients as previously described (Baltimore & Huang, 1968). These gradients showed that the released 35S component had a density of 1.40g/cc, which has previously been shown to be characteristic of ribonucleoprotein. Marker 74S ribosomes had a density of 1.52g/cc, as previously reported (Perry & Kelley, 1966). The 10S component was probably free RNA, since it did not reach equilibrium during the centrifugation.

Both the 35S and 10S components were shown to contain specific sequences transcribed from adenovirus DNA (Raskas, 1971), by hybridization

to immobilized adenovirus DNA as previously described (Fujinaga & Green, 1966). It was reported that 60% of the released RNA from the 30-40S component annealed with viral DNA. Ribonucleic acid from the 10S component annealed to "an appreciable but lower extent". It was suggested that this component contained host cell tRNA which has been shown to be synthesized late after infection (Raskas, Thomas, & Green, 1970). However, this material was phenol extracted, and it is not clear why tRNA should sediment at 10S.

The total RNA released after a 20-minute incubation was analyzed on polyacrylamide gels (Raskas, 1971; Raskas & Rho, 1973). The RNA contained low molecular weight species (4-7S) as well as RNA that was much larger. The 35S component contained RNA which had a size distribution similar to that found in KB cell polysomes late after adenovirus-2 infection (Raskas & Okubo, 1971; Thomas & Green, 1966). The 10S component contained all the low molecular weight RNA.

It was proposed that the mechanism of transport was relatively specific since only 5% of the nuclear protein or DNA was released under conditions which yielded 30-70% RNA release. The 5% DNA release seems very high, although this doesn't necessarily argue for lysis.

In vivo, new ribosome synthesis was not required for movement of adenovirus RNA to the cytoplasm, but the possibility exists that preexisting cytoplasmic ribosomes were necessary for RNA transport. However, in this in vitro reaction, nuclei were isolated from KB cells in which ribosome synthesis had been reduced 90% (Raskas, Thomas, & Green, 1970). Since the isolated nuclei released RNP that had a lower sedimentation rate and a lighter buoyant density (35S, 1.40g/cc), Raskas concluded that messenger transport in vitro doesn't require a ribosomal

subunit to "pull the RNA from the nucleus" (Raskas, 1971). This is in agreement with the previous finding (Raskas & Okubo, 1971) that adenovirus RNA which had recently emerged from nuclei in vivo does not co-sediment with the 45S ribosomal subunits.

Previous reports of complexes of mRNA and protein were often questioned because these complexes were isolated from total cytoplasmic extracts in which artifactual protein-RNA complexes can form during cellular fractionation (Girard & Baltimore, 1966; Baltimore & Huang, 1970). However, due to the absence of cytosol in this system (and other previously described systems), it is implied that the proteins attached to the RNA most likely originated in the nuclear preparation. Spirin and co-workers (Spirin, Belitsina, & Aitkhozhin, 1964) suggested that eukaryotic cells may contain proteins that function in mRNA transport, as have other investigators. Therefore, results in this system may be important in this regard.

Another system studied was that of Ishikawa and co-workers (Ishikawa, Kuroda, & Ogata, 1969; Ishikawa, Kuroda, & Ogata, 1970; etc.). Basically, this system has shown an ATP-dependent release of RNA. The incubation medium does not include cytosol preparations. The characteristics of RNA release were similar to those found in other systems.

It was shown that isolated rat liver nuclei released a rapidly labelled RNA in the presence of ATP (Ishikawa, Kuroda, & Ogata, 1969). A 45S component contained most of the rapidly labelled RNA, and this was shown to consist of RNA and protein in a ratio of 0.18:1. The RNA of the 45S component was shown to contain mRNA as a major component.

The protein moiety of the 45S ribonucleoprotein complex released in the presence of ATP was investigated (Ishikawa, Kuroda, & Ogata, 1970).

Polyacrylamide gel electrophoresis showed that the 45S component contained proteins which were less basic than ribosomal proteins and histones. The amino acid composition of the protein moiety was examined and the content of basic amino acids was found to be one-half the content of basic amino acids found in ribosomal subunits.

In another experiment, cells were labelled in vivo with ^{14}C-labelled algal protein hydrolysate (Ishikawa, Kuroda, & Ogata, 1970). Experiments with this labelled protein showed that the specific activity of the released fraction reached a value twice as high as that of the total nuclear proteins after one hour. It was also shown that the specific activity of the 45S component was lower than that of the 4S component. Ishikawa and co-workers concluded that the 45S component was an intermediate form of transport for mRNA, or at least that it had messenger-like properties (Ishikawa, Kuroda, Ueki, & Ogata, 1970). This component was found to contain specific carrier proteins which are less basic than ribosomal structural proteins, and have a high metabolic rate. However, as to the protein moiety, which has been designated as an "informofer" by Samarina and co-workers (Samarina, Lukanidin, Molnar, & Georgiev, 1968), little other evidence is available as to its chemical and metabolic properties.

Smuckler and co-workers (Smuckler & Koplitz, 1974; Smuckler & Koplitz, 1973; Shearer & Smuckler, 1972; Smuckler & Koplitz, 1976) developed a system with which to study RNA transport from isolated nuclei. They used rat liver nuclei isolated from normal rats and also treated rats with thioacetamide, a potent hepatocarcinogen (Fitzhugh & Nelson, 1948). The most interesting aspect of this system was that changes in the release of RNA with administration of the carcinogen could be

followed. It was shown that there were significant increases in RNA synthesis in many chemically induced cancers (Wu & Smuckler, 1971) and an altered capacity in these chemically induced cancers to restrict specific RNA species to an intranuclear location. In other words, these nuclei became "leaky", and RNA normally found only in the nucleus appeared in the cytoplasm (Smuckler & Koplitz, 1973; Shearer & Smuckler, 1971; Shearer & Smuckler, 1972). This effect was not due to toxic effects of the carcinogens used, as demonstrated by the use of analogues (Sheaere & Smuckler, 1971; Shearer & Smuckler, 1972).

The effects of thioacetamide were an enhanced energy-independent release. Rats were given an injection in vivo of ^{14}C-orotic acid, 45 minutes prior to sacrifice. The livers of the animals were removed, and the nuclei prepared by standard techniques. The isolated nuclei were suspended in a buffered system containing sucrose and ribonuclease inhibitor, isolated from a RNAase-cellulose column. No cytosol was used in this system. The concentration of the nuclear suspension was reported as 8.8mg/ml. When ATP was added to the incubation mixture, it was added at a concentration of 7.5mM. Incubations were carried out at 20C for 20 minutes (or up to 40 minutes). The in vitro effect of thioacetamide was also examined at 125 micrograms/ml, in addition to in vivo treatment.

The nuclei were shown to release approximately 10% of their RNA in 20 minutes at 20C in the absence of ATP. In the presence of ATP, they released approximately 20%. The released product sedimented at 35-45S.

The effects of AMP, ADP, GTP, UTP, CTP, pyrophosphate, EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, and 0.1% sodium deoxycholate treatments were examined. It was found that other triphosphates, when added to the incubation mixture, stimulated RNP release almost as efficiently as ATP

in control nuclei. AMP did not enhance release, although ADP in high concentration did. The properties of suspensions treated with NP-40 and TX-100 were found to be altered, although the release showed no differences when compared to controls. A premixing experiment was carried out with ATP and magnesium, in which EDTA and pyrophosphate were added to the incubation mixtures. Decreased magnesium concentrations due to chelation by ATP, EDTA, or pyrophosphate were associated with nuclear destruction.

The outer nuclear membrane appeared at least partially intact after isolation (Smuckler & Koplitz, 1974). Prominent morphological changes in thioacetamide-treated nuclei seemed to be restricted to the nucleolus when preparations were fixed in osmium tetroxide. After thioacetamide treatment, the nuclei appeared larger, the nucleoli were particularly prominent, and there was an accentuation of the nuclear-associated chromatin.

After administration of thioacetamide, there was a decrease in the quantity of RNP released in the presence of ATP. The decreased energy-dependent portion of the release was "compensated" for by an increased energy-independent release of RNP, in treated animals. It was concluded that a metabolic conversion of the carcinogen was necessary to achieve the altered release, since there existed a lag period prior to the appearance of the energy-independent release following in vivo treatment. The altered transport pattern was found to correspond to the development of tumors in treated animals (Smuckler & Koplitz, 1974).

Three weeks after the initiation of thioacetamide treatment, isolated nuclei showed a 12% release of RNA without added ATP, and a 14% release with added ATP. After 6 weeks, there was a 14% release of RNA without ATP added, and a 16% release with ATP added. Thus, a change in release

characteristics could be identified in animals that were fed thioacetamide long enough to produce liver tumors.

The RNP particles released from control nuclei and thioacetamide-treated nuclei, in both the presence and absence of ATP, showed identical characteristics on sucrose gradients. Similar ratios of absorbance at 260nm to absorbance at 280nm (A260/A280) were found (Smuckler & Koplitz, 1974). The RNA isolated from the major released component (35-45S) was similar when compared from the different systems, and consisted of 20-30S RNA with an irregular background.

The ATP-treated nuclei from thioacetamide-treated and control animals appeared similar ultrastructurally, and there was an enhanced aggregation of nuclear materials. Clumps of chromatin appeared to be randomly distributed throughout the nucleus, and the intervening spaces were relatively substructure-free. This was reported to resemble nuclear clumping after cell injury, and frequent clumping was reported about the nucleoli.

The nuclei were assayed for altered ATP concentrations, to ascertain whether altered release was related to changes in preexisting ATP concentrations in the isolated nuclei. Attempts were made to assay for nuclear ATP concentration prior to incubation, and ATP concentration dependence of release was examined (Smuckler & Koplitz, 1974).

The concentration of ATP in the quantity of nuclei used in their experiments could not be identified with thin-layer chromatography, which implied that the concentration of ATP added to the incubation medium by nuclei was less than 1 micromolar.

A direct assay for ATP concentration was performed in isolated nuclei using a luciferase assay. The results indicated that the thioacetamide-treated liver nuclei contained more ATP (either 2x or 1.5x,

depending on the treatment). The effective quantity of intranuclear ATP was 4.2 micromolar, a value three orders of magnitude less than that added as exogenous ATP to the assay medium. What these results show is obscure, since the overall concentration is not necessarily the crucial factor in local considerations.

The poly A content of the released RNA in this system has been examined (Smuckler & Koplitz, 1976). Approximately 17.5% poly A+ content was reported for control nuclei without ATP, with 19.7% with ATP being reported. Similarly, in thioacetamide-treated nuclei, 20.5% of the released RNA was poly A+ when no ATP was present in the incubation mixture, whereas 25.1% was poly A+ when ATP was present in the mixture with thioacetamide-treated nuclei. The poly A+ RNA was shown to consist predominantly of 9-16S species. A major component was the 9S RNA both in control and in treated animals released with and without ATP.

B. Genesis of Released Material

A great deal of work has gone into the identification of nuclear mRNA precursors and the processing of such precursors which occurs prior to (or during) transport. One such study, using a cellular slime mold, was undertaken by Firtel and Lodish (Firtel & Lodish, 1973).

It was shown that mRNA contained a sequence of about 100 adenylic acid residues at the 3' end (Firtel & Lodish, 1973). The slime mold nuclei, labelled under a variety of conditions, did not contain material which was directly analogous to the large heterogeneous RNA found in mammalian cells. It was reported that the majority of pulse-labelled nuclear RNA, that is not identified as rRNA precursor, contained at least one sequence of poly A. This RNA was found to have an average

molecular weight of about 500,000 daltons (1500 nucleotides), and appeared to be only about 20% larger than the cytoplasmic mRNA found in this system.

Pulse-labelling experiments showed that the nuclear poly A containing RNA was a precursor of mRNA (Firtel & Lodish, 1973). It had been shown that over 90% of the mRNA sequences are transcribed from non-reiterated DNA, and Firtel and Lodish showed that about 25% of the nuclear poly A containing RNA was transcribed from reiterated sequences (C_0t value of 10) and only 75% was transcribed from single-copy DNA (C_0t value of 10^3).

Evidence was presented that a large fraction of the nuclear poly A containing RNA contained, at the 5' end, a sequence of about 300 nucleotides that was transcribed from repetitive DNA and which was lost before transport of mRNA into the cytoplasm. In all, it was found that of the labelled nuclear RNA sedimenting between 6-18S, 60% was poly A containing.

Hybridization of both poly U and poly A to nuclear DNA indicated that approximately 0.33% of the nuclear DNA is complementary to either homopolymer. However, it was shown via depurination of ^{3}H-thymidine labelled DNA that no poly dT isotiches of greater than 25 nucleotides 2343 present. This was taken to suggest that the long poly A sequences were added post-transcriptionally, as has been shown to be the case in mammalian cell systems (Kates & Beeson, 1970B; Jelinek et al., 1973; Greenberg & Perry, 1972; Weinberg, 1973). In mammalian cells, as well as in the cells of this cellular slime mold, it was found that most of the mRNA molecules have sedimentation coefficients between 8S and 30S and contain poly A sequences at the 3' end (Jelinek et al., 1973; Weinberg, 1973; Lee, Mendecki, & Brawerman, 1971).

The situation is more complicated in the many other eukaryotic systems studied due to the diversity of these systems. An excellent review on nuclear RNA metabolism is available by Weinberg (Weinberg, 1973), and an excellent review on eukaryotic messenger RNA is available by Brawerman (Brawerman, 1974), and the subject will not be covered in detail here.

C. Characterization of Ribonucleoprotein Particles Released from Nuclei

A great deal of work has gone into the characterization of the ribonucleoproteins released from nuclei (for instance, Lukanidin, Georgiev, & Williamson, 1973). It was shown that nuclear D-RNA (HnRNA) was incorporated into ribonucleoprotein particles which were complexes of D-RNA with macroglobular protein particles, also referred to as informofers (Samarina, Lukanidin, Molnar, & Georgiev, 1968). Basically, a cell has two classes of mRNA forms, one being contained in free informosomes (Spirin, 1969) and the other form consisting of RNP particles bound to polysomes (Perry & Kelley, 1968). It has also been established that part of the D-RNA bound to the informofers in the nucleus is transported to the polysomes in the cytoplasm, and this portion is believed to correspond to the "true" mRNA (Drews, 1969). Apparently, mRNA is transported as informofers to the cytoplasm, although the intermediate form of mRNA during transport is not clear using this designation.

The question of whether informofers are actually involved in the transport of mRNA through the nuclear envelope was examined. This question was examined indirectly by comparing the proteins found in the nuclear complexes to those found in the cytoplasmic complexes. It was shown (Krichevskaya & Georgiev, 1973; Samarina, Lukanidin, Molnar, &

Georgiev, 1968) that nuclear particles contained only one electrophoretically homogeneous polypeptide component with a molecular weight of 40,000 daltons, for which the term informatin is used. Each informofer particle apparently contains several tens of informatin molecules. In the absence of mercaptoethanol several additional components were seen, apparently through the formation of S-S bonds. Studies have also shown that the polysomes contained proteins similar in electrophoretic mobility to informofer proteins (Olsnes, 1970; Scheweiger & Hannig, 1970; Lissitsky, Poiree, Cartouzou, & Grogorie, 1970). However, in these studies, there were complications due to contamination of the polysomes with nuclear material.

A study was undertaken (Lukanidin, Georgiev, & Williamson, 1973) to characterize the mRNP of polysomes of rabbit reticulocytes. This system contains no nuclei and the reticulocyte polysomes are very homogeneous. After the polysomes were dissociated with EDTA, mRNA was released in the form of homogeneous mRNP with a sedimentation coefficient of 14S. It was reported that the proteins of the 14S mRNP differed in their electrophoretic mobility from informatin.

To contrast the situation, the nuclear complexes containing D-RNA are found to contain the same electrophoretically homogeneous protein called informatin. In contrast to these nuclear particles, the mRNP's of reticulocyte polysomes are heterogeneous with respect to protein composition. In the reticulocyte polysomal mRNP, there are a large number of proteins, from which two basic ones have been isolated via DEAE-cellulose chromatography (Lukanidin, Georgiev, & Williamson, 1973).

One of these proteins has been shown to have similar (but not identical) electrophoretic behavior when compared to informatin. It

was hypothesized that when mRNA is transported to the polysomes from the nucleus, it is "reclothed" by replacement of the protein component.

The informofers released in Ehrlich's ascites carcinoma cells were also examined (Lukanidin, Kul'Guskii, et al., 1973). Previous studies have shown that nuclear complexes containing D-RNA were polysome-like particles in which RNA was bound to a series of globular protein particles called informofers as aforementioned. It was postulated that the D-RNA was located at the surface of the informofer. This was specifically indicated by the sensitivity of the nuclear particle RNA to RNAase (Samarina, Krichevskaya, et al., 1967) and by data which involved the attachment of free D-RNA to nuclear particles (Samarina, Krichevskaya, & Georgiev, 1967). Other data were obtained which confirmed this proposed model for the structure of the nuclear particles (Samarina, Lukanidin, & Georgiev, 1968).

A weak link in this postulated structure is that it had not proven possible to obtain informofers free of RNA. The treatment with RNAase was accompanied by the aggregation of the particles concerned. The study of the particles after removal of the RNA was further complicated by the fact that their protein component was labelled slowly in vivo, and it had been impossible to obtain highly radioactive protein-labelled particles.

However, in a study by Lukanidin and co-workers (Lukanidin & Kul'Guskii, 1973), protein-labelled particles were obtained by prolonged incubation of the cells with labelled amino acids or by an in vitro iodination technique. Through the use of these protein-labelled particles, it was possible to find conditions that provided complete dissociation of the RNA without destroying particle organization. Treatment with concentrated salt solutions enabled the authors to separate all of the D-RNA

from informofers without destroying them. The informofers were found to remain in solution in the form of globular macromolecular aggregates. The free informofers were homogeneous and were found to sediment at approximately the same rate as the original 30S nuclear particles. Their buoyant density was 1.34g/cc.

Electron microscopy was performed on these informofers (Lukanidin, Kul'Guskii, et al., 1973). Free informofers appeared as globules with a diameter of approximately 20nm. It was found that the addition of exogenous D-RNA to the informofers and the removal of the dissociating agent led to the restoration of RNP particles. Single particles or multiparticle aggregates were formed depending upon the molecular weight of the RNA added. Treatment with urea led to the dissociation of the informofers into homogeneous informatin polypeptide chains.

Much work has also gone into characterizing the mammalian proteins which show an affinity to polynucleotides (Schweiger & Mazur, 1974). In one study, Sepharose-poly A was prepared using the procedure of Wagner et al. (Wagner, Bugianesi, & Shen, 1971). This study showed that a small fraction of the soluble proteins of eukaryotic cells became associated with the insolubilized polynucleotides in vitro, and thus may be isolated by affinity chromatography on Sepharose-poly A or Sepharose-poly U columns. The results presented (Schweiger & Mazur, 1974) indicate that the specificity of at least some of the isolated proteins is not strictly directed to one of the polynucleotides used, but includes other homopolymers and presumably also ribonucleic acids.

Of the two groups of binding proteins which have been separated on the basis of different affinities to poly A columns, the high affinity proteins would be expected to comprise factors which specifically bind

to the poly A segment of the mRNA (Schweiger & Mazur, 1974). One stumbling block to the identity of these particles is that they are characterized only by their molecular weights in SDS-gel electrophoresis. It hasn't been shown how such proteins are related to other proteins that have been found associated with non-ribosomal RNA.

Of the low affinity cytosol proteins isolated by Sepharose-poly A columns, the one component isolated with a molecular weight of 43,000 daltons is (so far as has been characterized) identical to the "basic RNA-binding factor" characterized earlier (Schweiger & Spitzauer, 1972). The other major band appeared as a double band on gels, with a molecular weight of 45,000 daltons and was more acidic. These two proteins with closely related molecular weights also had very similar amino acid compositions. This was taken as evidence that certain binding proteins exist in different forms which are revealed as double bands on SDS-gel electrophoresis.

From the nucleosol preparations, the high affinity protein component with a molecular weight of 42,000 daltons was identical with one of the major protein species of the 30S RNP particles. Among the cytosol proteins was a component with a molecular weight of 77,000 daltons. Its molecular weight and the fact that it was eluted from the Sepharose-poly A column with the high affinity fraction suggested that this component was closely related to (or identical with) a protein which specifically binds to the poly A segment of mammalian polysomal mRNA (Blobel, 1973).

When disc gel electrophoresis patterns of the high affinity components (eluted with 1.0M KCl) from the nucleosol and cytosol fractions were compared, striking similarities were observed. The presence of

proteins in the two separate compartments of the cell was proposed to be of physiological significance and indicative of the proteins' possible role in the mechanism of nucleocytoplasmic transport of RNA.

Similarly, a protein of molecular weight 78,000 daltons was found bound to the polyadenylate region of eukaryotic messenger RNA (Blobel, 1973). Two distinct proteins were found tightly bound to heterogeneous mRNA associated with polysomes in mouse L-cells and in rat hepatocytes. The molecular weight of the larger of these was 78,000 daltons which was identical to that of the protein found associated with rabbit globin mRNA. Globin mRNA released from rabbit reticulocyte polysomes by puromycin-KCl (Blobel, 1971) was found to contain two proteins, one of molecular weight 78,000 daltons and the other of molecular weight 52,000 daltons (Blobel, 1972). These proteins appear to be tightly bound to the globin mRNA since they remained bound at 500mM KCl in the absence of magnesium, conditions in which substantial numbers of ribosomal proteins are dissociated from both the large and small ribosomal subunits (Blobel, 1972).

There were only two proteins in these mRNA-protein complexes (Blobel, 1973). This was taken as evidence to suggest that the association is specific and not an artifact caused by low salt concentrations (Baltimore & Huang, 1970). The proteins in this study were found to be bound to the poly A region of mRNA following digestion with RNAase. The molecular weight of the smaller protein component, 52,000 daltons, associated with mRNA from mouse hepatic and L-cell polysomes, was very similar to that found in globin messenger ribonucleoprotein (Blobel, 1972).

In a similar finding, two proteins were found to be bound to most species of polysomal RNA (Bryan & Hayashi, 1973). There exists some

evidence that at least one of these proteins is required for mRNA binding to the small ribosomal subunit (Lebleu, Marbaix, Huez, Timmerman, Burny, & Chantrenne, 1971; Ilan & Ilan, 1973).

Messenger RNP has been separated from ribosomal subunits of dissociated chick embryo cerebral polysomes. The mRNP was isolated and divided into fractions of 10-30S, 50S, and 70-200S. Electrophoresis in SDS-polyacrylamide gels revealed that two proteins of molecular weights 48,400 and 78,500 daltons were common to all three fractions. To study the protein bound to mRNA of the polysomes, it was necessary to wash off the large numbers of high molecular weight proteins which adhered to the polysomes during the standard isolation in hypertonic sucrose.

The phosphorylation of messenger RNA-bound proteins in HeLa cells has been studied (Auerbach & Pederson, 1975). Of two bands found upon electrophoresis of messenger-bound proteins (73,000 and 50,000 daltons), it was found that only the 50,000 dalton band was phosphorylated. The significance of this is unknown, although it is a possible control point in the regulation of transport.

Many proteins have also been found in association with the heterogeneous nuclear RNA (HnRNA) in eukaryotic cells (Pederson, 1974). It was shown that HnRNP particles have similar sedimentation velocities (40-300S) when isolated under physiological ionic conditions from a wide variety of mammalian cells. However, electrophoresis revealed a very distinct pattern of HnRNP proteins for each cell type. It was proposed by Pederson that the cell specificity reflects a situation in which HnRNA molecules that differ in nucleotide sequence are complexed with different sets of proteins, so that the resulting HnRNP particles are biochemically distinct at each genetic locus. Thus, binding proteins could allow for control of transport of HnRNP particles (or mRNP particles).

A comparison of the properties of cytoplasmic poly A containing RNA from polysomal and non-polysomal fractions from murine myeloma cells has also been made (MacLeod, 1975). It was found that approximately 40% of the cytoplasmic poly A containing RNA in exponentially growing myeloma cells was not found associated with polysomes. The RNA fraction was similar in size and poly A content to that of polysomal RNA.

Pulse-chase experiments were conducted in which cells were labelled for one generation time with ^{14}C-uridine, and then divided into 6 aliquots and labelled for different times up to 2 hours. A polysome gradient was then used to look at the different fractions and it was found that some of the non-polysomal poly A containing RNA was transferred into polysomes. The kinetics of accumulation of radioactive poly A containing RNA in cytoplasmic fractions was not consistent with a precursor-product relationship between non-polysomal and polysomal poly A containing RNA.

Failure to find sedimentation coefficients of 35S or 70S suggested that this RNA fraction was not contaminated with RNA species derived from endogenous C-type virus particles found in this system (Fan & Baltimore, 1973; Ihle, Lee, & Kenney, 1974). Mitochondria are known to produce a number of species of poly A containing RNA (Hirsch, Spradling, & Penman, 1974), but this was shown to account for, at maximum, less than 5% of the non-polysomal poly A containing RNA.

Scherrer and co-workers (Scherrer, Spohr, Granboulan, Morel, Grosclaude, & Chezzi, 1970) have suggested the designation messenger-like RNA (mlRNA) for the non-polysomal fractions with many other characteristics similar to polysomal mRNA. Two models are proposed to explain the regulation of mRNA to polysomes. In one model, a precursor-product relationship holds, but mRNA can exchange with mlRNA. In the other model, part

of the mlRNA is a precursor of mRNA, while the rest is inactive (not able to enter polysomes). The close similarity between the non-polysomal and polysomal poly A containing RNA in terms of size distribution, poly A content, and kinetics of appearance in the cytoplasm was taken as evidence that non-polysomal poly A containing RNA was inactive mRNA. That is, it is mRNA which is unable to initiate protein synthesis either temporarily (the first model) or permanently (the second model). Thus, the common feature of these models is the existence of translational control in eukaryotic cells.

MATERIALS AND METHODS

Cell Maintenance and Isotopic Labelling

MOPC-21 mouse myeloma tissue culture cells were maintained in Dulbecco's modified medium (Gibco) supplemented with 10% fetal calf serum. Release of both RNA and DNA was monitored. The MOPC-21 cells were labelled overnight with 2-^{14}C-thymidine (New England Nuclear, 54.7mCi/mmole, 0.05 microCi/ml), and labelled for 30 minutes just prior to isolation with 5,6-^{3}H-uridine (Amersham/Searle, 44Ci/mmole, 4.0 microCi/ml, 0.2 femtomoles/cell). In logarithmic cell releases, cells were labelled with thymidine at concentrations of approximately 3-4x10^{5} cells/ml. In stationary phase releases, cells were labelled with thymidine at concentrations of approximately 6x10^{5} cells/ml.

Isolation of Nuclei

Isotopically labelled cells were harvested from culture by centrifugation at 4C. The cells were washed with transport buffer (3mM $MgCl_2$, 8mM KCl, 4mM NH_4Cl, 10mM Tris-HCl, pH 7.6, 10mM 2-mercaptoethanol, 250mM sucrose), and resuspended in hypotonic reticulocyte standard buffer (RSB; 10mM NaCl, 3mM $MgCl_2$, 10mM Tris-HCl, pH 7.4). The cells were allowed to swell on ice for 10 minutes, and were lysed via Dounce homogenization. The nuclei were washed twice with transport buffer and collected by centrifugation. Cells were checked for lysis with 0.1% crystal violet solution (2.1% citric acid). Isolated nuclei absorb the dye, whereas intact cells do not.

Preparation of Logarithmic Phase Cytosol

Dialyzed cytosol was prepared from the 105,000g supernatant of logarithmically growing cells following Dounce homogenization. The 105,000g supernatant was dialyzed overnight against transport buffer, and then frozen at -80C in aliquots. All manipulations were performed at 0C.

Preparation of Stationary Phase Cytosol

This was prepared from the 105,000g supernatant of stationary phase MOPC-21 tissue culture cells following Dounce homogenization, using the procedure as described above.

Assay Procedure

Reaction mixtures of 4ml were prepared containing approximately $2x10^7$ nuclei, 1.2ml dialyzed cytosol (1.8-2.0mg progein as determined by the method of Lowry), which is equivalent to the cytoplasmic cytosol protein from $2x10^7$ cells, and transport buffer.

Each reaction contained approximately $4x10^6$cpm of TCA-precipitable ^{3}H-RNA. Release of isotopically labelled RNA from isolated nuclei was monitored for 30 minutes at various temperatures. Samples of 0.5ml were taken as follows:

1. An SDS-treated sample at 0 time, for total nuclear counts
2. A 0-time sample for ^{3}H-RNA in the supernatant
3. A 5-minute sample for ^{3}H-RNA in the supernatant
4. A 10-minute sample for ^{3}H-RNA in the supernatant
5. A 20-minute sample for ^{3}H-RNA in the supernatant
6. A 30-minute sample for ^{3}H-RNA in the supernatant

Nuclei were pelleted after release by centrifugation at 2500-3000g for 10 minutes, in a Sorvall RC2-B centrifuge. The post nuclear supernatant was withdrawn after addition of 0.5ml of transport buffer. Fifty

percent TCA was added to yield a final concentration of 10% TCA. The TCA precipitation was carried out on ice for 10 minutes, with bovine serum albumin (BSA) added to aid precipitation. The TCA precipitate was collected on a Whatman GF/C filter. The filters were dried, placed in toluene-omnifluor, and counted by liquid scintillation. Percent release was calculated as the total TCA-precipitable radioactivity which appeared in the supernatant, with the 0-time supernatant radioactivity subtracted (corrected).

Assays Performed

Release of TCA-precipitable RNA was assayed under the following conditions:

1. Release from logarithmically growing cells in logarithmic phase cytosol - monitored at 0C, 10C, 20C, 25C, 30C, and 37C
2. Release from stationary phase culture cells (over $1x10^6$ cells/ml) in logarithmic phase cytosol at 30C
3. Release from logarithmic phase cells in stationary phase cytosol at 30C
4. Release from stationary phase cells in stationary phase cytosol at 30C
5. Release from logarithmic phase cells, isolated by Dounce homogenization, treated with 0.5% Triton X-100 or with 0.5% Nonidet P-40 detergent, in logarithmic phase cytosol at 37C

RNA Isolation Procedure

Five percent SDS was added to the supernatant to a final concentration of 0.5%, with all subsequent centrifugation done at room temperature. To this an equal volume of phenol (saturated with buffer - 0.01M acetate, pH 6.0, 0.1M NaCl, 0.001M Na_2EDTA) was added. This mixture was vortexed for 3-5 minutes. An equal volume of $ChCl_3$ was added, and again the mixture was vortexed for 3-5 minutes. This mixture was centrifuged at 2500 rpm for

5-10 minutes. The organic phase was removed, and the $CHCl_3$ addition was repeated until a thin interface was obtained. The aqueous phase was removed, 2 volumes of 95% ethanol were added, and precipitation was allowed overnight in the freezer. For precipitation of RNA (and nucleoprotein particles) from very dilute solutions, the method of Dessev and Grancharov (Dessev & Grancharov, 1973) was employed, which employs a magnesium phosphate gel-like precipitation.

RNP Isolation Procedure

The material released after 30-minute incubation at 37C was concentrated by the method of Dessev and Grancharov (Dessev & Grancharov, 1973), and resuspended in buffer (10mM Tris-HCl, pH 7.4, 10mM NaCl, 10mM EDTA). This mixture was placed on 10-30% sucrose gradients and centrifuged at 50,000 rpm at 4C for 2 hours in a Beckman SW50.1 rotor.

Sedimentation Profiles

Sedimentation profiles were developed on linear sucrose gradients at 4C or 20C as noted, at the stated rpm and concentrations of sucrose, in a Spinco SW50.1 rotor in a Beckman L5-50 ultracentrifuge. The gradients were fractionated and counted in liquid scintillant.

Preparation of Nuclear RNA

Nuclei were labelled and isolated as previously described. The nuclei were washed with RSB, and resuspended at approximately $4x10^7$ nuclei/ml in 2ml RSB and 0.3ml of a mixed detergent solution (2 volumes 10% Nonidet P-40 or Triton X-100, and 1 volume of 10% deoxycholate). This mixture was vortexed for 5 seconds, and the nuclei were pelleted at 2000 rpm for 2 minutes. The supernatant was discarded, and the pellet resuspended in 2-3ml of HSB (High salt buffer, 0.5M NaCl, 0.05 $MgCl_2$,

0.01M Tris, pH 7.4). DNAase was added in a 40-microgram quantity to this mixture, and the mixture was incubated for 15-20 minutes at 37C. EDTA was then added to 30mM (pH 7.4), and SDS was added to 0.5%. An equal volume of phenol was added, the mixture incubated at 55C for 1 minute, and then vortexed for 10 seconds. This step was repeated, the same amount of chloroform was added, the mixture incubated for 1 minute at 55C, vortexed, and centrifuged. The organic phase was removed, and the aqueous solution was re-extracted with 2 volumes of chloroform, warmed to 55C, shaken, and centrifuged. The aqueous phase was removed, and precipitated with ethanol overnight. This mixture was pelleted at 10,000 rpm for 10 minutes, and resuspended in sterile, distilled H_2O (or SDS buffer).

Poly A Content Analysis

Poly A content analysis was performed using the supernatant obtained after 30-minute incubation at 30C (or with purified released RNA). The procedure was as follows.

Oligo-dT columns were used for poly A content analysis. The procedure used was essentially that of Aviv and Leder (Aviv & Leder, 1972), using application buffer consisting of 0.12M NaCl, 10mM Tris-HCl, pH 7.4, 0.2mM $MgCl_2$, and 0.5% SDS. The column was initially washed thoroughly with application buffer. Five percent SDS was added to the sample to yield a final concentration of 0.5% SDS. The sample was then mixed with equilibration buffer (4x, to yield the above concentrations). The sample was placed on the oligo-dT cellulose column. The poly A- fraction was collected with five 1-ml washes of the application buffer. The poly A^+ fraction (the bound material) was eluted with five 1-ml washes of buffer (10mM Tris-HCl, pH 7.4, 0.2mM $MgCl_2$, 0.5% SDS). The column was again rinsed with five 1-ml washes of the buffer. The column was periodically

stripped with 0.1N NaOH, and rinsed thoroughly with buffer. The collected fractions were made 10% TCA, BSA was added to enhance precipitation, and precipitation was allowed at 0C for 10 minutes. The precipitates were collected on Whatman GF/C filters, dried, and counted via liquid scintillation. An alternative procedure was sometimes employed using formamide. In this procedure, the sample was mixed with 4x equilibration buffer to 200mM NaCl, 50mM Tris-HCl, pH 7.4, 10mM EDTA, 3mM $MgCl_2$. The sample was applied to the column, and the A- fraction collected with this buffer. The A+ fraction was collected with the equilibration buffer containing 50% formamide. Prior to precipitation, the formamide was diluted to 8% to allow reassociation of proteins.

Phospholipid Extraction and Analysis

Lipids were extracted according to the procedure of Folch and coworkers (Folch, Lees, & Stanley, 1957). Phospholipid analysis was performed according to the procedure of Bartlett (Bartlett, 1959).

Preparation of Samples for Transmission Electron Microscopy

Samples of nuclei taken before and during transport assays (isolation methods noted) were fixed at 0C for 3-4 hours in potassium dichromate and acrolein (0.33% potassium dichromate, 2% acrolein, 2% sucrose, in H_2O, pH 7.4) (procedure according to Gary Hooper, Director, Electron Optics Laboratory, MSU). Fixed nuclei were dehydrated in a graded series of ethanol-water solutions (25%, 50%, 75%, 95%, and 100% ethanol), and embedded in Spurr's resin. Sections were cut on a Porter-Blum MT-2 ultramicrotome with a diamond knife. The sections were stained for 30 minutes with uranyl acetate in methanol-ethanol, and for 5 minutes in lead citrate, and visualized in a Phillips 300 electron microscope.

Preparation of Samples for Scanning Electron Microscopy

Nuclei were isolated (as noted) and fixed in 5% glutaraldehyde in phosphate buffer at 0C for 2 hours. The glutaraldehyde was removed, the pellet of nuclei rinsed for 20 minutes in 0.1M phosphate buffer, and the nuclei were post-fixed in 1% OsO_4 in 0.1M phosphate buffer at 0C for 2 hours. The specimen was rinsed in phosphate buffer for 20 minutes. The nuclei were dehydrated in a graded series of ethanol-water solutions (in increments of 10%), for 5 minutes in each solution. The specimen was then placed in a critical point dryer. Liquid CO_2 was flushed into the chamber numerous times, the chamber was heated and exhausted, and the dry tissue removed. The nuclei were then viewed in an ISI Super Mini-SEM.

As an alternative procedure, the nuclei were fixed and dehydrated as above. The nuclei pellet was frozen in liquid nitrogen, freeze-fractured with a razor blade, critical point dried, and viewed.

Procedure for Histochemical Localization of ATPase Activity for Transmission Electron Microscopy

Approximately $5x10^7$ cells were swelled in RSB for 10 minutes at 0C. The cells were lysed by Dounce homogenization. The suspension was centrifuged at 3000g for 10 minutes, pelleting the isolated nuclei. The pellet of nuclei was resuspended in transport buffer with sucrose, and centrifuged as above, and then resuspended again and centrifuged.

The pellet of nuclei was resuspended in 3% calcium formol and fixed for 5 minutes at 0C (calcium formol contained 1.4% calcium chloride, 10ml 37.4% formaldehyde, 100ml double-distilled H_2O). Transport buffer with sucrose was added to dilute the fixative, and the nuclei were centrifuged as above. The nuclei were resuspended in 2ml of transport buffer with sucrose, but not containing the usual 8mM KCl. This was divided into

two fractions. The ATP fraction was mixed with 1ml of a solution containing 2mM ATP, 0.5mM $Pb(NO_3)_2$, 3mM $MgCl_2$, 0.01M Tris-HCl, pH 7.6. The -ATP fraction was mixed with 1ml of an identical solution, containing 2mM NaH_2PO_4 in place of ATP. These mixtures were incubated at 37C for 30 minutes. The nuclei were pelleted, and fixed in potassium dichromate acrolein as described above.

RESULTS

A typical example of nuclear structure in an intact MOPC-21 cell is shown in Figure 1. The diameter of the nucleus is found to be approximately 6.9 micrometers (6900nm). The double-nuclear membrane, treated as described, is clearly defined. The outer nuclear membrane is commonly lined with ribosomes and is in direct continuity (or contact) with the rough endoplasmic reticulum of the cell. Pore structures are obvious in the nuclear envelope, with chromatin sometimes forming a "mouth" away from the pore. The inner nuclear membrane appears to be in contact with the chromatin. This area contains the amorphous lamina described by other investigators, although the treatment and staining used here do not allow visualization of it. A fine fibrillar structure is obscured in such nuclei; it is obscured by the nuclear membrane and the chromatin. Some fibrils may be seen upon close observation, however. In the cytoplasm, mitochondria, endoplasmic reticulum, and Russell bodies are present in the cells, and virus particles are commonly present in large numbers. Such control cells were isolated from logarithmic phase cell cultures.

A comparison was made between control nuclei in intact cells and nuclei isolated via Dounce homogenization, to assess the effect of the isolation procedure on ultrastructure (Figure 2).

As is evident upon examination of Figure 2, the outer nuclear membrane is partially intact, although discontinuities are apparent. The diameter of such Dounce isolated nuclei is found to be 6.9 micrometers

Figure 1. Transmission electron micrograph of an intact MOPC-21 cell, maintained as specified in Materials and Methods.

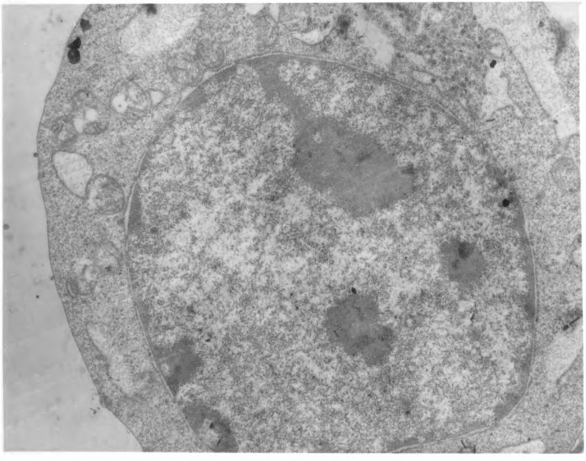


Figure 1.

Figure 2. Transmission electron micrograph of MOPC-21 nuclei isolated by Dounce homogenization, as described in Materials and Methods.

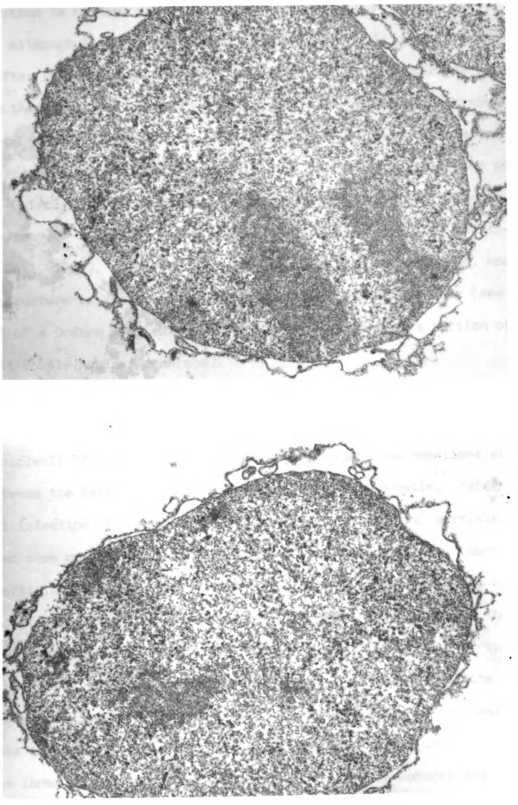


Figure 2.

(6900nm), identical with nuclear diameter in control cells. Cytoplasmic contamination is minimal after two washings in transport buffer and sucrose, although intranuclear detail remains unaltered. Pores remain intact after Dounce homogenization. The inner and outer membranes remain intact at the pore annuli, with breaks appearing in the outer membrane only at a distance from the pore complex. The inner and outer membranes seem to coalesce or merge at the pore complex, and appear attached to the pore annuli. Calculations from numerous micrographs show the pore diameter to be approximately 68nm, in good agreement with previous results (for instance, Gall, 1967). Ribosomes are numerous on the outer membrane, and a pore structure is shown. The fibrillar network is also visible (see Figure 3 of a Dounce homogenized nuclear membrane, showing a portion of an intact double nuclear membrane).

The nucleolar structure and heterochromatin structure are very similar in Dounce isolated and intact nuclei. Fine structural detail is apparent in the nucleoli of both types of nuclei, with connections sometimes evident between the heterochromatin and intranucleolar chromatin. Evidence of viral infection of the cells is apparent and intact viral particles are often seen on the outer nuclear membrane (see micrographs). Such virus particles have been found to have a diameter of 68nm and have been shown not to be a major factor in release experiments; the size of the released RNA is not identical with viral RNA, the buoyant density of the released particles is not the same as that of viral particles, and the release is unaffected by detergent treatment, which removes the nuclear membranes and essentially all of the virus particles with them.

The inner nuclear membrane is continuous in Dounce homogenized nuclei. The isolated nuclei appear identical whether viewed immediately

Figure 3. Transmission electron micrograph of nuclear membrane in a Dounce homogenized MOPC-21 nucleus, prepared and treated as in Materials and Methods.

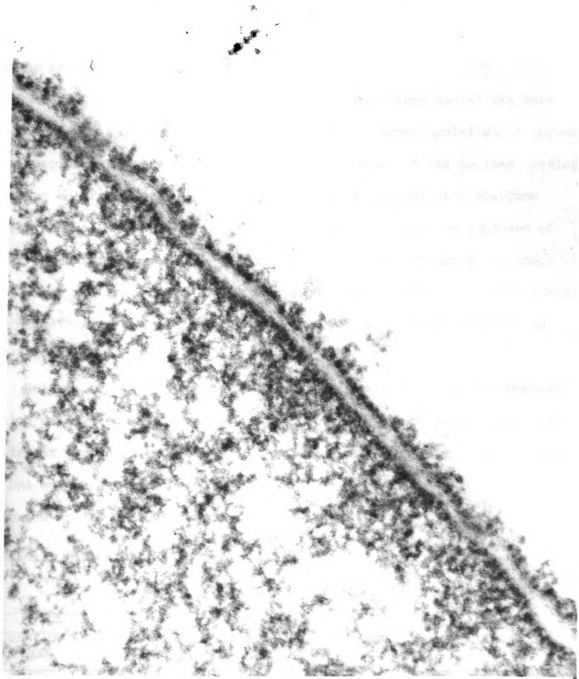


Figure 3.

after isolation, or after 45 minutes of incubation in transport buffer with sucrose, whether cytosol is present in the mixture or not. Thus, the stabilization factors reported by others (Schumm, McNamara, & Webb, 1973; Schumm & Webb, 1975) for stabilization of nuclei in noncancerous systems are not required in this system.

Scanning electron microscopy of Dounce homogenized nuclei was performed (Figure 4). Scanning electron microscopy shows nuclei which appear to be approximately round, with blebs or extensions of the nuclear envelope sticking out from the nucleus. Comparison with transmission electron micrographs maks it appear likely that these extensions are composed of outer nuclear membrane still attached to the nuclear envelope, although this outer membrane is not continuous around the periphery of the nucleus. The diameter was found to be approximately 6.7 micrometers (6700nm) to 6.9 micrometers (6900nm).

Release of ribonucleic acids from nuclei isolated by Dounce homogenization was tested under various conditions. Release of deoxyribonucleic acids was monitored as a control of nuclear condition, since essentially no DNA was released by intact nuclei. Table 1 lists the percentages released at the various temperature and time points.

TABLE 1. Percentage RNA released (with standard error of the mean) at various temperatures

INCUBATION PERIOD	TEMPERATURE OF INCUBATION				
	10C	20C	25C	30C	37C
5 minutes	0.59 ± 0.22	0.43 ± 0.28	0.28 ± 0.30	0.88 ± 0.14	4.69 ± 0.87
10 minutes	0.46 ± 0.16	0.97 ± 0.45	0.95 ± 0.68	2.40 ± 0.37	6.39 ± 0.92
20 minutes	0.70 ± 0.35	1.40 ± 0.61	2.63 ± 1.20	5.13 ± 1.00	6.97 ± 0.71
30 minutes	0.62 ± 0.41	3.79 ± 1.04	4.82 ± 1.75	6.26 ± 0.60	6.12 ± 1.42

Figure 4. Scanning electron micrograph of MOPC-21 nuclei isolated by Dounce homogenization, prepared and treated as described in Materials and Methods.

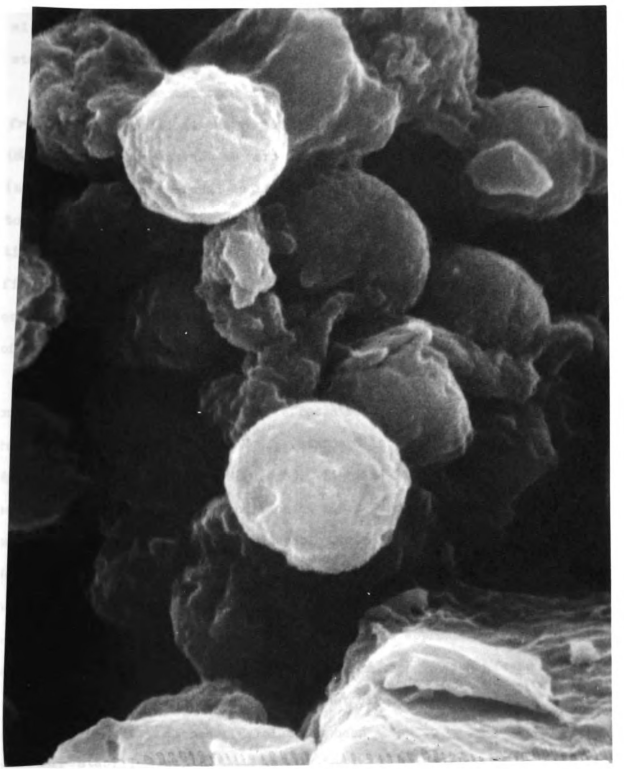


Figure 4.

This table shows release of TCA-precipitable RNA from logarithmic phase nuclei to logarithmic phase cytosol (assay procedure as described), at the temperatures as shown. Percentages transported are shown with the standard error of the mean for the individual measurements.

Figure 5 shows the time course of release of TCA-precipitable RNA from isolated nuclei at the various temperatures, as noted, corrected to 0% RNA in the supernatant at zero time. Typically, 1-2% of the total RNA (and DNA) counts are found in the supernatant at zero time, probably due to nuclear lysis, since approximately this proportion of the cell population would have the nuclear envelope dispersed. As can be seen from the figure, release proceeds rapidly at 37C, with a leveling off and a decrease in TCA-precipitable RNA occurring between 20 minutes and 30 minutes of incubation, probably due to degradation of released RNA by nucleases.

The release observed from isolated nuclei was found to be dependent on magnesium concentration. The effects of magnesium concentrations on release was tested with 3mM $MgCl_2$ (standard in transport buffer), 4mM $MgCl_2$, and 8mM $MgCl_2$ (see Figure 6). Eight millimolar $MgCl_2$ is often used as a control for nuclear condition at times. However, 8 millimolar $MgCl_2$ has been shown to cause a considerable shrinking of nuclei (Chatterjee & Weissbach, 1973). Upon light and electron microscopic examination, nuclei are observed to be smaller, and the chromatin is condensed, with loss of detail. The diameter of such nuclei is approximately 3.5 micrometers (3500nm), as compared to the 6.9 micrometers (6900nm) found in control nuclei. Calcium ion effects are not directly similar to the magnesium ion effects, although calcium has been shown to be necessary for nuclear stabilization in some cases (for instance, the rat liver nuclei system of Webb and co-workers). In all release systems tested to date,

Figure 5. Time course of release of TCA-precipitable RNA to the supernatant from logarithmic phase Dounce isolated MOPC-21 nuclei, assay as described in Materials and Methods, at temperatures as noted. 37C (●—●), 30C (★—★), 25C (○—○), 20C (✿—✿), and 10C (□—□).

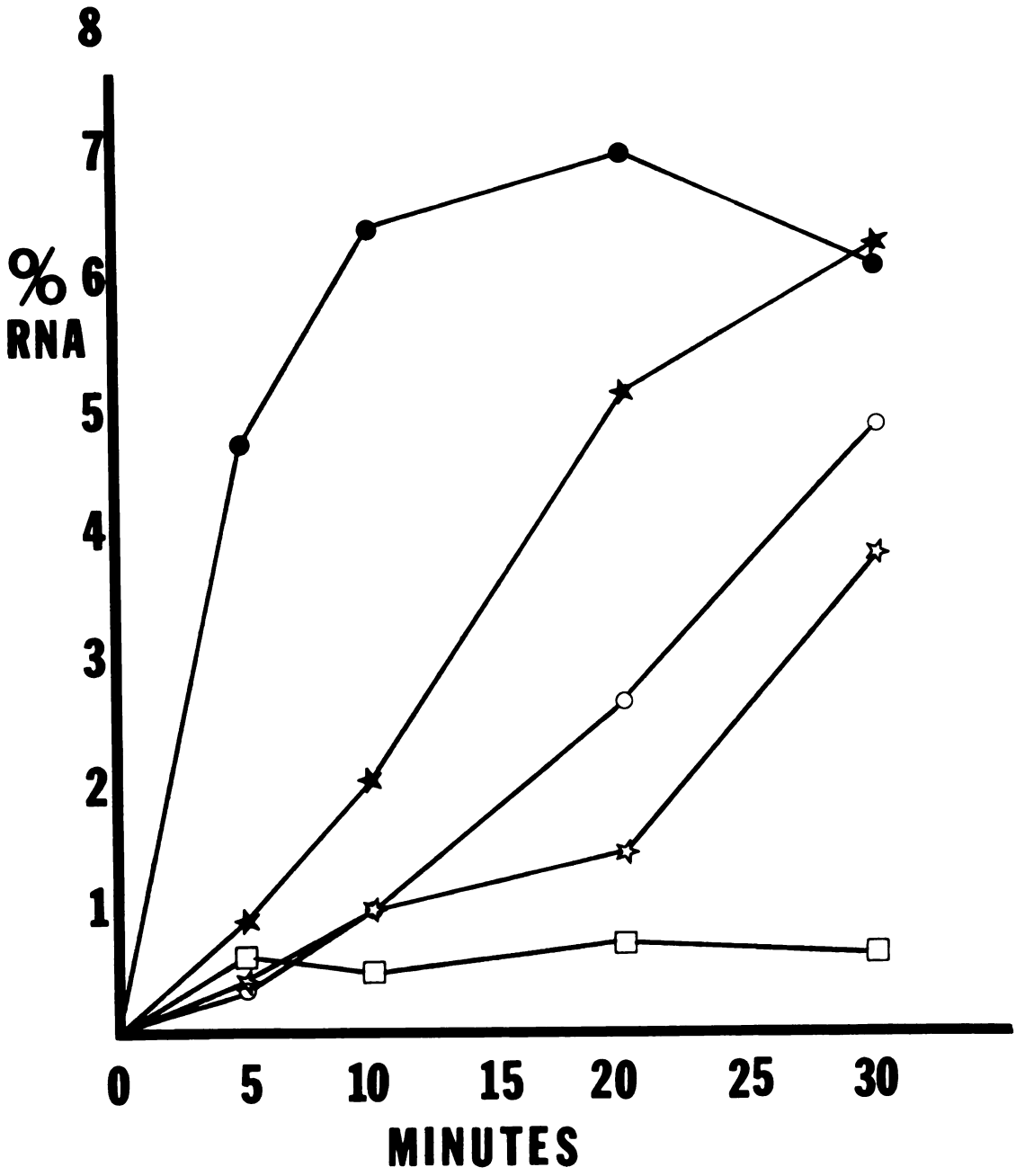


Figure 5.

Figure 6. Effects of magnesium concentration on release of TCA-precipitable RNA from Dounce isolated MOPC-21 cell nuclei. 3mM $MgCl_2$ (●—●), 4mM $MgCl_2$ (□—□), and 8mM $MgCl_2$ (✿—✿).

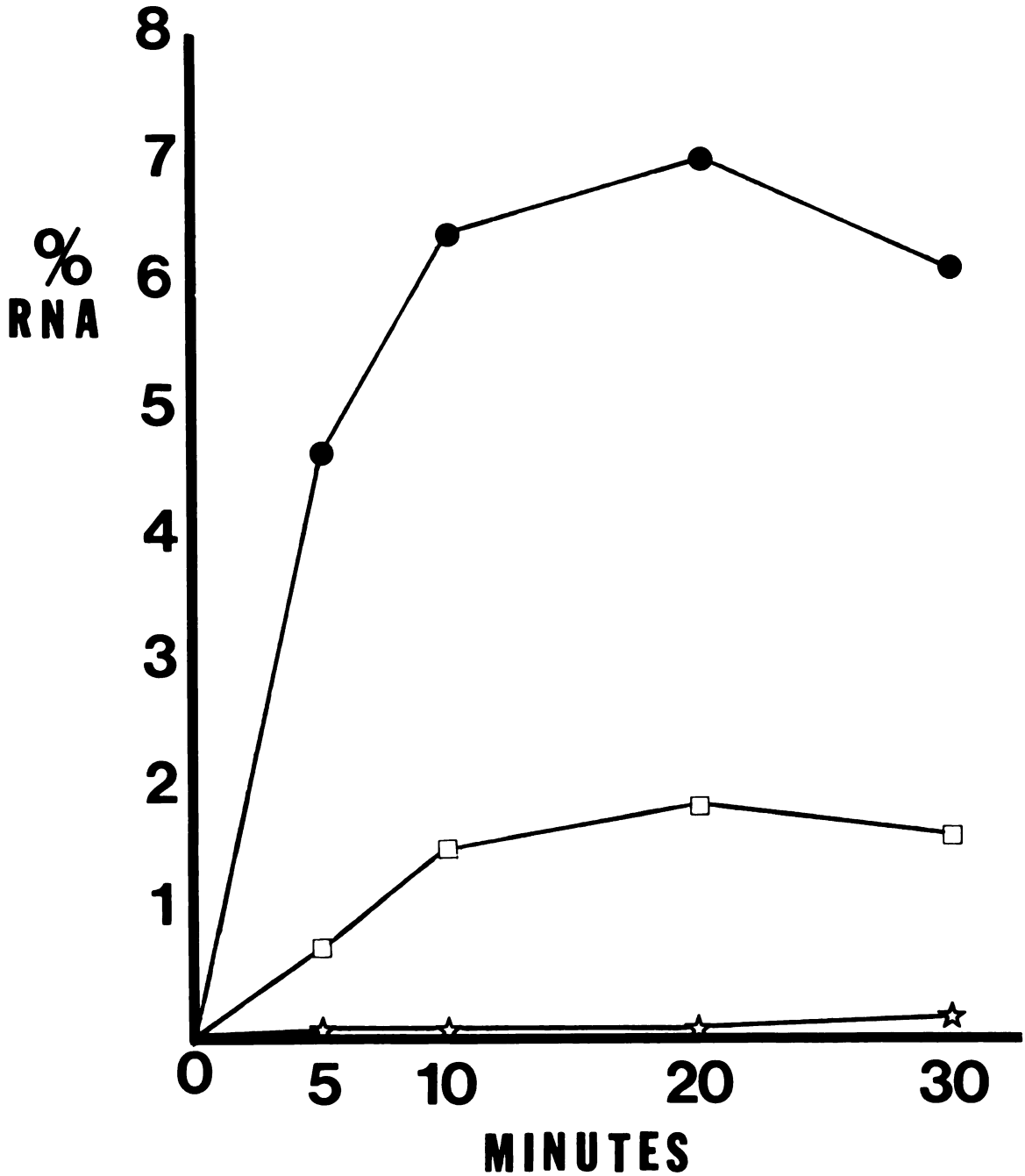


Figure 6.

however, the magnesium ion concentration has been crucial. Its effects and an explanation of its effects will be discussed.

An important method used for obtaining information concerning RNA release was measuring the rates of release of RNA, at different temperatures and otherwise similar conditions, from logarithmic phase nuclei. By choosing the linear rate of release from the graph obtained at the various release temperatures, and plotting these respective rates on an Arrhenius plot of - log (rate) vs. 1/T, an Arrhenius activation energy is obtained from the slope of the resulting line. As is shown in Figure 7, the activation energy from the linear rates is found to be approximately 20 kcals/mole uridine in TCA-precipitable RNA for the process of transport. As will be shown in the Discussion, this activation energy is independent of nuclease action.

Before the onset of this facilitated transport, a type of transport with a much lower activation energy is found; the activation energy for this diffusion-mediated release is approximately 2.9 kcals/mole uridine. The RNA released via this type of transport is small RNA, as can be shown using size distributions (from early time points in all release system combinations). The energy for this type of diffusion could easily be furnished by weak interactions (for instance, hydrogen bonding). These two basic types of transport, diffusion-mediated release and facilitated transport, will be more fully delineated in the Discussion section.

Numerous points should be clarified concerning this apparent activation energy prior to the discussion of it. One point concerns the process itself. The cell (or nucleus) is given labelled uridine in nucleoside form. Much of this labelled uridine is thought to be incorporated into RNA in the form of HnRNA, a very large precursor form of the various

Figure 7. Arrhenius graph of - log (rate of release) vs. 1/T, with linear rates of release taken from Figure 5.

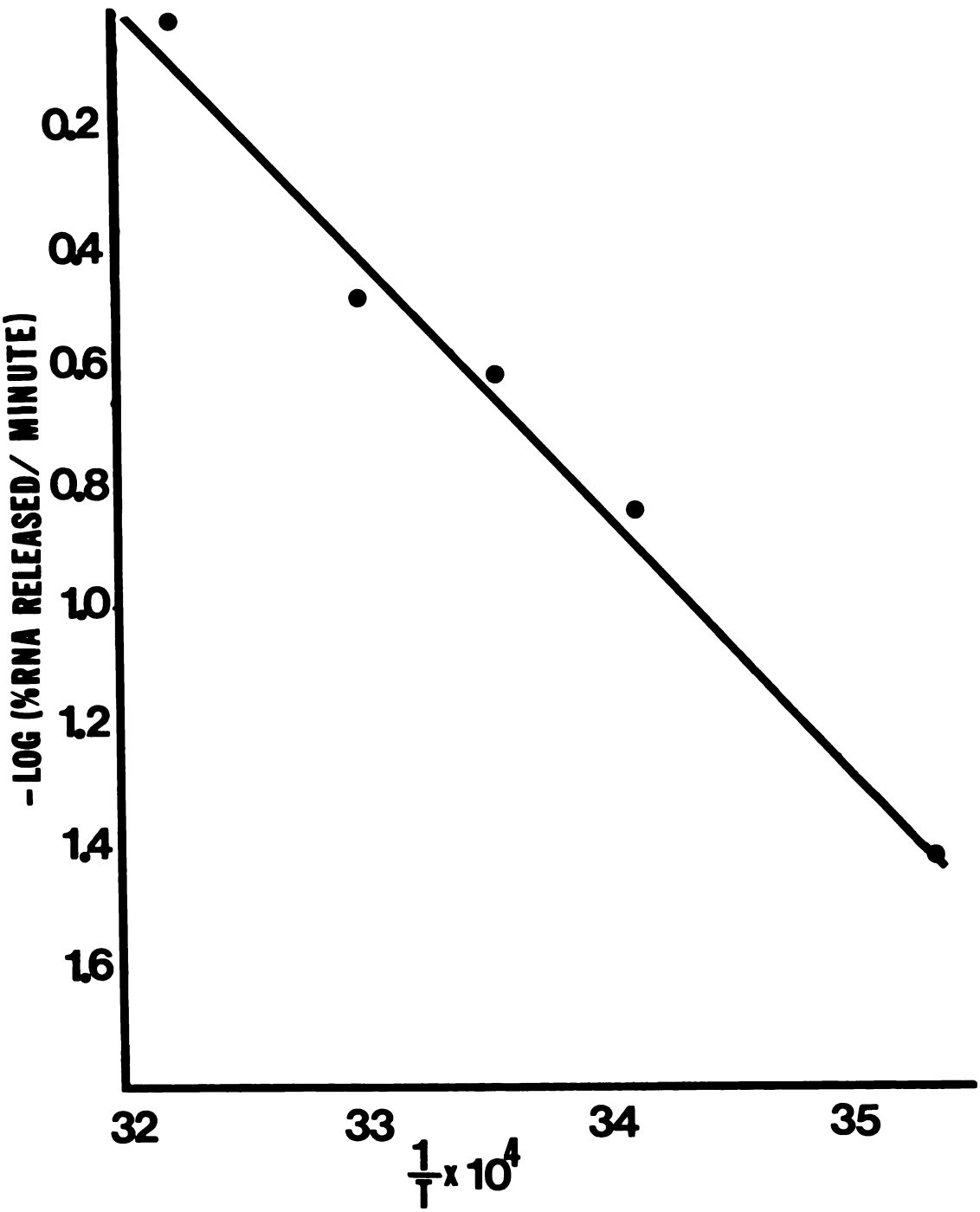

0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
-LOG (%RNA RELEASED/ MINUTE)
32
33
34
35
$\frac{1}{T} \times 10^4$

Figure 7.

types of RNA (mRNA, rRNA, etc.), with subsequent processing of the HnRNA to yield smaller RNA products (see Weinberg, 1973, for a review). After this processing, the RNA is transported out of the nucleus. Therefore, this apparent activation energy must include these various stages, and is not exclusively concerned with the transport component per se. The synthesis, processing, and transport components are difficult to uncouple, although this will be discussed at greater length. It thus appears that approximately 1 molecule of ATP is required per nucleotide in the TCA-precipitable RNA transported, with an efficiency of approximately 75% or greater.

A second major point derived from the Arrhenius data is based upon the domain of temperatures at which the release was measured. Previous work has shown that membrane phase transitions in similar organisms should be expected to occur somewhere between 15C-30C (Towers, Raison, Kellerman, & Linnane, 1972). Many transition points have been found at approximately 22C-24C in other types of membranes, although nuclear membranes have not been examined. The slope of the line obtained from the Arrhenius plot is a constant (straight line) indicating that RNA release is independent of any lipid phase transitions, if such transitions do occur in nuclear membranes. This correlates well with the results obtained with regard to detergent treatment.

Release of RNA was tested from nuclei treated with the detergent Nonidet P-40 or Triton X-100 (Figure 8). Release in the presence of magnesium was not detectable. The release is observed to proceed at essentially the same rate with or without the detergent treatment, within the standard deviation for the individual measurements. Nuclei were isolated and examined by Triton X-100 (Figure 9) treatment and by

Figure 8. Release of TCA-precipitable RNA from nuclei treated with detergent at 30C, as described in Materials and Methods. Dounce isolated (), Triton X-100 treated (), Nonidet P-40 treated (), percentage released from Dounce isolated nuclei shown with standard deviation units.

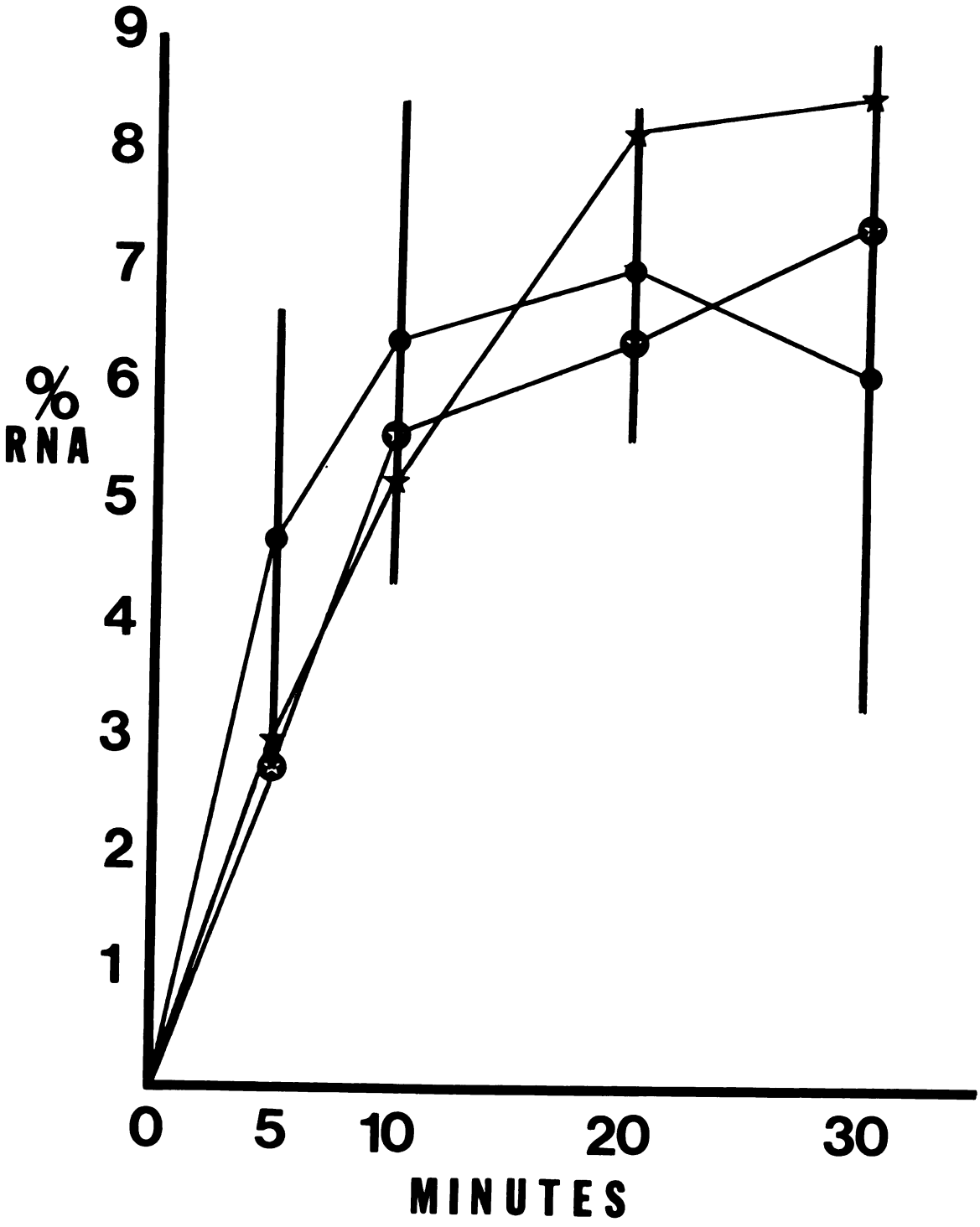


Figure 8.

Figure 9. Transmission electron micrograph of an isolated MOPC-21 nucleus treated with Triton X-100 detergent, as described in Materials and Methods.

Figure 10. Transmission electron micrograph of an isolated MOPC-21 nucleus treated with Nonidet P-40 detergent, as described in Materials and Methods.

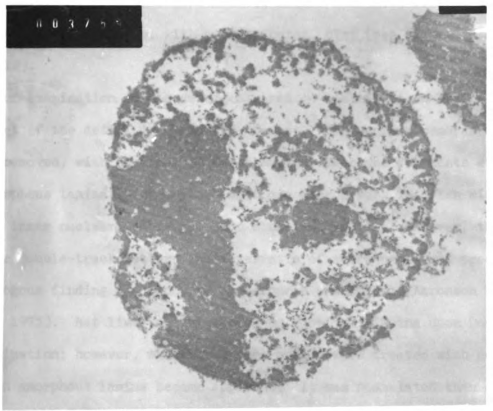


Figure 9.

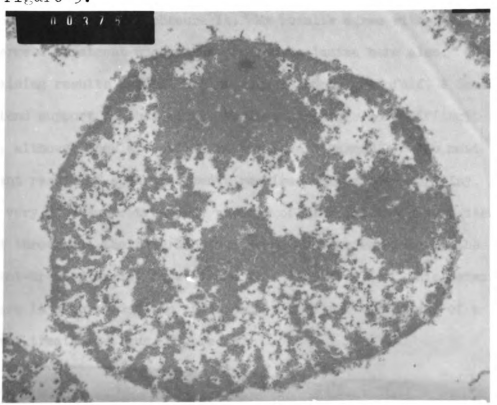


Figure 10.

Nonidet P-40 treatment (Figure 10). Higher magnification pictures of nuclear envelopes from detergent-treated nuclei show more clearly the structure of the envelope, with nuclear pores, etc. (see Figure 11 and Figure 12).

Upon examination of the detergent-treated nuclei, it is obvious that much of the detail is no longer present. The nuclear membranes appear removed, with no inner or outer nuclear membrane fragments evident. The amorphous lamina which appears in these micrographs is often mistaken for the inner nuclear membrane. Upon close examination, however, the familiar double-track pattern characteristic of membrane is not present. An analogous finding was reported by Aaronson and Blobel (Aaronson & Blobel, 1975). Rat liver nuclei showed no amorphous lamina upon Dounce homogenization; however, when these same nuclei were treated with detergent, an amorphous lamina became apparent. It was postulated than an amorphous lamina is always present, but the nuclear membranes and/or bound chromatin normally obscure it. My results agree with this finding, as detergent treatment uncovers an amorphous lamina here also. Similarly, the staining results of Chai and co-workers (Chai, Weinfeld, & Sandberg, 1974) lend support to this idea. Nuclear pores are more difficult to locate, although they are still present (see figures). The most important result of such treatments requires much closer scrutiny.

A very fine superstructural network of fine fibrils can be seen running throughout the heterochromatin and nuclear envelope in the detergent-treated nuclei (see Figure 13 and Figure 14). This network structure is also evident in the scanning electron micrograph of a detergent-treated nucleus (Figure 15).

Figure 11. Transmission electron micrograph of the nuclear envelope in a detergent-treated MOPC-21 nucleus. (Triton X-100 treatment)

Figure 12. Transmission electron micrograph of the nuclear envelope in a detergent-treated MOPC-21 nucleus. (Nonidet P-40 treatment)

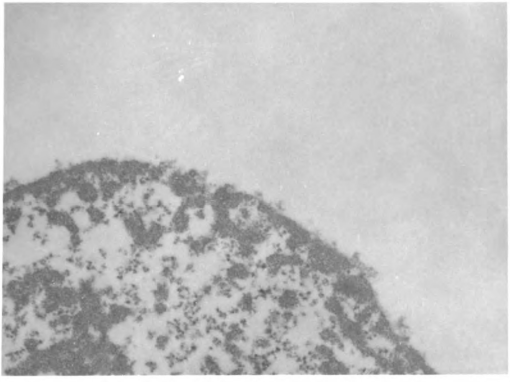


Figure 11.

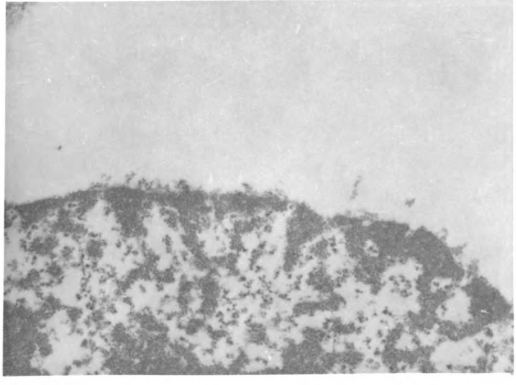


Figure 12.

Figure 13. Transmission electron micrograph of the microfibrillar network in a detergent-treated MOPC-21 nucleus. (Triton X-100 treatment)

Figure 14. Transmission electron micrograph of the microfibrillar network in a detergent-treated MOPC-21 nucleus. (Nonidet P-40 treatment)

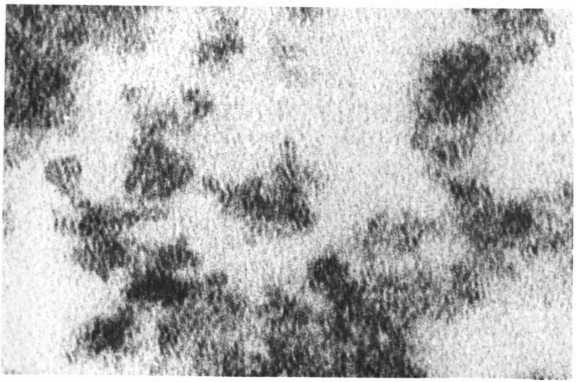


Figure 13.

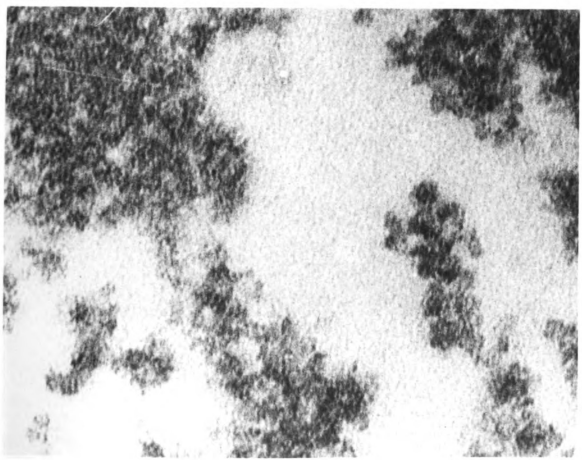


Figure 14.

Figure 15. Scanning electron micrograph of a detergent-treated MOPC-21 nucleus.

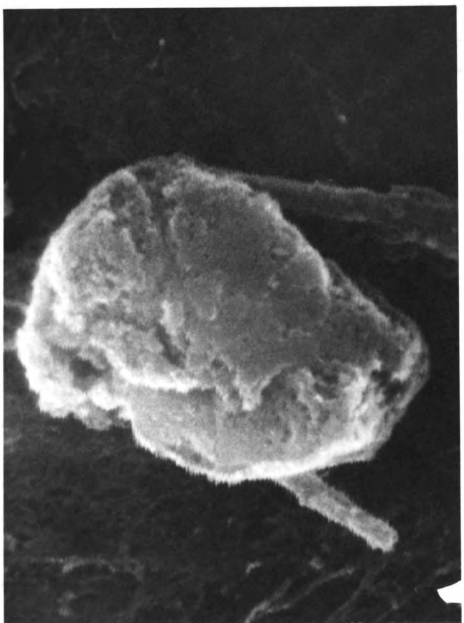


Figure 15.

The existence of some type of nuclear network has been proposed previously in HeLa cell nuclei, and this network was thought to allow formation of nuclear "ghosts" after detergent treatment and magnesium solubilization of chromatin. Riley and co-workers (Riley, Keller, & Byers, 1975) proposed that this network was composed of proteins. Electron micrographs of Dounce isolated nuclei were presented before treatment by these investigators. However, no micrographs of treated nuclei were presented to support their contention that a fibrous network existed. Gel electrophoresis was performed on ghost components, which showed enrichment in two bands. In this instance, only the periphery showed evidence of a network structure, since their treatment destroyed any intranuclear structure. Similarly, Berezney and Coffey (Berezney & Coffey, 1974) reported a fibrous protein network which extended throughout the nucleus. A function for this network will be proposed in the discussion section. The diameter of the fibrils seen in this study is approximately 6.0-7.0nm, in excellent agreement with the results of Aggarwal and Sodhi (Aggarwal & Sodhi, 1973) for microtubules appearing near nuclei. This fine fibrillar network, similarly to the amorphous lamina, is somewhat obscured by heterochromatin and membrane normally present. Microfibrils have also been mentioned in conjunction with sheets of chromatin.

Other treatments were examined for their effects on this fibrillar network, such as treatment with trypsin (Figure 16) and treatment with RNAase and DNAase (Figure 17) for 10 minutes with conditions as specified. Upon close observation, nuclei treated with trypsin still show the fibrillar structure, whereas RNAase and DNAase treated cells do not. It is possible that the fibrils are tightly bound with chromatin and are protected from trypsin action by this binding, or that the proteins are

Figure 16. Transmission electron micrograph of a MOPC-21 nucleus isolated by Dounce homogenization and treated with trypsin.

Figure 17. Transmission electron micrograph of a MOPC-21 nucleus isolated by Dounce homogenization and treated with RNAase and DNAase as described in Materials and Methods.

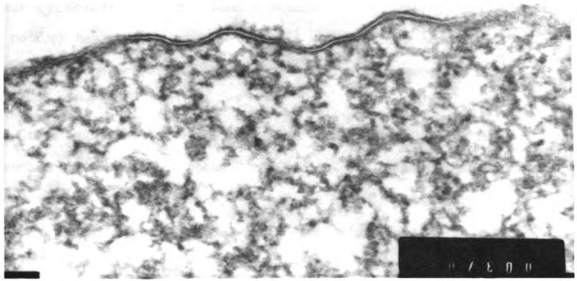


Figure 16.

Figure 17.

tightly folded, although it is also possible that the composition of these fibrils may in fact be predominantly ribonucleic and deoxyribonucleic acids and not protein, thus being immune to the action of trypsin. RNAase and DNAase treatment seems to remove all of this network, although the network has previously been proposed as protein in composition. I believe that the network is basically tightly folded protein, as will be proposed.

Since magnesium ion effects and the transport of RNA are independent of membrane lipids (to be shown), the site of action of Mg^{++} must exist elsewhere than in the membrane. In support of this conjecture is the fact that detergent-treated nuclei are similarly inhibited in release by high concentrations of Mg^{++} ions (see Figure 8). A hypothesis which I formulated is that Mg^{++} binding to the fibrillar network is responsible for the inhibition of release through contraction or conformational change in the fibrillar network, a process also responsible for gross shrinking of the nuclei in 8mM $MgCl_2$. Nuclei were examined for shrinkage in magnesium concentrations as specified. The calculation of the gross size of nuclei in high magnesium concentrations gives a diameter of approximately 35 micrometers (3500nm), in comparison to the 6.9 micrometer (6900nm) diameter of control nuclei. A detailed discussion will follow in the Discussion section.

The argument may be advanced that the detergent treatment is not effectively removing the lipids from the nuclear membrane. To test this, nuclei were isolated by Dounce homogenization, and their phospholipids extracted and analyzed. Also, the nuclei were Dounce homogenized and subsequently treated with detergents, and their phospholipids extracted and analyzed. The results are presented in Table 2.

TABLE 2. The effects of detergent treatment on phospholipid composition in isolated MOPC-21 nuclei

COMPOSITION & PHOSPHOLIPID	TREATMENT		
	DOUNCE HOMOGENIZATION	TRITON X-100 TREATMENT	NONIDET P-40 TREATMENT
SUPERNATANT	29.3%	86.3%	88.7%
PELLET	70.7%	13.7%	11.3%

As is evident from these results, approximately 90% of the phospholipids were removed by detergent treatment.

Using these results and the results obtained from the data on the release of RNA at various temperatures, the release of RNA from nuclei is shown to be independent of nuclear membrane phospholipids (lipids). That is, no transition point manifests itself in the release data from 10C to 37C, and when the lipids are removed by detergent treatment, the release is unaffected.

Studies were also undertaken to determine the effect of cell culture state (density) on release. In the medium employed, the cells grow logarithmically from about $1x10^5$ cells/ml to about $9x10^5$ cells/ml, with a doubling time of approximately 16-18 hours. Above $9x10^5$ cells/ml a stationary phase is reached, with culture growth slow thereafter and with cell death occurring at $1.4\text{-}1.6x10^6$ cells/ml.

Nuclei were isolated from stationary phase culture (see Figure 18). The nuclei isolated from stationary phase cultures appeared different when compared to nuclei isolated from logarithmic phase cultures. The peripheral chromatin is not as distinctive in stationary phase nuclei, the heterochromatin is much less dense and much more uniform in staining

Figure 18. Transmission electron micrograph of a stationary phase MOPC-21 nucleus isolated by Dounce homogenization as described in Materials and Methods.

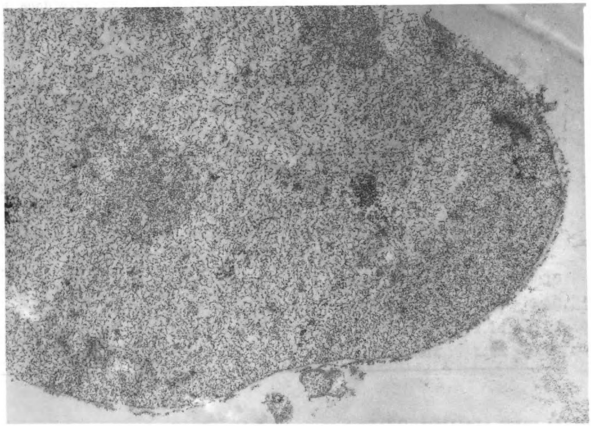


Figure 18.

intensity, and there are few (if any) nucleoli present in such cells. A double nuclear membrane is present.

Nuclei were isolated from such stationary phase cell cultures and tested for release of RNA. Cytosol preparations were also obtained from stationary phase cells, and tested for release properties. The results of such experiments are summarized in Table 3 (presented graphically in Figure 19 and Figure 20).

TABLE 3. RNA release from combinations of various nuclei and cytosol types. Percentages released shown with standard error of the mean.

INCUBATION PERIOD	INCUBATION SYSTEM @ 30C NUCLEI TYPE CYTOSOL TYPE			
(minutes)	LOGARITHMIC LOGARITHMIC	LOGARITHMIC STATIONARY	STATIONARY LOGARITHMIC	STATIONARY STATIONARY
5	0.88 ± 0.14	0.72 ± 0.12	0.68 ± 0.13	0.41 ± 0.10
10	2.04 ± 0.37	1.14 ± 0.17	0.91 ± 0.11	0.60 ± 0.10
20	5.13 ± 1.00	1.59 ± 0.19	1.50 ± 0.13	1.18 ± 0.22
30	6.26 ± 0.60	2.42 ± 0.25	1.76 ± 0.14	1.56 ± 0.27

As is apparent, the logarithmic phase nuclei/logarithmic phase cytosol system was the system which showed the highest release of RNA. Logarithmic phase nuclei incubated in stationary phase cytosol released less RNA than this system, although more than either system containing stationary phase nuclei.

When stationary phase nuclei were incubated with much higher concentrations of logarithmic phase cytosol, the release of RNA approximated the release of RNA obtained in the logarithmic/logarithmic system in total amount (data not shown). However, a significantly higher release of DNA

Figure 19. Release of TCA-precipitable RNA from logarithmic phase nuclei to logarithmic (●——●) and stationary phase (✪——✪) cytosol, assay procedure as described in Materials and Methods.

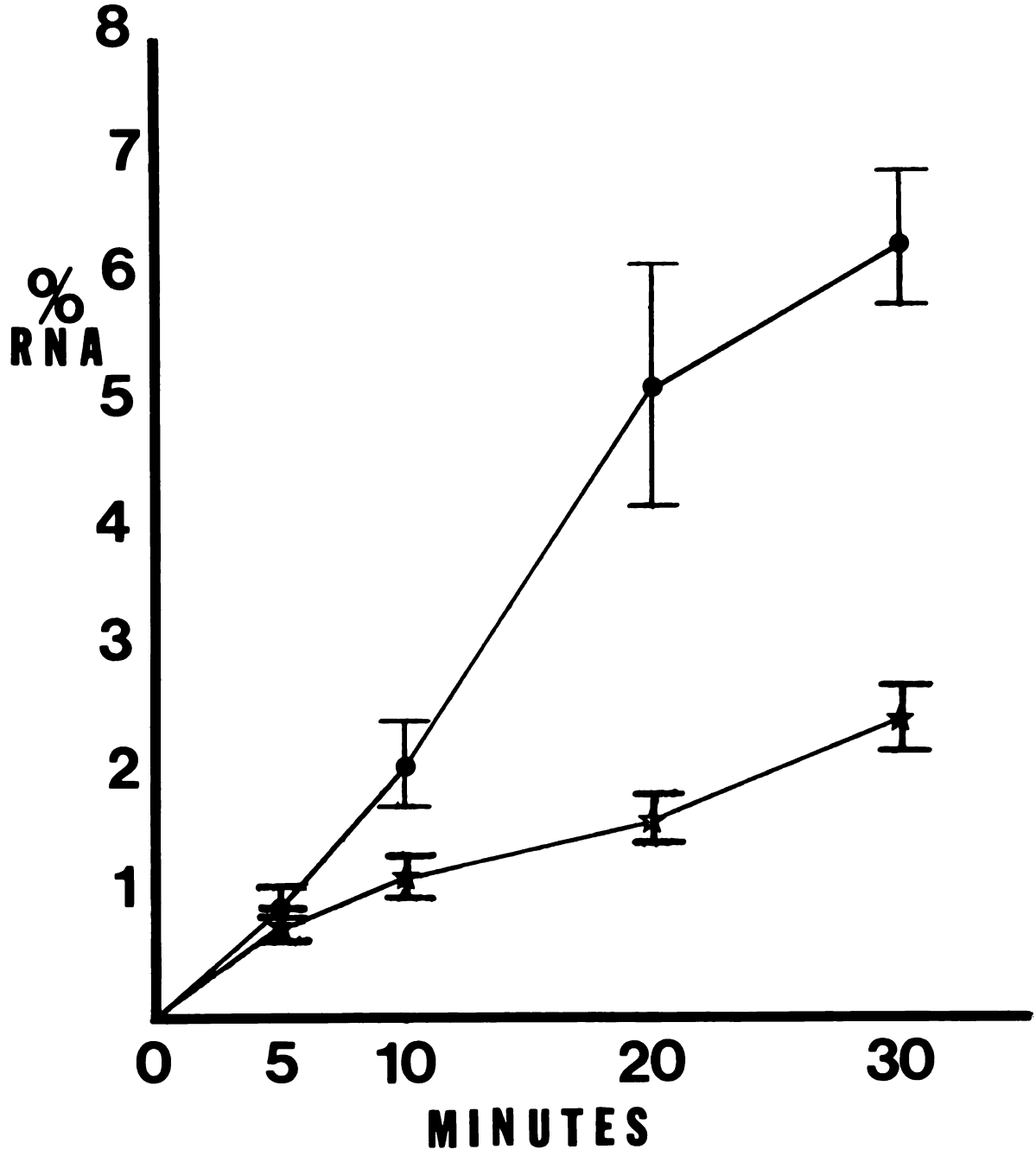
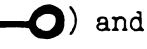


Figure 19.

Figure 20. Release of TCA-precipitable RNA from logarithmic phase nuclei to logarithmic () and stationary () phase cytosol, assay procedure as described in Materials and Methods.

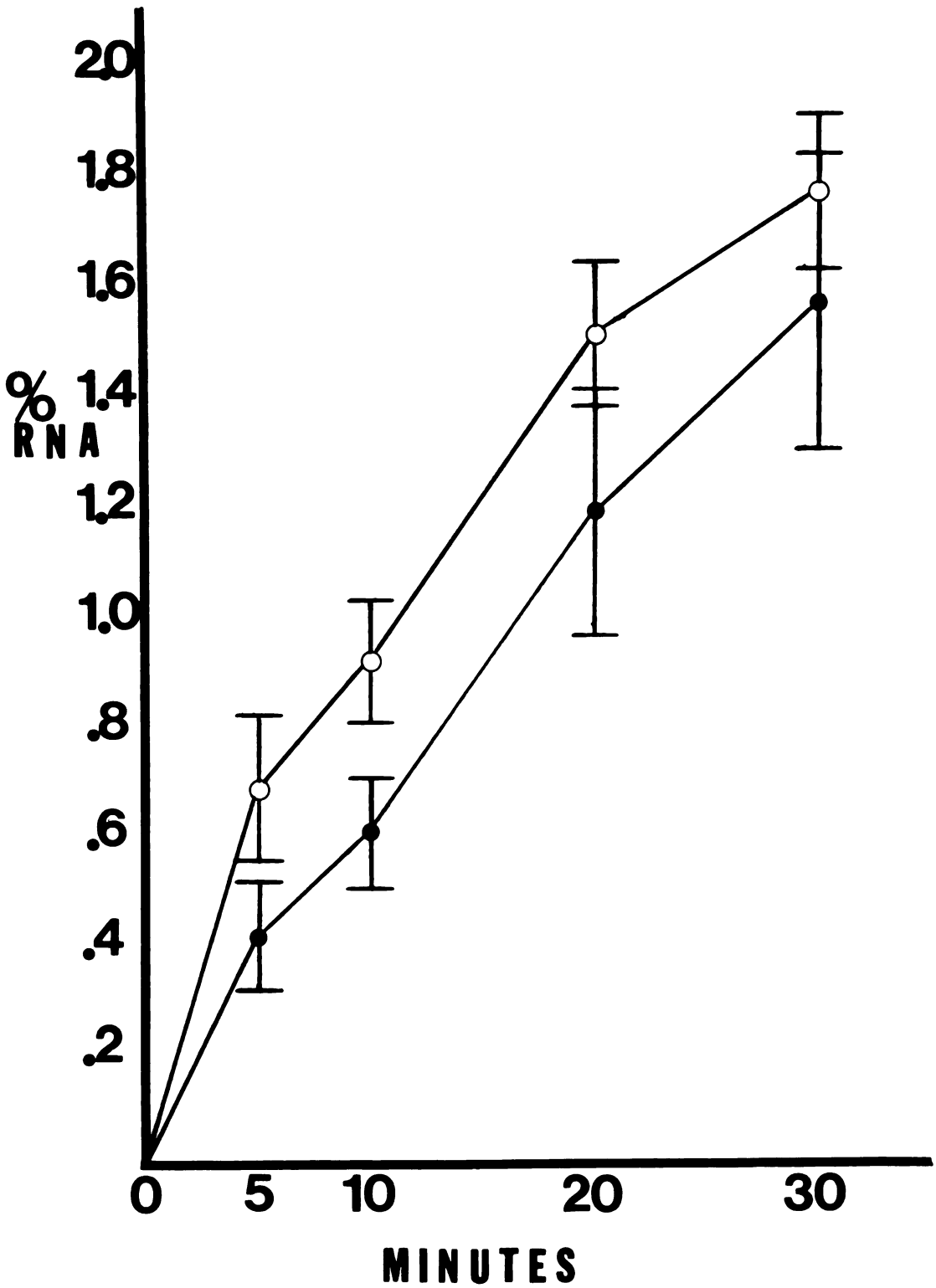


Figure 20.

was observed in such experiments (approximately 1-2% over controls), indicative of lysis or leakiness (loss of restriction). What happens in this case is unclear, although presumably nuclei are lysing.

Many conclusions can be drawn from such release combinations. When logarithmic phase nuclei are incubated in stationary phase cytosol, the release is inhibited. When the logarithmic phase nuclei are then incubated in higher concentrations of stationary phase cytosol, the release is not further elevated and is usually found to be further inhibited. This argues against a reduction in release being caused by a lack of transport factors in stationary phase cytosol, since the concentration of these would be increased in such a supplemented system. It appears necessary to postulate an inhibition of the transport process (at the synthesis, processing, or transport stage) in the stationary phase cytosol preparations. It should be pointed out that the initial rates of release are approximately equal in all the various combinations with the exception of a minor drop in the stationary phase nuclei/stationary phase cytosol system. Also, the RNA transported is small molecular weight RNA, which is presumably diffusible (see Discussion).

With release characterized as above, I turned to a characterization of what is being released in this system, since such information is vital to an understanding of the transport process and for comparison with other systems and in vivo results.

The distribution of the RNP particles released from logarithmic phase culture nuclei to logarithmic phase cytosol was examined (Figure 21), with conditions as specified. The distribution was heterogeneous, with much of the RNP appearing in the range where messenger RNP has been reported (Schweiger & Mazur, 1974; Raskas & co-workers, Henshaw, 1972; Schumm &

Figure 21. Sucrose gradient analysis of released RNP particles from logarithmic phase MOPC-21 nuclei, isolated by Dounce homogenization, to logarithmic phase cytosol, assay procedure as described in Materials and Methods.

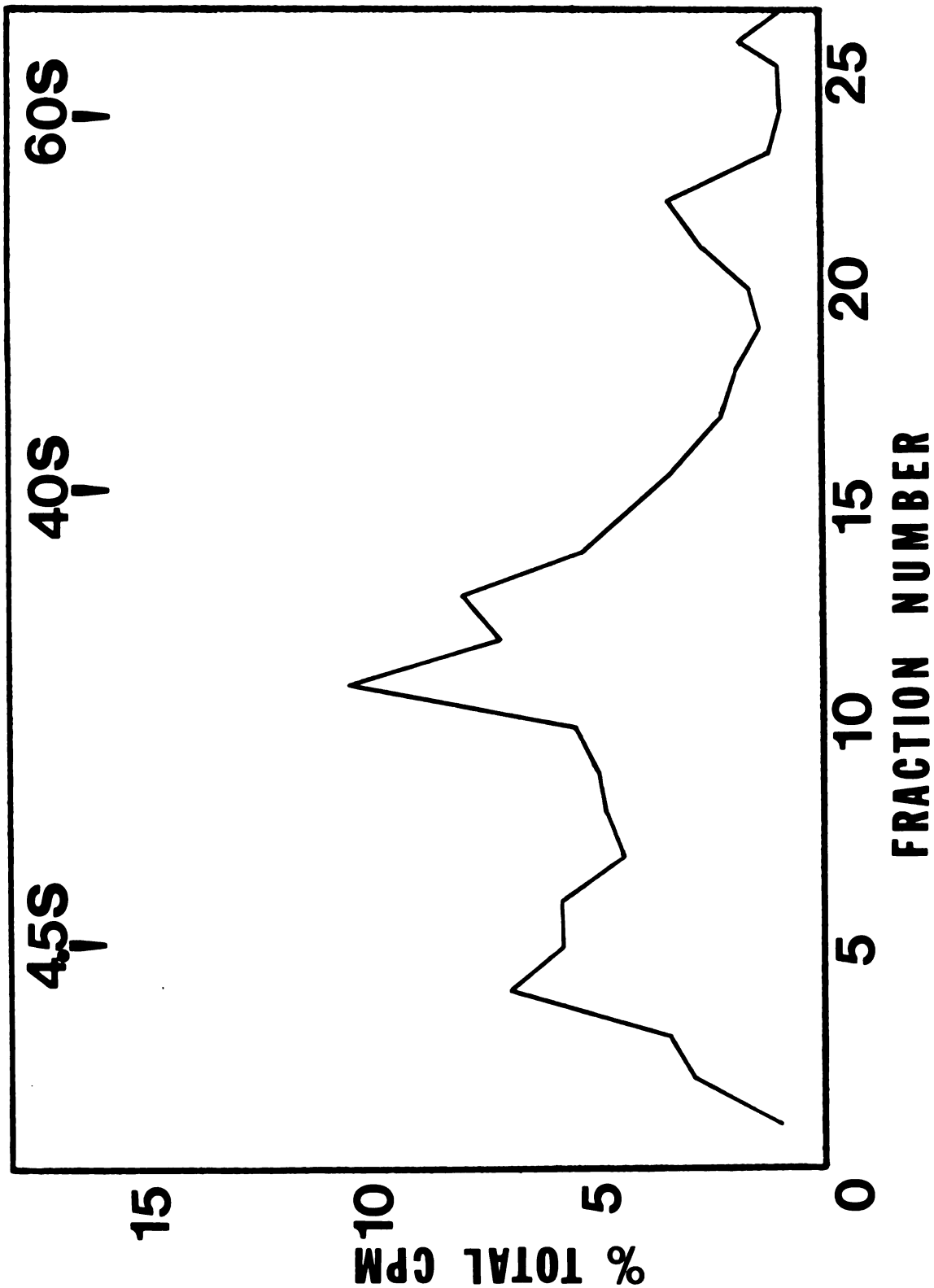


Figure 21.

Webb, 1972; Ishikawa & co-workers; Lukanidin & co-workers, etc.). Many other components were present also, as can be seen from the graph of the distribution, including tRNA.

To examine the RNA released from the nuclei, the RNA was extracted (as described) from the released RNP (Figure 22). The distribution released from logarithmic phase nuclei to logarithmic phase cytosol was homogeneous, with tRNA, rRNA, and much presumed mRNA appearing in the released material. This distribution is not identical with the distribution of RNA found in the cytoplasm of these myeloma cells in vivo (shown in Figure 23, work done previously in our lab). However, the labelling times were different (see legend), and the turnover times of the various types of RNA are widely different, and hence identical distributions would be surprising. A 30-minute in vivo chase is not as effective as a 30-minute in vitro chase, so the results are not clear cut. In comparison, the RNA released from logarithmic phase nuclei to stationary phase cytosol is of smaller size than the RNA released from these nuclei to logarithmic phase cytosol (data not presented). Much RNA in the size range of tRNA, snRNA, etc., is present. This is compatible with the idea that facilitated transport is not operative here.

The nuclear RNA in logarithmic phase nuclei and in stationary phase nuclei was examined, to see if gross differences exist in the distributions (Figure 24). As is evident, the stationary phase nuclei are not readily synthesizing HnRNA, and most of the labelled RNA is present as very small 4S RNA, in comparison to the rather heterogeneous distribution of HnRNA seen in logarithmic phase cells. It is obvious from the figures that synthesis of RNA in stationary phase nuclei is drastically curtailed in comparison with logarithmically growing cells.

Figure 22. Sucrose gradient analysis of RNA purified from the released RNP from Dounce isolated logarithmic phase nuclei to logarithmic phase cytosol.

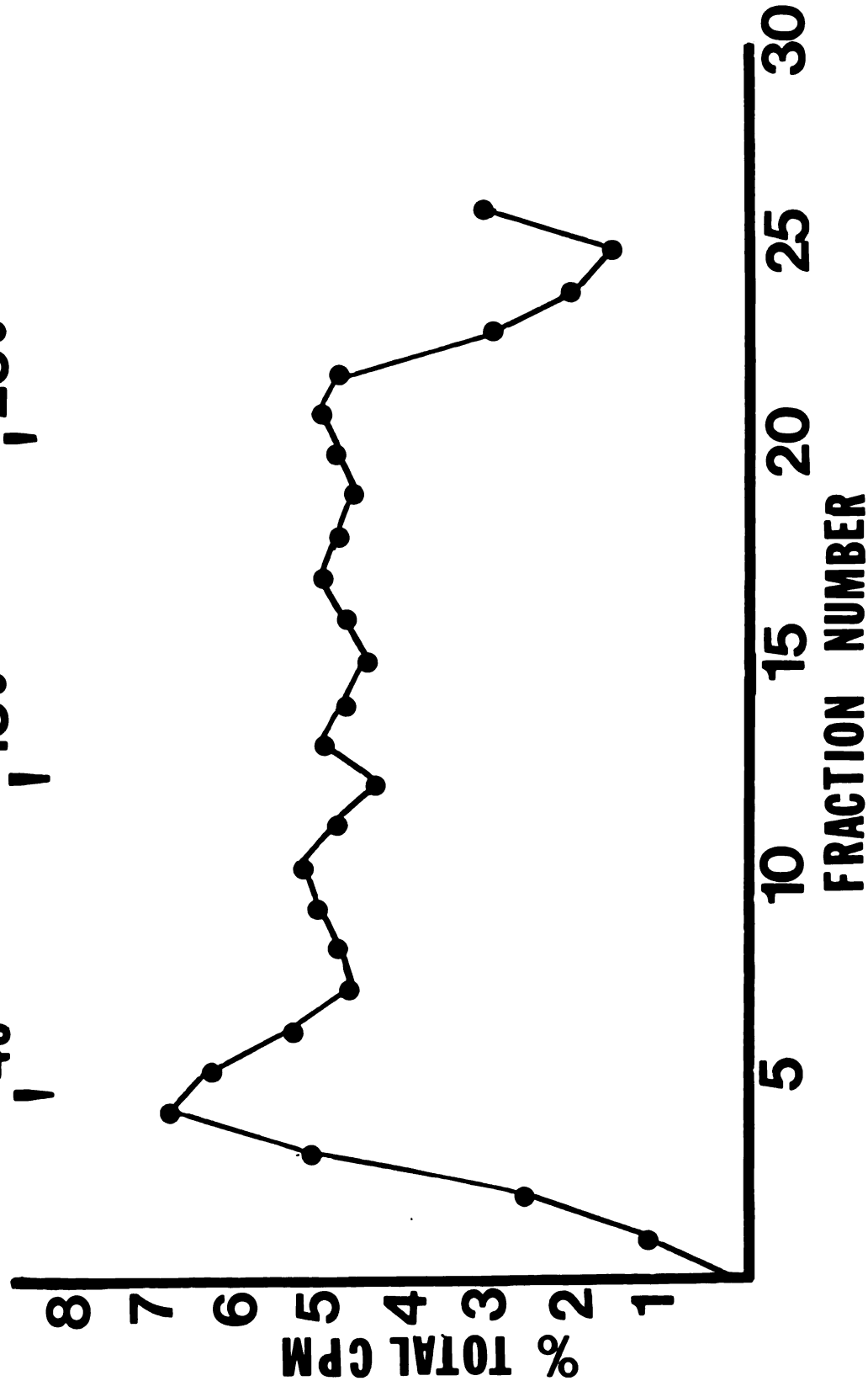


Figure 22.

Figure 23. Sucrose gradient analysis of cytoplasmic RNA found in intact MOPC-21 cells.

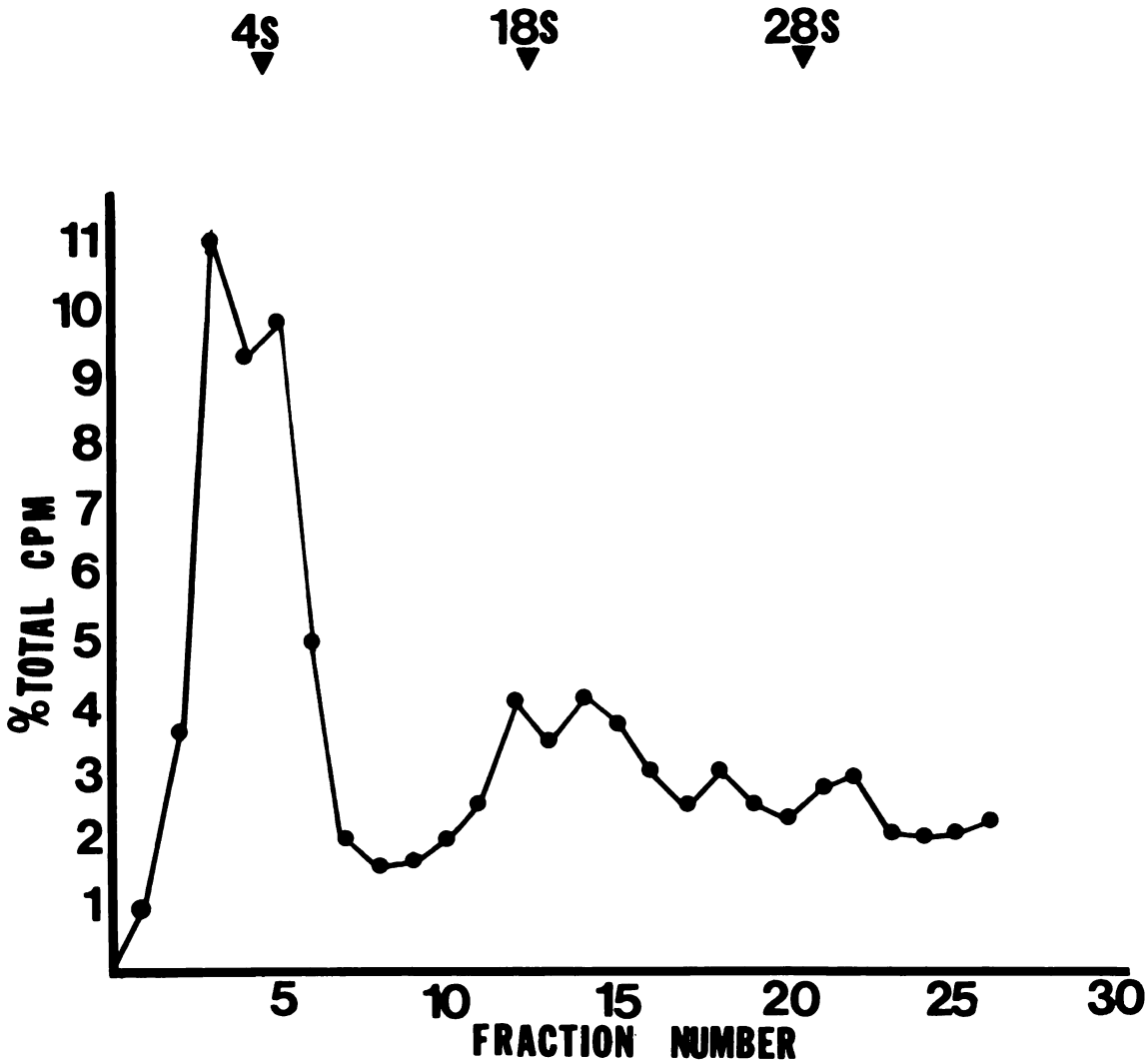
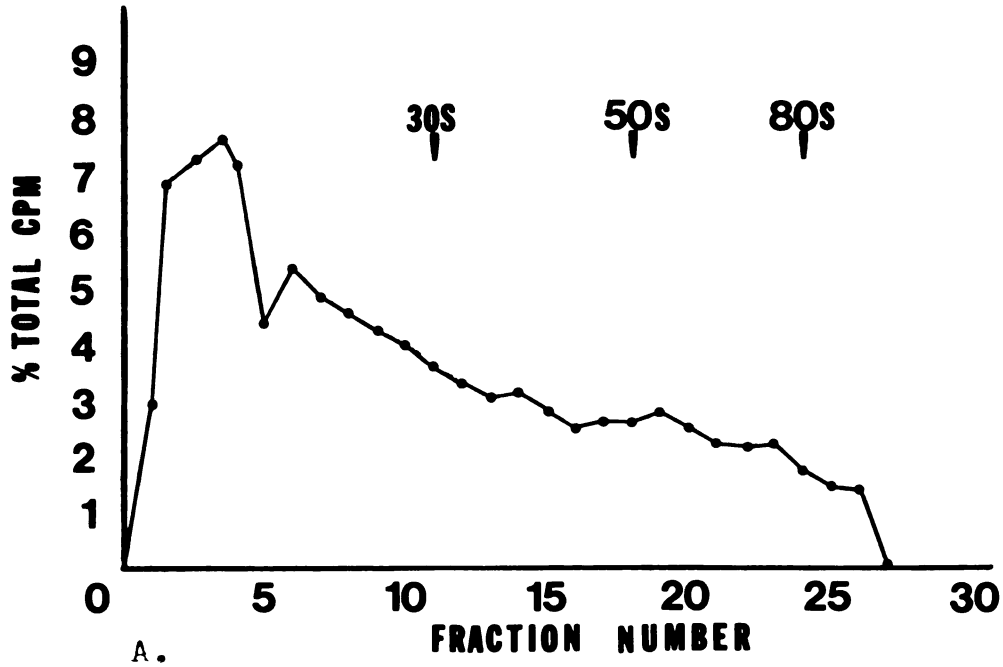
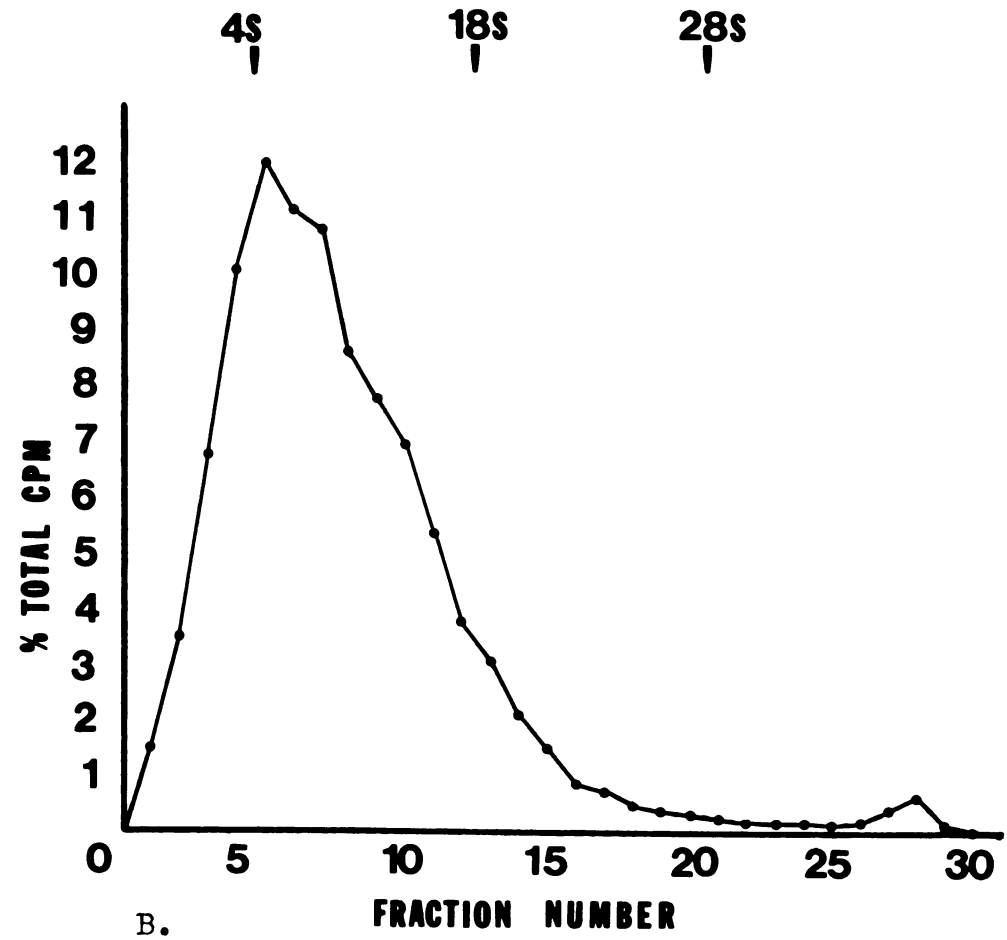

4S
18S
28S
%TOTAL CPM
11
10
9
8
7
6
5
4
3
2
1
5
10
15
20
25
30
FRACTION NUMBER

Figure 23.

Figure 24. Sucrose gradient analysis of the nuclear RNA found in logarithmic phase nuclei (A) and stationary phase nuclei (B), isolation as described in Materials and Methods.


30S
50S
80S
% TOTAL CPM
0
1
2
3
4
5
6
7
8
9
0
5
10
15
20
25
30
FRACTION NUMBER

A.


4S
18S
28S
% TOTAL CPM
0
1
2
3
4
5
6
7
8
9
10
11
12
0
5
10
15
20
25
30
FRACTION NUMBER

B.

Figure 24.

This nuclear RNA data seems to correlate well with the finding that the initial rates of release from logarithmic phase nuclei and stationary phase nuclei in logarithmic phase cytosol are nearly the same, since it is contended that small RNA is transported immediately via passive diffusion. No evidence of increased degradation of RNA by nucleases was found in stationary phase cytosol, as is consistent with the release curves obtained. The data also correlate well with the electron micrographs of stationary phase nuclei, showing that few or no nucleoli exist on these cells.

A further characterization of the released RNA was performed, indicative of mRNA, by examining the poly A content of the released RNA, as measured by binding of RNA to oligo-dT cellulose columns. Table 4 presents the percentages of poly A+ RNA found in the RNA released from the various release system combinations.

TABLE 4. Poly A content of RNA released in various release systems. Percentages shown with standard error of the mean for measurements.

CLASSIFICATION OF TRANSPORTED RNA (BINDING TO OLIGO-dT CELLULOSE	INCUBATION SYSTEM NUCLEI TYPE CYTOSOL TYPE			
	LOGARITHMIC LOGARITHMIC	LOGARITHMIC STATIONARY	STATIONARY LOGARITHMIC	STATIONARY STATIONARY
POLY A-	64.4 ± 6.7	58.3 ± 4.7	75.3 ± 1.7	72.6 ± 9.0
POLY A+*	35.6	41.7	24.7	27.4

*The poly A+ figures are obtained by addition of separate washes, and consequently are not shown with standard errors.

For comparison, the size distribution of polysomal poly A+ RNA was examined (Figure 25, previous work done in our lab). The size distribution is compatible with the size distribution of released RNA in the

Figure 25. Sucrose gradient analysis of polysomal poly A+ RNA isolated from intact logarithmic phase MOPC-21 cells.

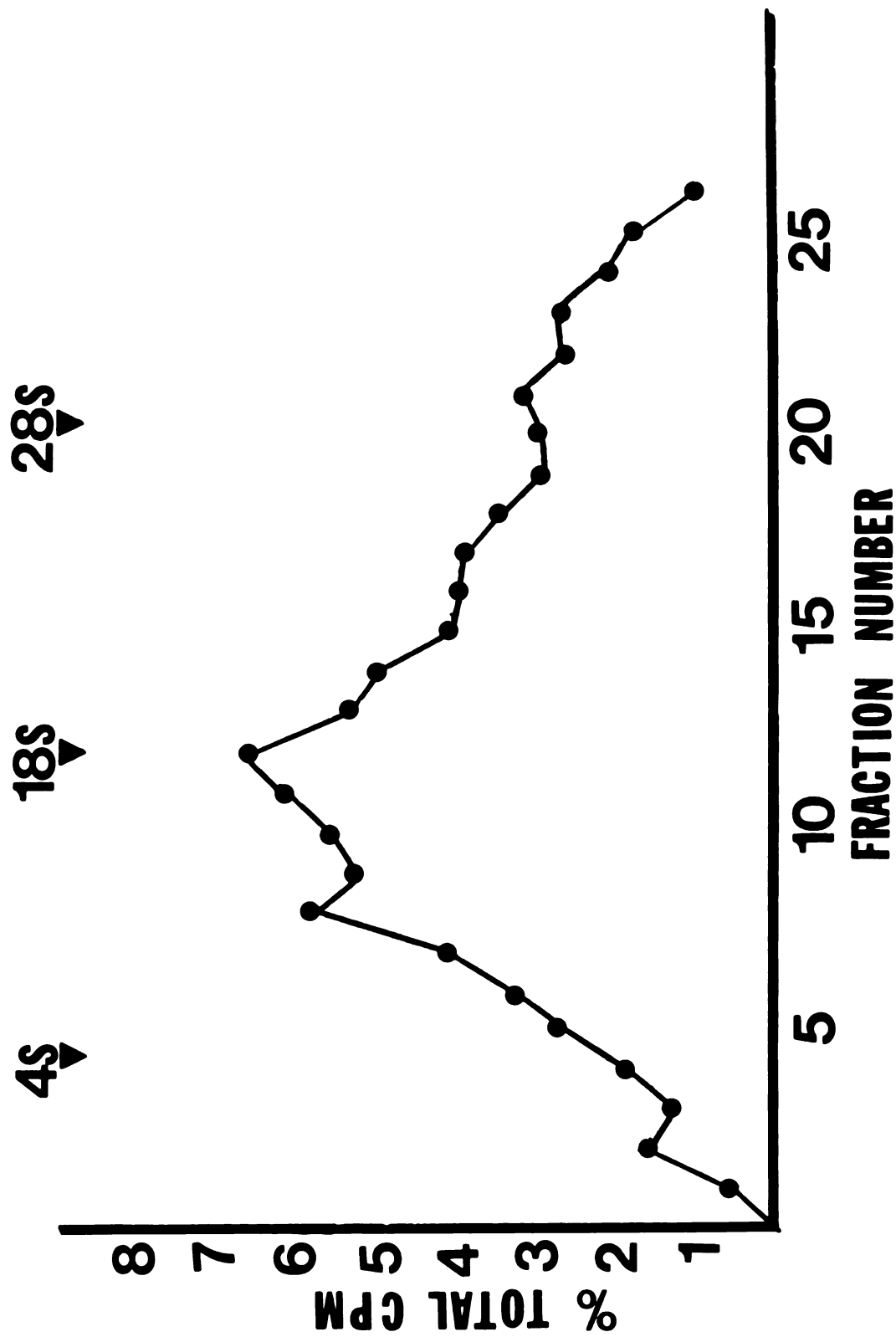


Figure 25.

logarithmic/logarithmic system, keeping in mind the percentage poly A+ of the released RNA.

Two results from the poly A data are particularly evident. First, the percentage of poly A+ RNA released from logarithmic phase nuclei is higher (by 11-14%) than the percentage of poly A+ RNA released from stationary phase nuclei to the same cytosol preparations. It should be mentioned that some experiments showed yields of TCA-precipitable label greatly in excess of predicted yields based on amounts of RNA found in the samples, and these experiments were disregarded. Some experiments showed obvious lysis of nuclei (via ^{14}C-thymidine and ^{3}H-uridine labelling) and were also disregarded.

Second, an interesting property of this system is that stationary phase cytosol apparently stimulates transport of RNA containing a higher percentage of poly A+ RNA than does logarithmic phase cytosol, although the total amount of RNA transported is greatly reduced. This difference is immediately obvious in the case of stationary phase nuclei, where the difference is +2.7% higher poly A+ content in the RNA released to stationary phase cytosol as compared to logarithmic phase cytosol.

In the case of logarithmic phase nuclei, a +6.1% difference is shown. This apparent difference may be due to the fact that a larger proportion of the RNA transported is large RNA, and does not diffuse passively. When the experiments were performed simultaneously with both types of cytosol preparations using logarithmic phase nuclei isolated from the same culture, the difference in percentage poly A+ was found to be +2.6% $\pm$ 0.6% (shown with the standard error of the mean). This result agrees well with the results previously mentioned with regard to poly A+ content of RNA released from stationary phase nuclei. Again, it

should be mentioned that the total RNA released from logarithmic phase nuclei to stationary phase cytosol is much reduced when compared to the RNA released from these nuclei to logarithmic phase cytosol, and the RNA released from stationary phase nuclei is smaller in size than that released from logarithmic phase nuclei (data not presented).

DISCUSSION

As a result of transmission electron microscopic studies, it has been demonstrated that Dounce homogenized nuclei are relatively intact after their isolation. That is, the inner nuclear membrane is fully continuous, the nuclear pores with their accompanying annuli are intact, the outer nuclear membrane is attached at the pore annulus though not continuous around the periphery of the nucleus, the chromatin structure is similar to the structure observed in intact cells, and the nucleolar structure is finely detailed and identical to the analogous structures seen in controls. When the transport of RNA was monitored from such Dounce isolated nuclei, numerous points were demonstrated which merit discussion.

Two basic types of transport of RNA molecules were demonstrated. A diffusion mediated release of small molecular weight RNA molecules was demonstrated, with an activation energy of about 2.7 kcals/mole uridine. Molecules involved with this type of release are those molecules which are capable of passively diffusing through nuclear pores. A facilitated transport of larger RNA molecules has also been demonstrated, with an activation energy of about 20 kcals/mole uridine. Basically, RNA molecules small enough to diffuse through 4.5nm pores do so. The larger RNA molecules, such as rRNA and mRNA, cannot freely diffuse through the pores, and thus these are the molecules which require facilitated transport, via a mechanism to be proposed. These two types of transport will now be discussed.

The facilitated transport of RNA (as was mentioned), through an Arrhenius graph using the linear rates of release of RNA obtained at various temperatures, was shown to have an activation energy of about 20 kcals/mole uridine in TCA-precipitable RNA. This use of linear rates of facilitated transport is shown to yield an activation energy which is independent of nuclease action.

The rate constant k may be written as $k = Ae^{-(E/RT)}$, where A is the y-intercept obtained from the Arrhenius graph, E is the calculated activation energy, R is the gas constant, and T is the absolute temperature. Using the A=13.9 obtained, and T=30C for example, k is found to be 0.35% RNA released/minute. Thus at 30C, approximately 10.5% of the RNA should be released in 30 minutes incubation. Samples made 0.5% SDS after 30-minute incubations showed that 72.8% of the RNA in the mixture remained TCA-precipitable, yielding 27.2% degradation. It is expected that nuclease action in the cytoplasm and nucleus are approximately equal, although further experimentation is underway to verify this point. Also, 6.3% TCA-precipitable RNA was found in the supernatant. Thus, the predicted 10.5% release corresponds quite closely with the actual 8-10.2% accounted for in the supernatant, and this shows that the activation energy calculated from this Arrhenius graph is actually independent of nuclease action.

The initial rates of release of RNA were also examined at the various temperatures, and a different type of transport was demonstrated. Using an Arrhenius graph, at temperatures below about 24C an activation energy of about 2.9 kcals/mole uridine was obtained. Above this temperature, a much higher activation energy was obtained, approximately 10 times higher. However, it is suggested that the release observed at 30C and 37C was

caused by facilitated transport, with larger RNA molecules released, accompanied by the appearance of poly A tracts, whereas these moieties did not appear at the lower temperatures initially. Thus, the two portions of the Arrhenius graph are not directly comparable. It should be noted that the activation energy of the line obtained from 30C to 37C is higher than the activation energy obtained for facilitated transport. This is due to a slightly delayed onset of facilitated transport at 30C as compared to 37C.

The discontinuity in the slope of the lines occurs at 23-25C. Such discontinuities (in the identical temperature domain) have been shown to correspond to phase transitions in lipids of mitochondrial membrane (Raison, Lyons, Mehlhorn, & Keith, 1971) and of rough endoplasmic reticulum (Towers, Raison, Kellerman, & Linnane, 1972).

It is thus suggested that the rapid initial release obtained at temperatures below 24C is mediated by simple diffusion of small RNA molecules (and "driven" by weak interactions such as hydrogen bonding). It has been reported that tRNA appears in the cytoplasm with no lag period (Weinberg, 1973), snRNA appears in the cytoplasm with little delay (Weinberg & Penman, 1969), and even histone message appears in the cytoplasm with little delay (Brawerman, 1974), thus supporting the idea that small RNA molecules are transported immediately after synthesis. There seems to exist a lag period before facilitated transport becomes prominent at lower temperatures. In general, the lower the temperature, the more delayed is the appearance of facilitated transport. At approximately 0C, facilitated transport is inhibited.

Thus, the discontinuity found in the Arrhenius graph of initial rates is apparently a reflection of the differential onset of facilitated

transport, and presumably also is a reflection of the membrane control over passive diffusion. The inward passage of cytosol components and small RNA molecules is inhibited by the non-fluid nuclear membranes at temperatures below 24C, and facilitated transport is initiated only after this cytosol component can gain entrance to the nucleus.

Therefore, the Arrhenius graph is divided into two portions, a high activation energy component which occurs above 24C, and a low activation energy component which occurs below 24C initially. This high activation energy component is obtained since facilitated transport is the overwhelming component above 24C even initially, and the low activation energy component is the diffusion-mediated release of small RNA which occurs before the onset of facilitated transport.

It should be mentioned that only the onset of facilitated transport is dependent on membrane fluidity via inward passage of cytosol factors. The Arrhenius graph obtained using the linear rates of release implies that facilitated transport is not dependent on membrane fluidity, and in conjunction with this finding is the fact that detergent-treated nuclei exhibit release comparable to the release obtained from non-detergent-treated nuclei.

It may be inferred that the initiation of facilitated transport is dependent on cytosol factors which are basically enzymatic in nature: That is, once these components enter the nucleus, the release is no longer dependent on continued entrance or exit of more such components, since such passage would again depend on membrane fluidity and since facilitated transport does not depend on membrane fluidity. Such an enzymatic component could be a cytoplasmic and nuclear nuclease, an enzyme for

attachment of poly A sequences of mRNA to nuclear envelope structures, or many other moieties.

As was mentioned, the process of transport is initiated at the incorporation of labelled uridine in HnRNA and is terminated as labelled uridine appearing in TCA-precipitable RNA in the supernatant. The question of how the activation energy is divided into the various phases of synthesis, processing, and transport must be posed.

Due to the lack of a lag period in the release of small molecular weight RNA at temperatures below 24C, and to the low activation energy obtained for such release, it seems probable that synthesis and processing do not require large amounts of energy. Similarly, the results of Marzluff and co-workers (Marzluff, Murphy, & Huang, 1973) indicate that synthesis is not highly energy dependent. The results of Brunner and Raskas (Brunner & Raskas, 1972) showed that ATP seemed to stimulate release of RNA molecules only after they underwent cleavage. Similarly, the results of Webb and co-workers (Racevskis & Webb, 1974) suggested that nuclear processing was not the limiting factor in transport of RNA, since aliquots of nuclei incubated at OC had an initial rate of release at 37C comparable to the initial rate of release obtained from aliquots incubated first at 37C (where processing occurred), although the probable coupling of transport and processing obscures the meaning of this experiment. So, it appears that most of the energy required for facilitated transport is required at the level of transport per se.

The question of what structure the RNA is transported through should now be posed. Previous work has demonstrated that an ATPase activity was present at the nuclear pores (Chardonnet & Dales, 1972). Thus, a reasonable possibility is that approximately one molecule of ATP is hydrolyzed

at the nuclear pore level for every nucleotide in the RNA being transported from the nucleus. With a ballpark figure for the approximate amount of energy required for transport, a mechanism for facilitated transport may be proposed, in which molecules of RNA too large to passively diffuse through pores are moved out through the pores an average of one nucleotide length as the energy obtained from the hydrolysis of each molecule of ATP is utilized.

Some of the crucial characteristics of nuclei and nuclear envelopes which must be explained by any proposed facilitated transport mechanism are the following. Nuclei are capable of shrinking approximately 50% in high concentrations (8-10mM) of magnesium ions. Nuclei are inhibited in all transport of RNA molecules by high concentrations of magnesium. Nuclei are capable of swelling to approximately 1.7-2.0 times their volume in RSB, heparin, etc. Nuclei transport RNA to the cytoplasm with an activation energy for the process of approximately one molecule of ATP per nucleotide in the RNA, and such transport is independent of nuclear membrane. Nuclei are observed to undulate in living cells. Nuclei are observed to contain a fine fibrillar network in their nuclear envelopes.

To explain the abovementioned phenomena, I propose that an actomyosin-like network exists in the nuclear envelope, with physical parameters similar to the actomyosin system found in muscle.

Some of the basic properties of this proposed system are as follows. Magnesium and calcium in low concentrations are necessary for stabilization of this network. Magnesium in higher concentrations induces conformational changes in the network which restrict passage through the pores, even in diffusion-mediated transport, although more will be said about this point. By analogy, maximum contraction of the actomyosin

network in muscle is approximately 50%. This is precisely the observed contraction of the nuclear radius in high concentrations of magnesium. As long as the magnesium remains, the network remains fully contracted and will not expand to its resting dimension, thus inhibiting the transport mechanism. The actomyosin-like network in nuclear envelopes contracts about 3.0nm per molecule of ATP hydrolyzed. In the analogous actomyosin network in muscle, one molecule of ATP is hydrolyzed for a contraction of the network by one G-actin subunit, or approximately 3.0nm. A network contraction of 3.0nm at the nuclear periphery (circumference) corresponds to a contraction of about 0.45nm in nuclear radius. This figure represents approximately the linear distance occupied by each nucleotide in an RNA molecule. Therefore, the hydrolysis of one molecule of ATP causes a contraction of the nuclear radius by about the linear dimension of a nucleotide in an RNA molecule. This corresponds precisely with the relationship derived independently from the Arrhenius graph, showing that one molecule of ATP was required for facilitated transport of each nucleotide in TCA-precipitable RNA to the cytoplasm.

Another property of this actomyosin-like network in nuclear envelopes is that this network is capable of approximately 20% expansion. In the actomyosin system in muscle, full extension of the network represents a 20% increase in length. A 20% increase in peripheral distance corresponds to a 20% increase in radial distance, both being first order with respect to the radius. This increase implies that a volume increase of 1.75X is possible, which is the observed maximal swelling of nuclei in RSB.

The mechanism for facilitated transport of RNA must also be asymmetric with respect to RNA passage, allowing passage of large RNA molecules outward to the cytoplasm, but not permitting passage of large RNA

molecules inward. However, diffusion-mediated transport is not unidirectional, since it is a diffusion process. Also, the results of Wise and Goldstein (Wise & Goldstein, 1973) indicate that small RNA molecules (4-6S) can shuttle back and forth between nucleus and cytoplasm, indicating the bi-directional aspect of this diffusion-mediated transport. On the basis of these properties, the following mechanism of action is proposed for facilitated transport.

Mechanism for Facilitated Transport of RNA

As an average, for each molecule of ATP hydrolyzed at the ATPase site on the molecular envelope (possibly the nuclear pores), the actomyosin-like network contracts about 3.0nm, causing an average 0.45nm contraction in nuclear radius. The RNA molecule, which is freely passable outward toward the cytoplasm, remains stationary as the nuclear envelope constricts inward 0.45nm. As the network relaxes again (expands), the RNA does not move relative to the nuclear envelope, since such movement is possible only in the outward direction. The net effect of this cycle or undulation is that the RNA molecule is expulsed an average of one nucleotide for each molecule of ATP hydrolyzed at the ATPase site in the nuclear envelope. The cycle outlined then starts again, with the complete facilitated transport of an RNA molecular dependent upon its size and the rate of hydrolysis of ATP.

As the ATP molecules are hydrolyzed over numerous sites on the surface of the nucleus, the nuclear surface contracts rhythmically through the summation of such local perturbations, and the overall effect of this rhythmic contraction and expansion is an "undulating" nuclear surface, as has been reported (Roberts & Northcote, 1970).

It does not seem reasonable to expect a pore ATPase action to be sufficient to account for such a macroscopic effect, especially since only about 10^3 pores would exist on a nucleus of the size used in this study (6900nm). My results suggest that an ATPase activity is probably present over the entire nucleoplasmic side of the inner nuclear membrane location (the amorphous lamina), as will be discussed.

In the muscle actomyosin system, myosin is a long asymmetrical molecule, with a head at one end which possesses an ATPase activity (at two sites) and two -SH groups. One of the -SH groups is readily reducible and such reduction stimulates the ATPase action. The other -SH group is not as readily reducible, and reduction of this group completely inhibits ATPase action. Brief trypsin treatment cleaves the myosin molecule in about the middle of its long tail, leaving two long segments. However, longer trypsin treatment cleaves the molecules near the head moiety. The dimension of this remaining portion of the head molecule after such trypsin treatment is about 9nm. The myosin molecules can be removed in this system by treatment with 0.6M $MgCl_2$.

From my results, it is likely that a myosin-like molecule exists in the nuclear system. Referring to Figure 16, a darkly staining band is evident on the interior of the inner nuclear membrane after trypsin treatment, which is not evident in in vivo controls. This darkly staining band is about 9nm thick, suggesting that it is the location of the heads of the myosin-like molecules. I therefore propose that the myosin-like molecules are oriented with their heads at the nucleoplasmic side of the inner nuclear membrane, and that the amorphous lamina is actually composed of such myosin-like molecular tails. Trypsin treatment removes the amorphous lamina, a procedure which is also used to extract myosin molecules

from the actomyosin system in muscle. I similarly propose that the fine fibrillar network shown in the Results section (for instance, Figure 14 and Figure 15) consists of F-actin-like molecules, polymers of the analogous subunit. Such polymers of G-actin seen in the muscle actomyosin system are seen to have a diameter of 6.0nm, in excellent agreement with the 6.0-7.0nm diameter seen in my micrographs. An intranuclear network of fibers can also be seen in some areas, extending in from the nuclear envelope. The presence of any intranuclear network depends upon the presence of RNA and/or DNA, since treatment with a mixed solution of RNAase and DNAase removes all intranuclear structure. Attempts to assay for ATPase activity, which would be associated with the heads of myosin-like molecules, were made, via the lead method. Preliminary results showed the expected overall deposition of reaction product along the membrane, although the results were very inconclusive. More work is currently underway to confirm this ATPase distribution. Similarly, experiments are underway to determine whether actin and myosin molecules exist in the nuclear membrane, via labelled antibody, although it is only claimed that the molecular components in this system are similar to actin and myosin, and not necessarily identical with them.

In the muscle actomyosin system, Ca++ ions provide the stimulus for contraction. In the nuclear system, it seems reasonable to postulate that Mg++ ions provide the stimulus for contraction, due to the singular effects of Mg++ ions upon this system.

Magnesium inhibition of facilitated transport of RNA has been observed in all release systems designed. Previously, investigators have suggested that magnesium functions by chelating ATP molecules, thus removing the necessary energy source (Schneider, 1959; Sauerman, 1974, for instance).

My results similarly show that Mg++ ions inhibit transport, even after detergent treatment has removed the nuclear membranes. When nuclei were examined in the presence of relatively high concentrations of magnesium (8-10mM), a conformational change was apparently induced in the fibrillar network structure, since the nuclei were greatly reduced in size (50%). Thus, Mg++ ions have profound effects on the nuclear structure.

If Mg++ ions were chelated by ATP, causing a reduction in available ATP, then the cross-bridges between myosin and actin filaments would be stabilized in the absence of ATP, and the network would "freeze". However, it is unclear why the network should remain fully contracted. A result which is more damaging to this proposed explanation is why diffusion-mediated release is inhibited by magnesium, since this release does not require an energy source.

It seems much more reasonable to assume that Mg++ has its effects through a binding to a nuclear structure. Since (1) transport is independent of nuclear membranes, (2) magnesium inhibits transport independently of the nuclear membranes (3) magnesium inhibits diffusion-mediated release of small molecular weight RNA independently of any energy requirement, and (4) magnesium apparently induces conformational changes in the fibrillar network (to the extent of gross size changes in nuclei), it appears plausible to postulate that the mode of action of Mg++ inhibition of RNA release is through the induction of conformational changes in the fibrillar network. Magnesium binding in the analogous actomyosin system in muscle is required for hydrolysis of ATP. The ratios of Mg++ to ATP reported by many other investigators which best stimulate release may just be manifestations of the binding of these moieties to the network structures.

It has previously been postulated that the amorphous lamina of the chromatin "holds" the pores in position. From my studies, it seems more reasonable to hypothesize that the fine fibrillar network holds the nuclear pores in position. Therefore, Mg^{++} inhibition occurs when Mg^{++} binding to the fibrils causes contraction of these structures, resulting in gross shrinking of the nuclei, and which affects the pores sterically, hindering exit of RNA through these pores. The nature of such a conformational change might be a constriction of the pore diameter, a tilting of the pore complex relative to the tangent plane at its location, fibrillar blockage of entrance to or exit from the pores, or any number of similar possibilities. Again it is tacitly assumed that RNA passes out to the cytoplasm through nuclear pores, as is the most likely alternative. From my results, it is only mandatory that the transport process proceed through some structure in the nuclear envelope which remains intact after removal of the lipid membranes. Nuclear pores are the most notable candidates, but any structure meeting these criteria and in direct physical contact with the fibrillar network would work equally well.

Many release systems require the addition of exogenous ATP (non-cancerous systems) or other nucleotide triphosphates for the transport of RNA from the nucleus to the cytoplasm. In our system and in other cancerous systems, however, the addition of exogenous ATP is no longer required. Thus, the question arises "where does the energy required for transport in such systems originate?".

One possible answer is that cancerous cells store more ATP than their non-cancerous counterparts and that this elevated concentration of ATP furnishes the energy required for transport. Work by Smuckler and co-workers (Smuckler & Koplitz, 1974) showed that ATP concentrations are

higher in cancerous cells (ATP-independent system) than in the analogous non-cancerous system (ATP-dependent) by a factor of 1.5X to 2.0X. This difference in stored ATP, however, was found to be about three orders of magnitude lower than the amount of ATP that is required as exogenously added ATP.

The presence of more ATP is important, because it could be taken as evidence that more high energy compounds in general are present in cancerous cells. It could be postulated that a binding site for ATP exists or is modified in such cancerous cells, so as to allow binding of ATP with greater precision. Thus, it could be argued that the effective concentration of ATP where it is needed is much greater than the overall effective concentration found in such systems. Some difference in pore phosphatases in cancerous vs. non-cancerous cells has been shown to exist, due to the differential effects of beryllium ions on release, as noted earlier (Schumm & Webb, 1975; Cutler, Chaudhry, & Montes, 1974), so differences in binding properties could go hand in hand with this difference.

However, another more plausible possibility appears directly applicable to the nuclear system. It is the concept of phosphagens from the analogous muscle actomyosin system. Mammalian muscle would require hydrolysis of about 10^{-3} moles of ATP per gram of muscle per minute during activity, but the amount of ATP actually present is about $5x10^{-6}$ moles/gram. Careful analysis of the ATP content and ADP content in muscles before and after a single contraction shows that there is essentially no change in the concentration of either (Lehninger, 1970). This concentrational difference between "existing" stores and requirements is almost identical to the situation found in cancerous vs. non-cancerous systems. That is, the amount of exogenously added ATP required by non-cancerous

release systems is three orders of magnitude higher than the concentration increase in ATP found in the ATP-independent cancerous systems.

In the muscle actomyosin system, it was found that phosphagens were present in muscle tissue (for instance, phosphocreatine), which are phosphate compounds other than ATP with high energies of hydrolysis, which can be used to rephosphorylate ADP to ATP. It is plausible to assume that the energy required for transport in cancerous systems is furnished by phosphagens occurring in higher concentrations in cancerous systems than in non-cancerous systems. This would explain why the ATP concentration increase in cancerous systems doesn't compensate for the ATP-independence seen in such systems.

Another finding is that facilitated transport of RNA from the nucleus to the cytoplasm is independent of the nuclear membrane. This leads directly to the question "what is the function of the nuclear membranes?", since they do not function in control of the flow of genetic material to the cytoplasm. Work by others (Horowitz and co-workers) has shown that the diffusion of small solutes into the nucleus is best explained in terms of passage of the solute molecules through nuclear pores of diameter 4.5nm, with the basic assumption that the nuclear envelope is not freely permeable to such solute molecules.

Thus, it appears that the function of nuclear membranes is to control the passage of small solute molecules. The nuclear membrane functions by allowing concentration asymmetries to exist with regard to small solute molecules, which allows for compartmentalization of functions within the nucleus as opposed to the cytoplasm. That such a function is performed by the nuclear membrane is suggested also by the transition point manifested in the Arrhenius graph of initial rates of release, where it was

concluded that the onset of facilitated transport required the membrane-governed transport of some cytosol components into the nucleus.

From this type of study with small solutes (hydrated dextrans), however, Horowitz and co-workers have extrapolated their diffusion domain to include macromolecules and have arrived at the conclusion that concentration asymmetries existing in the cell are attributable to passive processes. This conclusion is seemingly in direct conflict with the results of many other investigators, as was noted in the review of the literature. However, this conflict can be resolved. Horowitz and co-workers are examining a process which is dependent upon the nuclear membrane state, and are incorrectly extrapolating their conclusions to include transport of materials not necessarily governed by the same rules, such as facilitated transport of RNA, herein shown to be independent of the nuclear membranes.

The existence of the fibrillar network in the nuclear envelope can be utilized to explain previous results. The results of Monneron (Monneron, 1974), showing no redistribution of labelled nuclear membrane components by diffusion, can be explained on the basis of such a network existing in the membrane structure. Diffusion is restricted because this network of fibers blocks such diffusion, much the same way that a net suspended in shallow water would restrict motion of floating objects to the individual compartments of the net.

Another result of this study was the finding that the state of the cell is important to transport of RNA, both with regard to the total amount of RNA transported and with regard to the poly A content of the released material, although the results are difficult to interpret.

Using logarithmic phase nuclei, the initial rates of release of RNA are very similar with the different cytosol types. The logarithmic phase cytosol facilitates the same initial rate of release as the stationary phase cytosol (5-minute period), after which the releases are drastically different. This initial release represents what is basically diffusion-mediated release of small molecular weight RNA, although facilitated transport has already started at this time.

Using stationary phase nuclei, a slightly greater initial release is stimulated by logarithmic phase cytosol than by stationary phase cytosol, after which the rates of release are identical. It therefore appears that cytosol properties are due to the components which are necessary for the transport of RNA. An inhibitor of these transport components is apparently formed, since larger amounts of stationary phase cytosol do not stimulate logarithmic phase nuclei to release more RNA (without lysis), and in fact the addition of more stationary phase cytosol usually results in further depression of release.

When the release from logarithmic phase nuclei into logarithmic phase cytosol is compared with the release from stationary phase nuclei into logarithmic phase cytosol, the initial rates of release are approximately the same (5-minute period), indicative of diffusion-mediated transport. After this initial period, the rate of release from stationary phase nuclei is very much reduced in comparison. Thus, it can be concluded that there is no significant difference between the stored small molecular weight RNA ready for immediate transport in the logarithmic phase and stationary phase nuclei. That the release is drastically curtailed after this initial period can be predicted on the basis of the nuclear RNA distribution found in stationary phase nuclei, showing a major

reduction in the amounts of larger molecular weight RNA synthesized. This also correlates well with the fact that the poly A content of the material released by stationary phase nuclei is reduced by about 30%.

As has been alluded to, another result concerns the poly A content of the transported RNA. Logarithmic phase nuclei transport more total RNA and a higher percentage poly A+ RNA than do stationary phase nuclei. With logarithmic phase cytosol used in the assay mixture, logarithmic phase nuclei release RNA 10.9% higher in poly A content than do stationary phase nuclei. With stationary phase cytosol in the assay mixture, logarithmic phase nuclei release RNA 14.3% higher in poly A content than do stationary phase nuclei.

As regards cytosol preparations, it appears that stationary phase cytosol stimulates release of RNA which is approximately 2.7% higher in poly A content than does logarithmic phase cytosol. This result is valid for both types of nuclei. In the case of logarithmic phase nuclei, a +6.1% difference is shown. As was explained, however, this higher percentage may be due to differences in the individual preparations, since when the experiments were run simultaneously, the difference was shown to be +2.6% $\pm$ 0.6% (standard error of the mean). In general, as the cells were grown to stationary phase, it was found that the stationary phase nuclei/stationary phase cytosol system showed a poly A content about 8% lower than the logarithmic phase nuclei/logarithmic phase cytosol system, with total RNA transported being reduced about 75% or more.

SUMMARY

The facilitated transport of RNA was shown to be independent of the nuclear membranes. The existence of a fine fibrillar network was demonstrated, which controls the transport of RNA, presumably through contraction and relaxation cycles, forcing RNA through the nuclear pores. The activation energy for facilitated transport of large RNA molecules was found to be approximately 20 kcals/mole uridine in TCA-precipitable RNA. A diffusion-mediated transport for small RNA molecules was also found, with an activation energy of about 2.9 kcals/mole. Cell growth state was found to be important for release, in total amount of RNA transported, in RNA synthesized in nuclei from cultures at different cell densities, in the poly A content of the released RNA, and even in the gross size of such nuclei.

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