SEPARATION OF FREE AND MEMBRANE-BOUND POLYRIBOSOMES FROM MINERAL OIL PLASMACYTOMA 21

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
PAUL JEFFREY FREIDLIN
1976


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ABSTRACT

SEPARATION OF FREE AND MEMBRANE-BOUND POLYRIBOSOMES
FROM MINERAL OIL PLASMACYTOMA 21

By

Paul Jeffrey Freidlin

Polysomes were obtained from the myeloma cell line MOPC-21 which synthesizes and secretes an IgG-like molecule. Differential centrifugation of the total polysome population resulted in a supernatant which contained free polysomes and a pellet which contained membrane-bound polysomes. By this technique the membrane-bound polysomes were contaminated with free polysomes. Membrane-bound polysomes were separated from contaminating free polysomes by a combination of partition separation in a dextran-methylcellulose aqueous polymer two-phase system and differential centrifugation. The absorbance profile of the membrane-bound polysomes varied in a manner which appeared to be related to the physiological state of the cells. Free and membrane-associated polysomes were incubated in a cell-free system for protein synthesis. The polypeptide products were chromatographed on an anti-myeloma affinity column, but with the particular affinity chromatography system employed, the presence of newly synthesized myeloma polypeptide could not be unequivocally demonstrated.

SEPARATION OF FREE AND MEMBRANE-BOUND POLYRIBOSOMES FROM MINERAL OIL PLASMACYTOMA 21

By

Paul Jeffrey Freidlin

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1976

TO MY PARENTS,

AND TO DEDICATED, RESPONSIBLE PEOPLE EVERYWHERE...

ACKNOWLEDGEMENTS

Throughout this study I was buoyed by the encouragement and aided by the patient counsel of Dr. Ronald J. Patterson.

The Department of Microbiology and Public Health helpfully provided me with financial assistance.

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INTRODUCTION

Well defined free and membrane-associated fractions of polyribosomes (polysomes) are necessary for studies on the post-transcriptional control of immunoglobulin synthesis. The composition of these fractions may be examined to gain insight into the factors which cause some polysomes to be attached to membrane while others are not (for review see Rolleston, 1974). The fractions can also serve as a source of reactants for experiments that involve systems for cell-free protein synthesis (Uenoyama and Ono, 1972; Pryme, 1974; for review see Rolleston, 1974).

It is generally accepted that membrane-associated polysomes in eukaryotic cells are responsible for the synthesis of proteins secreted from the cell and possibly for proteins incorporated into cell membrane, while free (unattached) polysomes produce intracellular proteins (for review see Rolleston, 1974). The myeloma cell line Mineral Oil Plasmacytoma 21 (MOPC-21) has been shown to secrete an immunoglobulin-like (IgG) gamma globulin and to produce excess light chains which are degraded intracellularly instead of being secreted (Baumal and Scharff, 1973). Cell lines which secrete immunoglobulin have been shown to contain more membrane-bound RNA than nonsecreting variants (Kimmel, 1969). Cioli and Lennox (1973) and Pryme (1973) reported that polysomes associated with membrane are perhaps the only polysomes involved in immunoglobulin biosynthesis. Thus a

reasonable approach to purifying myeloma messenger RNA would be to first isolate a well defined fraction of membrane-bound polysomes.

The conventional technique used to separate membrane-bound from free ribosomes is that of zonal centrifugation in a discontinuous sucrose gradient (Bloemendal, 1974). With this technique free polysomes, shown to have a density of about 1.55 grams/cc (Dissous *et al.*, 1974), have been defined as those which sediment through 2M sucrose of density 1.26 grams/cc (Bloemendal *et al.*, 1974), while membrane-bound polysomes should float over 2M sucrose (Cioli and Lennox, 1973).

There are many problems with the use of discontinuous sucrose gradients to isolate free and membrane-bound polysomes. Long periods of ultracentrifugation are required, and Blobel and Potter (1967) have demonstrated that the conditions of centrifugation (such as length of time centrifuged) must be critically monitored in order to effect complete separation of membrane-bound and free polysomes. Cioli and Lennox (1973) and Dissous *et al.* (1974) found that the efficiency of the separation of membrane-associated from free polysomes depended on the physiological state of the cells used as the source of the polysomes. The pellet of free polysomes which results from the conventional discontinuous sucrose gradient technique can be resuspended only with great difficulty (R. Patterson, personal communication).

Another common method used to separate free from membrane-associated polysomes is differential centrifugation. Kimmel (1969) centrifuged a post-nuclear supernatant at 27,000 x g (max) for 5 minutes and used the pellet as a source of membrane-associated

polysomes, while the polysomes in the supernatant were designated as free polysomes. Parameters such as the length of time a sample is centrifuged, and the g force employed, have been shown to affect the amount of membrane-associated polysomes which pellet in fractionation steps intended only for the removal of mitochondria (Blobel and Potter, 1967). Differential centrifugation has been described as an extension of classical Svedberg considerations, one important point being that complete separation of one class of polydisperse biological molecules from another such class is not possible by differential centrifugation alone (for review see de Duve, 1975).

Consideration of the limitations of zonal and differential centrifugation led to a search for another method of separating membrane-bound from free polysomes. Albertsson (1959, 1960, pp. 177-182) used a dextran-methylcellulose aqueous two-phase system to partition free ribonucleoprotein particles from particles attached to membrane. The partition behavior of biological macromolecules has been shown to depend on many complex factors such as size, surface area, and chemical nature of the macromolecule, and type of polymer used and ionic composition of the polymer (Albertsson, 1971, pp. 314-320). It was reasoned that a combination of (1) differential centrifugation and (2) partition separation in an aqueous non-ionic polymer two-phase system would separate polysomes by their surface area in addition to the Svedberg parameters of density and molecular weight. Since a membrane-polysome complex has more surface area than a polysome alone, the combination of differential centrifugation and partition separation should provide

a more complete separation than that afforded by conventional techniques.

This study documents the successful separation of free from membrane-bound polysomes by use of a combination of differential centrifugation and partition separation in a dextran 68-methylcellulose 4000 aqueous polymer two-phase system. Attempts were made to show the presence of myeloma messenger RNA by analysis of the product made in a cell-free system for protein synthesis, but the results were equivocal.

LITERATURE REVIEW

Isolation and Description of Free and Membrane-Associated Polyribosomes

In 1955, Palade (1955) described small, 100 to 150 Å, particles with high RNA content which had affinity for endoplasmic reticulum and the second (outer) nuclear membrane. These particles did not exhibit an affinity for other cellular membranes. The particles were preferentially associated with endoplasmic reticulum in cell types that seemed to have in common a high degree of differentiation, whereas in other cell types, characterized by rapid proliferation or a less differentiated state, the particles occurred more or less freely distributed in the cytoplasm (Palade, 1955). These observations were extended to over forty different mammalian and avian cell types (Palade, 1955b). Palade and Siekevitz (1956) showed by electron microscopy and other means that membranous vesicles with attached RNA-rich particles, obtained by homogenization of rat liver cells and subsequent differential centrifugation, were derived from rough surfaced (studded with particles) endoplasmic reticulum.

When cells are homogenized in sucrose containing buffers, fragments of rough endoplasmic reticulum with attached particles form vesicles termed microsomes. The term microsome was coined by Hanstein in 1880 and applied by Claude circa 1943 to a product rich in ribonucleic acid and phospholipid found during differential

centrifugation (for review see Bernhard, 1954; Palade, 1956; Hendler, pp. 154-201, 1968; Palade, 1975). Palade and Siekevitz (1956) observed that when microsomes were treated with the detergent deoxycholate (DOC), their membranes were disrupted and solubilized leaving the RNA-rich particulate components.

Wettstein *et al.* (1963) treated a rat liver post-mitochondrial supernatant (PMS) with DOC and then centrifuged it through a discontinuous sucrose gradient to separate the particulate components, by this time known as ribonucleoprotein particles or ribosomes, from other cellular material. They showed that protein synthetic activity resided mainly in ribonuclease-sensitive aggregates of ribosomes (polysomes). Webb *et al.* (1964) observed that even without DOC treatment some polyribosomes would sediment through the 2M sucrose of a discontinuous gradient. This effect was markedly enhanced when homogenates from hepatomas were used in place of the usual rat liver homogenates. Webb *et al.* (1964) attributed this difference between cancerous and normal rat liver to differing amounts of polysomes associated with the RER, or to a difference in the type of association.

Blobel and Potter (1967) demonstrated with rat liver homogenates that many factors must be taken into consideration when discontinuous sucrose gradients are used to obtain a quantitative estimate of free and membrane-associated polysomes in a cell type. They showed that the low centrifugal force used to remove nuclei from the homogenate also removed about 35% of the cellular RNA. Washing the nuclei could not reduce the loss below 25%. Since the nuclei contained about 5% of the RNA, 20% was reasoned to be RER either

attached to the nuclei or large enough to co-sediment. Blobel and Potter (1967) found that when differential centrifugation was used to separate mitochondria from microsomes and free polysomes, a varying amount of microsomes pelleted with the mitochondria. Thus the PMS contained a variable amount of microsomes, and the quantitative estimate of the percentage of free and membrane-associated polysomes, obtained by centrifugation of the PMS through a discontinuous sucrose gradient, varied correspondingly. The amount of microsomal material co-sedimenting with the mitochondria depended on the degree of homogenization (with increased homogenization fewer microsomes pelleted with the mitochondria) and on the force used to effect separation (with increased force more microsomes pelleted with the mitochondria). Blobel and Potter (1967) also showed that depending on the force used, a certain minimum amount of centrifugation time was necessary to completely pellet the putative free polysomes in a discontinuous sucrose gradient.

Cioli and Lennox (1973) tagged membrane with ^{14}C-labeled choline. They showed that some membrane-associated polysomes from MOPC-21 tissue culture cells would pellet through 2.0 M but not 2.3 M sucrose. They suggested, based upon additional work, that this phenomenon might be common among rapidly proliferating cells. Dissous *et al.* (1974) found that rat liver polysomes of two different densities pelleted through 2.0 M sucrose. During starvation, the proportion of higher density polysomes increased. Polysomes which did not pellet through 2.0 M sucrose had a third characteristic density. Ribonuclease treatment of all three polysome fractions resulted in monoribosomes which had a unique fourth density.

Kimmel (1969), using a modification of the differential centrifugation method of Attardi (1967), centrifuged a post-nuclear supernatant (PNS) at 27,000 x g for 5 minutes and used the pellet as a source of membrane-associated polysomes, while the polysomes in the supernatant were designated as free polysomes. Differential centrifugation has been described as an extension of classical Svedberg considerations, one important point being that complete separation of one class of polydisperse biological molecules from another such class is not possible by differential centrifugation alone (for review see de Duve, 1975).

Albertsson (1959; 1960, pp. 177-182) used a dextran-methylcellulose aqueous polymer two-phase system to partition free ribonucleoprotein particles from particles attached to membrane. The partition behavior of biological macromolecules has been shown to depend on many complex factors such as size, surface area, and chemical nature of the macromolecule, and type of polymer used and ionic composition of the polymer (for discussion see Albertsson, 1971, pp. 314-320). Aqueous polymer two-phase systems have recently been used to isolate plasma (cell surface) membranes (dextran-polyethylene glycol, Lesko *et al.*, 1974; Brunette and Till, 1971) and chromosomal deoxyribonucleoprotein (dextran-polyethylene glycol, Turner *et al.*, 1974), and to perform sensitive assays for antibody and different conformations of antigen with nanogram amounts of material (dextran-polyethylene glycol, Reese *et al.*, 1973).

In summary, free and membrane-associated polysomes have been operationally defined by differences in their behavior upon zonal centrifugation, differential centrifugation, and partition separation

in polymer two-phase systems. They have also been visualized by electron microscopy. Functional differences, that is, differences in the types of protein produced by each fraction, also exist as will be discussed later in this review.

Different Modes of Attachment of Polyribosomes to Microsomal Membrane

Palade and Siekevitz (1956) treated microsomes obtained by differential centrifugation of rat liver homogenates with versene (ethylenediaminetetraacetate-EDTA) and ribonuclease (RNase). When the microsomes were reisolated by differential centrifugation, it was found that approximately 50% of the microsomal RNA had been released from the microsomes. Electron microscopy revealed that the loss of RNA was correlated with a loss of the particulate fraction (ribosomes) attached to the microsomal membrane. Washing and reisolation of untreated microsomes removed a smaller number of ribosomes from the membrane.

The concentration of the divalent cation Mg^{++} is critically important for polysomal stability--at concentrations lower than 1.0 mM ribosomes begin to dissociate into their subunits, while polysomes begin to aggregate at concentrations higher than about 5 mM (Palade and Siekevitz, 1956; Hamilton and Petermann, 1958; Girard *et al.*, 1965; Breillatt and Dickman, 1966). Palmiter (1974) found that approximately 10% of the total amount of ribosomes would precipitate at 5 mM Mg^{++}, and he has used higher concentrations of Mg^{++} to quantitatively precipitate polysomes which were recovered in undegraded, biologically active form. Girard *et al.* (1965) found that EDTA treatment dissociated ribosomes and polysomal

associated ribosomes into their two subunits (presumably by chelating Mg^{++} to a concentration too low to maintain stability). These subunits, however, sedimented at reduced S values. If the EDTA treated subunits were returned to a cytoplasmic extract at 1.5 mM Mg^{++}, the subunits again sedimented at their higher S values. The cause of the shift in S value was not determined, but it seemed to require cytoplasmic extract and was not only a Mg^{++}-induced effect.

Sabatini *et al.* (1966) brought about the stepwise dissociation of ribosomes from guinea pig hepatic microsomal membrane with increasing concentrations of EDTA. Almost all of the small subunits were released first, and then increasing amounts of large subunits were released. The initial release of small subunits suggested that ribosomes were attached to membrane by their large subunits. They supported this interpretation with electron microscopy findings. Approximately 30% of the RNA remained bound to microsomal membrane even after treatment with high concentrations of EDTA. This residual RNA was found to be mainly associated with large subunits. The particles resistant to detachment by EDTA contained approximately 70% of newly synthesized protein.

Rosbash and Penman (1971) used EDTA, puromycin and RNase to dissociate HeLa cell ribosomes and mRNA from membrane. The released material was called "loose", in contrast with the "tight" material which remained attached to membrane. Ribosomes resistant to the action of EDTA were also resistant to the action of puromycin and RNase. A combination of EDTA and puromycin released more mRNA than EDTA alone. The loose ribosomes had a higher density (1.55 gm/cc) than the tight ribosomes (heterogeneous, mean density 1.49 gm/cc)

(Rosbash and Penman, 1971b). Bleiberg *et al.* (1972) incubated mouse myeloma cells with NaF, an inhibitor of protein synthesis, and found that approximately 25% of the membrane-associated ribosomes were released during the incubation. Addition of 1 M KCl to an homogenate from NaF treated cells increased the amount of released ribosomes to nearly 100%, while KCl alone released about 50% of the ribosomes. EDTA treatment released all the small subunits and approximately 50% of the large subunits. EDTA and 1 M KCl each released approximately 40% of the bound mRNA, while little or no mRNA was released by incubation with NaF. Incubation with NaF appeared to release a fraction of the same ribosomes released by EDTA. Sarma *et al.* (1972) treated rats with etionine or CCl_4. Hepatic microsomes isolated from these rats released a larger amount of ribosomes on exposure to 0.4 M KCl than microsomes from saline treated rats. Sarma *et al.* (1972) also studied ribosomal attachment to mouse liver microsomal membrane. High salt plus puromycin was more effective than high salt alone in detaching ribosomes from membrane. Neither RNase nor puromycin alone could effect release of ribosomes; moreover, high salt plus RNase was not more effective than high salt alone. High salt treatment with other monovalent cations, Li^+, Na^+, and Ca^+, had the same effect as treatment with high K^+ concentration.

Rosbash and Penman (1971a), Bleiberg *et al.* (1972), and Sarma *et al.* (1972) reported that ribosomes were released from membrane in the form of monomers or subunits, but not polyribosomes.

Adelman *et al.* (1973a) used a combination of differential centrifugation and zonal centrifugation to obtain a pure but degraded

fraction of rat liver microsomes. In a companion paper (Adelman *et al.*, 1973b), they reported that up to 40% of ribosomes (in the form of subunits) could be released by high KCl concentrations, with an additional 40% released when puromycin was included with the KCl. Treatment with puromycin at low salt concentration did not result in ribosome detachment. A fraction, approximately 15%, of the ribosomes could only be released from membrane under conditions which caused extensive unfolding of ribosomal subunits.

Segregation of Polyribosomes into Free and Membrane-Associated Fractions

The preceding sections of this review illustrate that it has been fairly well documented and accepted that some polyribosomes are bound to endoplasmic reticulum while others are not, and that there are at least two classes of membrane-associated polysomes--a tightly and a loosely bound class as defined by various criteria (for review see Rolleston, 1974). The molecular events which are necessary and sufficient to cause a selected fraction of polysomes to become membrane-associated have not yet been elucidated. The remainder of this review documents other approaches taken to gain insight into the molecular events affecting polysomal segregation.

i. Functional Differences

It is generally accepted that membrane-associated polyribosomes are responsible for polypeptides destined to be secreted or incorporated into cell membrane, while free polyribosomes produce polypeptides for intracellular use (except incorporation into intracellular membrane). The data gathered so far support the above generality (for comprehensive review see Rolleston, 1974).

Whether tight or loose membrane-associated polysomes produce the same polypeptides remains an unresolved question. Tanaka and Ogata (1972) found that a distinct amount of ribosomes, approximately 25%, was released by RNase treatment of rat liver microsomes. Nascent serum albumin was found almost exclusively on the tight (RNase resistant) ribosomes. Nascent immunoglobulin has also been reported to be almost exclusively on tight (KCl resistant) ribosomes (Zauderer and Baglioni, 1972). Harrison *et al.* (1974a,b) found that both loose and tight (KCl released, unreleased, respectively) fractions contained immunoglobulin mRNA. Zauderer *et al.* (1973) showed that high salt treatment, EDTA exposure, and repeated washing through 15% to 30% sucrose gradients released approximately equivalent fractions of ribosomal RNA. In contrast to other investigators, they found a significant amount of loose RNA to be polysomal instead of monosomal or in the form of subunits. Histone mRNA (7-9S RNA) was found in the free and loose fractions, but was essentially excluded from the tight fraction. Rosbash (1972) reported that inhibition of protein synthesis with cycloheximide prevented attachment of 35% to 50% of newly synthesized mRNA.

ii. Affinity of Ribosomes for Membrane

A great deal of research has been directed towards finding differences between the affinities of free and membrane-associated ribosomes for membrane. Baglioni *et al.* (1971) found that 60S subunits of mouse myeloma cells attached to membrane even when protein synthesis was inhibited by cycloheximide, and that these 60S subunits were not in equilibrium with free 60S subunits. Borgose *et al.*

(1973) demonstrated that membrane-bound rat liver ribosomes would exchange small subunits but not large subunits with free ribosomes. Ekren *et al.* (1973) and Rolleston (1972) determined that the binding capacity of stripped RER was greater for large ribosomal subunits than for small subunits (ribosomes must be removed from RER before new ribosomes can be added, Pitot and Shires, 1973). Rolleston (1972) found no difference in binding to stripped RER between free and membrane-associated ribosomes. Rolleston and Mak (1973) reported no difference in binding to stripped RER between free and membrane-associated polyribosomes. Binding to smooth endoplasmic reticulum (SER) was sensitive to ionic strength, moderate binding occurring at 25 mM KCl and very low binding at 100 mM KCl. Pitot and Shires (1973) also found no difference between the binding of free or membrane-associated polyribosomes to membrane. They classified binding reactions into two types, a temperature-independent type observed only on derivatives of RER, and a temperature-dependent type which requires incubation of the polysomes with membrane at 25-37°C. They also advanced the concept of a *membron*, a "functioning, regulatable, translating polyribosome complex with a specific surface area of membrane." Rolleston and Lam (1974) reported that smooth endoplasmic reticulum binds ribosomes with the same affinity as RER, but has fewer binding sites.

iii. Protein Composition of Subunits

Another line of investigation has been directed towards finding differences in the protein make-up of ribosomal subunits from free and membrane-associated ribosomes. Borgese *et al.* (1973) found that

the protein complement of subunits from rat liver free and membrane-associated ribosomes was similar except for one band in polyacrylamide gel electrophoresis (PAGE) which was more intense in free large subunits. Hanna *et al.* (1973) found an additional protein in rat liver free monosomes analyzed by two-dimensional PAGE; however, the protein pattern for free and membrane-associated monosomes was identical when both were treated with DOC. Fehlmann *et al.* (1975) used two-dimensional PAGE to analyze rabbit reticulocyte ribosomal proteins. They found two proteins which seemed unique to the free monosomes and four proteins which seemed to belong only to the membrane-associated monosomes. Kaulenas and Unsworth (1974) reported a difference in the protein complement of the large subunits of ribosomes from adult mouse liver and kidney. The difference became apparent from 11 days to 14 days of gestation, coinciding with the time of initiation of kidney differentiation. Starvation (Hanna and Godin, 1974) and hepatectomy with accompanying regeneration (Sheinbuks *et al.*, 1974) resulted in a change in the protein complement of both subunits of rat liver ribosomes. Hanna and Godin (1974) reported that the free and membrane-associated ribosomes underwent an identical change when rats were starved.

Differences have been sought in the pattern of phosphorylation of free and membrane-associated ribosomal proteins. Egly *et al.* (1972) found that newly phosphorylated protein was mainly associated with DNA-like RNA, but could often be co-isolated with polysomes due to the sedimentation characteristics of the ribonucleoprotein particle. Pierre *et al.* (1974) reported that rat liver ribosomal phosphoproteins occurred primarily in the small subunits. They found

a difference in the pattern of phosphorylation of free and membrane-associated polysomes.

iv. Non-Ribosomal Proteins Associated with Ribosomes

Non-ribosomal proteins associated with ribosomes have been studied only with great difficulty, due to suspected artifacts of non-specific adsorption inherent in isolation procedures. Olsnes (1970) observed the adsorption of solubilized RER proteins to polysomes. The adsorbed proteins could be removed by DOC alone, or by a combination of Triton X-100 and high salt concentration. Henshaw *et al.* (1973) and Hirsch *et al.* (1973) reported the isolation of two types of Ehrlich ascites small ribosomal subunits, those found free in the cytoplasm and those derived from polyribosomes. The small subunits found free in the cytoplasm were shown to be combined with substantial amounts of non-ribosomal protein, and would not join with large subunits until this protein had been removed by treatment with 0.5 M KCl. Shires *et al.* (1971) found that trypsin treatment under mild conditions destroyed the ribosome binding capacity of stripped RER. Kreibich and Sabatini (1974) reported that smooth and rough endoplasmic reticulum had similar sets of proteins with the exception of the ribosomal-associated proteins on RER.

v. Relation Between Nascent Polypeptide and RER

Since functional differences have been found between free and membrane-associated polysomes, it is reasonable to consider the relationship between nascent chain and RER as a possible factor in

the attachment of polysomes to RER. Relevant studies which employed puromycin to release nascent chain have already been presented in the section on different modes of attachment of polyribosomes to microsomal membrane.

Other studies have examined the fate of polypeptides synthesized by membrane-associated polysomes. Vectorial discharge, through membrane into a cisternal space, of polypeptides synthesized on ribosomes attached to RER or microsomal membrane has been fairly well documented and accepted (Redman and Sabatini, 1966; Redman *et al.*, 1966; for review see Palade, 1975). Sabatini and Blobel (1970) reported that controlled proteolysis of microsomes resulted in two broad categories of nascent chain fragments: smaller carboxy-terminal segments which were protected by the large subunit and larger membrane-associated fragments which depended on intact membrane for their protection. Kreibich *et al.* (1973) showed that the vesicular content of microsomes could be released by low levels of detergent insufficient to produce an extensive change in microsomes other than the reversible formation of openings in membrane which may allow leakage of contents.

vi. Kinetics and Composition of Free and Membrane-Associated RNA

Free and membrane-associated ribosomal and messenger ribonucleic acids have been examined for differences in kinetic behavior or base composition. Loeb *et al.* (1967) reported that the base composition and rate of synthesis of ribosomal RNA was the same for both free and membrane-associated ribosomes in rat liver. Mishra *et al.* (1972) found the same decay pattern and half-life for free

and membrane-associated rat liver ribosomal RNA. Murty and Sidransky (1972) reported that for hepatic mRNA, free polysomal mRNA appears to have a short half-life as compared with mRNA of membrane-associated polysomes. Storb (1973) examined the half-life of mRNA (poly(A)+ RNA) in myeloma cells and found that microsomal poly(A) + RNA had a longer (by a factor of about 4) half-life than free poly(A)+ RNA.

Attardi and Attardi (1967) showed that HeLa cell membrane-associated mRNA had a base composition very different from that of free polysomal mRNA. The membrane-associated mRNA was especially high in adenylic acid. Baglioni *et al.* (1972) found that myeloma cell free and membrane-associated mRNA contained similar poly(A) sequences. Stevens and Williamson (1972) isolated poly(A)-containing immunoglobulin mRNA from the cytoplasm and nucleus (hnRNA) of myeloma cells. This mRNA contained messengers for both heavy and light chains. Poly(A) sequences may be involved in the attachment of polysomes to microsomal membrane (Milcarek and Penman, 1974; Lande *et al.*, 1975).

vii. Proteins Bound to mRNA

Messenger RNA, independent of ribosomal subunits, has been found associated with protein. The relationship of this protein to RER or to ribosomes has not yet been clearly established. Whether membrane-associated ribonucleoprotein (RNP) complexes contain the same or different protein complements as free RNP complexes is still unresolved.

Baltimore and Huang (1970) reported that a heterogeneous group of HeLa cell soluble cytoplasmic proteins could bind to all types

of RNA. The bond was unstable at high ionic strength. Lee *et al.* (1971) released mRNA from polysomes by starvation of mouse sarcoma 180 ascites cells and showed that the mRNA was complexed with specifically bound protein--the protein could not be removed by high salt concentration, nor would it significantly bind to nuclear RNA. Milcarek and Penman (1974) reported an association of HeLa cell microsomal membrane and poly(A). Lande *et al.* (1975) found an association between human diploid fibroblast microsomal membrane and mRNA at or near the 3' poly(A) end. Kwan and Brawerman (1972) found a particle of apparent protein composition attached to the poly(A) segment of mouse sarcoma 180 mRNA. Two proteins, a larger one of about 78,000 Daltons and a smaller one of about 50,000 Daltons, have been reported bound to ribosome-free polysomal mRNA of mouse L cells and rat hepatocytes (Blobel, 1973) and chick cerebra (Bryan and Hayashi, 1973). Blobel (1973) found that the larger protein was bound to the poly(A) sequence of mRNA. Gander *et al.* (1973) prepared a post-ribosomal supernatant from which mRNA-protein complexes were isolated. They demonstrated that none of the complexed proteins was identical to any of the proteins associated with polysomal mRNA, and that some of the proteins were phosphorylated and contained phosphoserine. Immunoglobulin has been reported to bind to mRNA (for review see Stevens and Williamson, 1974), and to ribosomes (Moav and Harris, 1970; Eschenfeldt and Patterson, 1975).

viii. RNA Other than mRNA and rRNA

Little work has been done on the role of transfer RNA (tRNA) in the segregation of free and membrane-associated polysomes. Jones

and Mach (1973) reported that met-$tRNA_F$ provides N-terminal methionine while met-$tRNA_M$ provides internal methionine in mouse myeloma cells. Darnbrough *et al.* (1973) showed that in reticulocyte cell lysates, met-$tRNA_F$ can combine with the small ribosomal subunit independently of mRNA. Gerlinger *et al.* (1975) examined the translation of oviduct and reticulocyte polysomal RNA in a tRNA dependent Krebs-II ascites cell-free system. They found that translation proceeded better when both tRNA and polysomes were from the same tissue than when the tRNA came from a heterologous source. Shafritz (1973a,b) demonstrated that a significant amount of mRNA in polysomes is not translated in rabbit liver cell-free systems.

A low molecular weight RNA, termed translational control RNA (tcRNA), has been found which affects the binding of mRNA to small subunits, and met-tRNA to polysomes, in a rabbit reticulocyte cell-free system (Kennedy *et al.*, 1974).

ix. High Salt Wash "Factors"

A heterogeneous mix of initiation factors, tRNA, diverse proteins and other unassigned material is obtained by exposing free and membrane-bound fractions to high concentrations of salt (usually KCl). After exposure to high salt, the ribosomes are pelleted and the supernatant dialyzed to a salt concentration compatible with polysomal stability. This dialysate is termed a high salt wash.

Membrane-associated polysomes (derived from detergent solubilized microsomes) from rabbit liver (Shafritz and Isselbacher, 1972) and myeloma cells (Abraham *et al.*, 1974) have been reported to show a marked preference for homologous high salt wash (that is,

a high salt wash from microsomes as opposed to free polysomes) as assayed by increased translation in cell-free systems for protein synthesis. A corresponding preference by free polysomes for homologous wash was not observed by Abraham *et al.* (1974). Uenoyama and Ono (1972) mixed free and membrane-associated ribosomes, mRNA, and high salt wash in different combinations in a cell-free system for protein synthesis. When the components of the cell-free system were homologous, better translation resulted than when the components were not all from the same source. Abraham *et al.* (1974) showed that chain initiation on membrane-associated polysomes was stimulated only by a microsomal high salt wash, whereas initiation on free polysomes was stimulated by either free or microsomal wash. Pryme (1974) reported that membrane-associated polysomes from myeloma cells (MPC-11) in the G1 phase could be stimulated in a cell-free system for protein synthesis by either G1 or G2 high salt wash, but that G2 membrane-associated polysomes could only be stimulated by G2 wash.

MATERIALS AND METHODS

Cells

Mouse plasmacytoma cells maintained in tissue culture were used in this study. The myeloma cell line Mineral Oil Plasmacytoma 21 (MOPC-21) secretes an immunoglobulin-like (IgG) gamma globulin and produces excess light chains (of the k class) which are degraded intracellularly instead of being secreted (Baumal and Scharff, 1973). Three other cell lines were employed. The XC.1 cell line neither secretes nor contains intracellular immunoglobulin. The S49.1 cell line consists of lymphoma cells derived from thymus (T) cells which do not produce detectable amounts of gamma globulin. Cells of the S180 line are not part of the immune system and do not produce gamma globulin. All lymphoid cell lines used in this study were kind gifts of the Cell Distribution Center, Salk Institute.

The S180 cell line was maintained in ascites form in Swiss Webster mice. MOPC-21 cells, in addition to being grown in tissue culture, were also maintained in ascites form in BALB/c mice. The MOPC-21 cells were passaged in the peritoneum at approximately 7- to 10-day intervals.

The MOPC-21, XC.1, and S49.1 cell lines were maintained *in vitro* in tissue culture. The culture medium consisted of Dulbecco's Modified Eagle Medium (GIBCO) supplemented with antibiotics (75 micrograms/ml streptomycin, 100 units/ml penicillin, 40 units/ml

mycostatin) and 10% fetal calf serum. Tissue culture cells were maintained in roller bottles which were flushed with 95% air, 5% CO_2, sealed and incubated at 37°C at a rotation speed of one-half revolution per minute. Typically, MOPC-21 cells were grown to a density of 4 to 8 x 10^5 cells/ml and diluted to 2 to 4 x 10^5 cells/ml with fresh medium.

The MOPC-21 cell line was periodically checked for antibody (myeloma protein) production. MOPC-21 cells were grown in tissue culture and pelleted by centrifugation. The supernatant medium received ammonium sulfate to 50% saturation in order to precipitate the gamma globulin fraction. The precipitate was centrifuged (3500 x g max for 30 min) resuspended in distilled water, and reprecipitated with 40% saturated ammonium sulfate. This precipitate was centrifuged, resuspended in distilled water, and reprecipitated with one volume of saturated ammonium sulfate added dropwise by burette. The final precipitate was centrifuged, resuspended in distilled water and dialyzed against buffer (20 mM Tris, pH 7.4, 150 mM NaCl). Ouchterlony immunodiffusion revealed one line against specific antisera.

The MOPC-21 cell line was routinely checked for mycoplasma contamination. The method used, observation for cytoplasmic incorporation of radioactive thymidine, yielded results which were consistently negative (that is, indicative of no contamination).

Enzyme Assays

Assay parameters (proper reagent concentrations, temperatures, etc.) for the assay of 5'-mononucleotidase and glucose-6-phosphatase were generously provided by Dr. Walter J. Esselman.

Glucose-6-phosphatase is a conventional marker for microsomal membrane. To assay for it a stock solution was prepared of EDTA (pH 6.5), histidine (pH 6.5), glucose-6-phosphate (adjusted to pH 6.5 with 1 N HCl), and deionized 1X glass distilled water. When combined with the proper volume of enzyme fraction, this stock solution gave the following final concentrations of reagents: 1 mM EDTA, 7 mM histidine, and 40 mM glucose-6-phosphate. The total volume of the reaction mixture was 0.5 ml. The reaction mixture was incubated at 37°C for 20 minutes, at which time the reaction was stopped by the addition of 2.5 ml of 10% TCA. The mixture was then cooled on ice for 15 minutes. Any precipitate was removed from the mixture by filtration (with a Swinney adapter and a 10 ml syringe) through Whatman GF/C glass fiber filters (2.4 cm). Alternatively, the precipitate was removed by centrifugation at 1,500 x g (max) for 15 minutes in an International refrigerated centrifuge. Phosphate was determined by the method of Fiske and SubbaRow by using a Fisher Gram-Pac (Fisher A974 ANS) pre-mix of 1-amino-2-naphthol-4-sulfonic acid--dry mixture. One and eight-tenths milliliter of the precipitate-free enzyme reaction mixture was added to 2.5 ml of deionized 1X glass distilled water. To this 0.5 ml of 25 gm/l ammonium molybdate in 5N H_2SO_4 was added followed by 0.2 ml of Fisher A974 ANS. After 10 minutes the spectral absorbance at 660 nm was read. The blank was an identically treated sample which had received buffer in place of buffer plus enzyme fraction.

The assay for 5'-mononucleotidase, a conventional marker for plasma membrane, depended on a different stock solution but otherwise followed the same procedure as the glucose-6-phosphatase assay.

The stock solution for the 5'-mononucleotidase assay was prepared with AMP (adjusted to pH 7.0 with 1.0 N NaOH), glycine (pH 9.1), $MgCl_2$, and deionized 1X glass distilled water. When combined with the proper volume of enzyme fraction this stock solution gave the following final concentrations of reagents: 5 mM AMP, 100 mM glycine, and 10 mM $MgCl_2$.

A standard curve was constructed using Na_2HPO_4 in buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris, pH 7.4, 0.25 M sucrose, 100 µg/ml heparin; this buffer is abbreviated as RSB[such]). Two standard concentrations of Na_2HPO_4 were included with each experiment.

Construction of the Aqueous Two-Phase Polymer System

A 10% (w/w) solution of dextran 68 (D68) (Sigma Type 2000) was made according to the method of Albertsson (1960, pp. 28-29). The undried dextran was first wetted and mixed to a paste with a small amount of sterile, deionized 1X glass distilled water. The rest of the water was then added and the dextran dissolved by stirring and slowly heating the mixture to boiling. The flask was then covered and the solution allowed to cool. Typically this step involved 5 gm of D68 plus 45 gm of water. A 1% (w/w) solution of methylcellulose 4000 (MC4000) (a kind gift of Dow Chemical Company) was also prepared according to the method of Albertsson (1960, pp. 31-32). The methylcellulose was dried at 110°C for 16 to 24 hours. Two hundred grams of a 1% (w/w) solution of MC4000 was prepared in the following way: 2.0 gm of dry MC4000 were weighed into an Erlenmeyer flask and 100 gm hot (80-90°C) sterile, deionized 1X glass distilled water added. The flask was closed and shaken

vigorously for a few minutes in order to wet the powder. Ninety-eight grams of cold water were then added, the flask shaken and allowed to stand with occasional stirring until it reached room temperature. The powder then swelled and dissolved slowly. Care was taken not to allow material to sediment to the bottom of the flask. The solution was then cooled to 4°C and kept at this temperature.

To make the stock solution the following constituents were added in the given order to an approximately 200 ml capacity bottle with rectangular sides: (1) 13.6 gm of a 10% (w/w) solution of D68; (2) 72.0 gm of a 1% (w/w) solution of MC4000; (3) 0.5 gm of 0.2 M $MgCl_2$; (4) 0.5 gm of 0.2 M K_2HPO_4; and (5) 13.4 gm of water (after Albertsson, 1959). The bottle was then placed almost horizontal and parallel to the body of the person making the stock solution, at which time the bottle was rotated 21 rotations toward the person and 21 rotations away from the person, at a speed of about 1 rotation/sec, to mix the components. Directions for mixing were worked out during the course of this study and should be followed because formation of the phases is a highly empirical process. The stock solution, routinely stored at 4°C in a standard refrigerator, kept well for at least 4 months with only minimal attention given to sterile technique. This stock solution, when diluted 1:1 with a polysome fraction, gave the desired phase system.

To construct the phase system, the stock solution was mixed as described above and 2.5 gm were withdrawn with a 10 ml pipette and deposited in a 15 ml Corex centrifuge tube. The following components were added in the order given to the 2.5 gm of stock: (1) 0.05 ml

of heparin at 10 mg/ml; (2) 0.8 ml of 15% (w/v) RNase-free sucrose in water; (3) 0.01 ml of 0.2 M K_2HPO_4; (4) 0.64 ml of RSB made up with 100 µg/ml of heparin [RSB(HEP)]; and after mixing the Corex tube 10 rotations in a manner similar to that described in mixing the stock solution, (5) 1.0 ml of crude 27,000 x g membrane pellet (MP1) suspended in RSB(such).

The Corex tube was then rotated in a manner similar to that described above. The tube was then lowered to an approximately 45° angle with an axis perpendicular to the floor, and subsequently rotated around the axis 10 times clockwise and 10 times counter-clockwise. Finally, the tube was rocked from almost perpendicular to almost horizontal position 10 times. This mixing algorithm was important to follow, since other mixing procedures did not allow consistent formation of the two phases.

After mixing, the aqueous polymer system was centrifuged for 10 min at 1,400 x g max in an International refrigerated centrifuge to separate the phases. It should be mentioned that all procedures were done at 4°C in order to minimize RNase degradation of polysomal material. This low speed centrifugation consistently allowed formation of two phases from a properly mixed aqueous polymer system.

The final weight of the aqueous polymer two-phase system was 5.0 gm, which was equivalent to 5.0 ml since the density of the system was very near that of water. The bottom phase was of course denser than the top phase but, curiously, it was much less viscous. The final concentration of each component in the system was 0.68% (w/w) D68, 0.36% (w/w) MC4000, 133 µg/ml of heparin/ml, 1.4 mM

Mg^{++}, 0.9 mM K_2HPO_4, 3.2 mM Tris, pH 7.4, 3.2 mM NaCl, and 0.12 M RNase-free sucrose.

Isolation of Free and Membrane-Bound Polysomes

Figure 1 presents a general schematic of the procedure used to isolate free and membrane-bound polysomes. Cells were grown to a density of usually 5 to 9 x 10^5 cells/ml. If labeled polysomes were desired, 0.5 or 1.0 µC/ml of ^{3}H-uridine was added 10 to 20 hours before termination of the incubation period. At the end of incubation, cells were poured over crushed frozen saline and pelleted for 8 minutes at 500 x g (max) in an International refrigerated centrifuge. Unless otherwise indicated, all procedures were done at 4°C. The cells were resuspended in RSB(hep) and allowed to swell for 15 minutes. They were then recentrifuged and resuspended in approximately one volume of RSB(such) in a 7 ml Kontes Dounce homogenizer. Immediately after resuspension in the sucrose buffer, the cells were Dounced 10 times with a tight pestle. This resulted in greater than 90% lysis of cells; however, if the cells were allowed to equilibrate in the sucrose buffer for about 10 minutes, the efficiency of lysis was reduced to 50 to 70%. The nuclei were then pelleted at 900 x g (max) for 5 min and washed once with RSB(such). The wash was added to the first supernatant and the total supernatant was coded PNSW (post-nuclear supernatant plus wash). The PNSW was then spun at 27,000 x g (max) for 5 minutes in a Sorval RC2-B centrifuge. The supernatant (PMS1--first post-mitochondrial supernatant) was decanted. It contained crude free polysomes. The pellet (MP1--first pellet), which contained the crude microsomal material, was resuspended

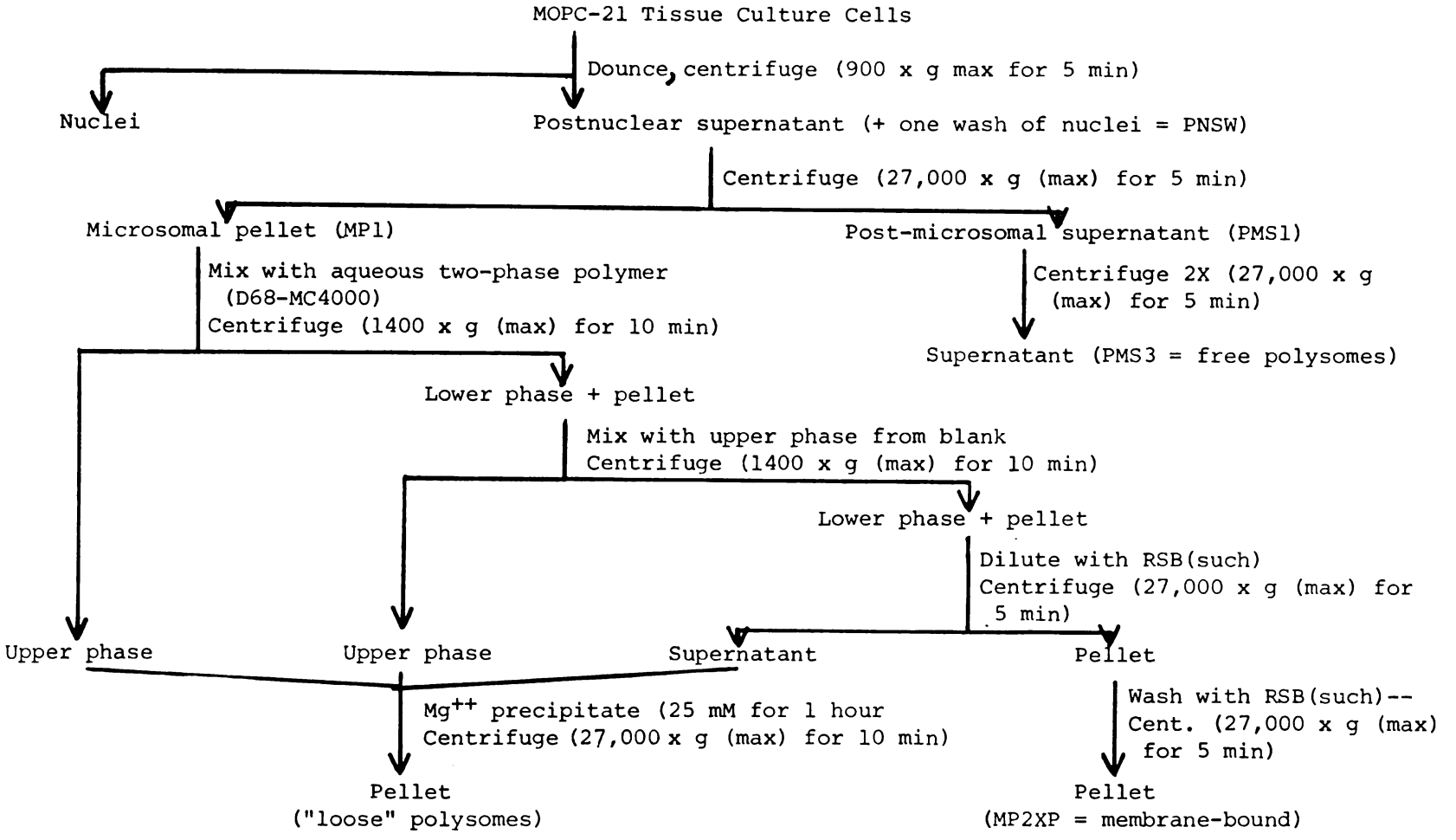

Figure 1. General schematic of the procedure used to separate membrane-bound from free polysomes.

in RSB(such) and frozen at -80°C for later use. The supernatant was then centrifuged two more times at 27,000 x g (max) for 5 minutes. This supernatant (PMS3) was used as a source of free polysomes. For early experiments, including those in which enzyme assays were performed, the PMS1 was chromatographed on Sepharose 6B (Pharmacia). The procedure was similar to that of Eschenfeldt (thesis, 1975, p. 27), except that a different elution buffer was used, namely RSB(such). A column (1.5 x 15 cm) was poured and washed with at least 10 bed volumes of RSB(such). PMS1 was applied to the column in a volume of 2.5 ml or less. The polysomes were eluted at a flow rate of approximately 10 ml/hr with RSB(such). Polysomes were eluted in the void volume. The cloudy, white fractions were combined and designated PMS,S1 (the extra S indicated that the PMS1 had been chromatographed on Sepharose 6B). The PMS,S1 was then centrifuged two more times (27,000 x g (max) for 5 minutes), labeled PMS,S3, and used as a source of free polysomes. Storage was at -80°C. After thorough washing with RSB(such), the column could be reused. The column was periodically treated with a solution of 0.1% diethylpyrocarbonate (Calbiochem) in RSB(such).

The previously frozen MP1 was routinely processed within a few days of freezing. MP1 was partitioned as described in the section "Construction of the Aqueous Two-Phase Polymer System." The bottom phase was consistently cloudy and contained the membrane-bound polysomes. Some material would always pellet. After the first partition separation, the clear top phase was withdrawn with a 10 ml pipette and saved. To the bottom phase was added the top phase of a blank made with RSB(such) in place of MP1. Without resuspending

the pellet, the new system was mixed and centrifuged as described previously. The clear top phase was again withdrawn and saved. The bottom phase was then diluted with 5 ml of RSB(such), and the contents mixed with a 10 ml pipette, resuspending the pellet in the process. The contents of the tube were then distributed evenly to other 15 ml Corex tubes so that no one tube would hold more than 3 ml. The tubes were then centrifuged for 5 minutes at 27,000 x g (max) in a Sorvall RC2-B centrifuge. The supernatants were decanted and combined with the previous two top phases. The pellets were combined and resuspended in 2 ml of RSB(such) and recentrifuged at 27,000 x g (max) for 5 minutes. The supernatant was decanted and added to the other supernatant plus top phases. This mixture was distributed evenly among Corex tubes (15 ml size) so that no tube would hold more than 6 ml. The supernatant polysomes were then Mg^{++} precipitated as described in the section "Mg^{++} Precipitation of Polysomes." These supernatant polysomes were termed "loose"; they were found in the crude 27,000 x g (max) pellet but they did not stay with the microsomes upon partitioning and further differential centrifugation. The pellet was resuspended in approximately 1 ml of RSB(such). Resuspension of membranous material was commonly accomplished with the aid of a small (approx. 2 ml) homogenizer. The polysomes in this final pellet were termed "tight" and were coded MP2XP for membrane-bound polysomes, twice partitioned. The MP2XP fraction was either frozen immediately or Mg^{++} precipitated and then stored at -80°C.

Mg^{++} Precipitation of Polysomes

The polysome fraction to be precipitated was made 25 mM with $MgCl_2$ and allowed to sit for one hour at 4°C. If no membrane was desired, the fraction was first given 100 µg/ml heparin, then treated for 5 minutes with 0.5% TX-100 (v/v), and then precipitated as above. The precipitate was centrifuged for 10 minutes at 27,000 x g (max), then resuspended in 20 mM HEPES (Sigma) buffer, pH 7.4. The polysomes were then stored at -80°C until needed. The magnesium precipitation procedure is a variant of a technique presented by Palmiter (1974).

System for Cell-Free Protein Synthesis

The system was a modification of a system developed by Marcu and Dudoch (1974). Reactions were done in a total volume of 0.5 ml or 1.0 ml unless otherwise noted. Final concentrations of reactants were as follows: (a) 100 mM KCl, 7 mM Mg acetate, 20 mM HEPES (pH 7.4), 6 mM 2-mercaptoethanol; (b) 1 mM ATP, 0.02 mM GTP, 8 mM creatine phosphate; (c) 2.325 or 4.65 units/ml creatine phosphokinase; (d) 25×10^{-6} M amino acids other than those in the ^{3}H-L-amino acid mix which was added to 4 µC/ml; (e) high speed supernatant at approximately a final concentration of 0.1 mg protein/ml and 0.006 mg nucleic acid/ml; and (f) varying amounts of polysomes generally ranging from 1 to 5 A_{260} units/ml. Reactant groups (a) and (b) were stored as pre-mixed stock solutions at -20°C. All reactant groups were 10-fold concentrated and thus were diluted 1:10 for the reaction. For reactant groups (b) and (d), the pH was adjusted to about neutrality (7-7.5). Creatine phosphokinase was usually diluted in RSB(such) and stored at -20°C.

High speed supernatant was prepared by centrifugation of a PNSW, or a PMS, at 250,000 x g (max) for 90 minutes at 4°C in a Beckman SW 50.1 rotor in the Beckman Model L5-50 ultracentrifuge. The supernatant was dialyzed against two changes of 100 volumes of buffer adjusted to about pH 7.4 (20 mM HEPES, pH 7.4, 2 mM 2-mercaptoethanol, 2.5 mM Mg acetate, and 100 mM KCl). The supernatant was then stored at -80°C. It retained activity (stimulated the synthesis of polypeptide) for about 5 weeks.

Reactions were incubated at 37°C for 40 minutes in a New Brunswick Shaker Bath (model G76). After incubation, the tubes were removed to ice, aliquots were taken for TCA precipitation, and the remainder was saved to use in affinity column studies. TCA precipitation was followed by heating at 90°C for 15 minutes. The tubes were then cooled on ice for 15 minutes, and the precipitate collected by vacuum filtration on Whatman GF/C filters which were counted in 5 ml of toluene plus omnifluor.

Mg^{++} and K^{+} optimums were obtained by varying the concentrations of the respective salts in group (a). pH optimums were obtained by varying the pH of the buffer in group (a). As such, the pH optimum was empirically an optimum for the pH of group (a), but might not have been an accurate value for the pH of the whole reaction.

Affinity Chromatography Columns

Protein was coupled to Sepharose 6B (Pharmacia) by the method of Cuatrecasas (1970). The method in detail can also be found in the master's thesis of Eschenfeldt (1975, p. 15). Briefly stated,

the method allows protein to couple to cyanogen bromide activated Sepharose. Sepharose 6B coupled with normal rabbit gamma globulin and Sepharose 6B coupled with anti-myeloma protein were used.

Columns were poured (at room temperature) in disposable Pasteur pipettes to a final packed volume of 0.3-0.5 ml Sepharose. They were washed twice with buffer (25 mM Tris, pH 7.6, at 4°C, 175 mM NaCl, 5 mM $MgCl_2$, and 100 µg/ml sodium heparin). The columns were washed three times with a total of about 4.0 ml of 10% fetal calf serum to saturate nonspecific binding sites. They were then rewashed three times with buffer. Sample was applied in 0.4 ml aliquots. Unbound sample was eluted with about 4.0 ml of buffer. Eluate was precipitated with 10% trichloroacetic acid (TCA). The precipitate was collected by vacuum filtration on Whatman GF/C glass fiber filters (2.4 cm) and counted in 5 ml or 10 ml of toluene-Omnifluor (New England Nuclear) scintillation fluid.

Functional Assays

Polypeptides were prepared from polysome fractions in a system for cell-free protein synthesis by methods described elsewhere in this thesis. The reaction mixture containing the ^3H-labeled polypeptides was brought to 33 mM EDTA with 100 mM Na_2EDTA in water, pH 7.0, to dissociate ribosomes into subunits and release nascent chains. After 10 minutes on ice, 1 M KCl in RSB was added to a final concentration of 250 mM. Sample was then applied to an anti-myeloma protein column and a control column of normal rabbit gamma-globulin (NRGG). The method of application and preparation of eluate (unbound material) for counting has already been described.

For each experiment control columns were prepared which were tested against purified ^{14}C-labeled myeloma protein.

Sucrose Gradient Analysis

Polysomes were analyzed on 15-45% (w/v) linear sucrose gradients in the SW 50.1 rotor (5.2 ml sucrose per gradient). Centrifugation was at 45,000 rpm (250,000 x g [max]) for 35 minutes (occasionally this was changed to 40 minutes). Gradients were collected from the top with continuous monitoring of absorbance at 254 nm. For some experiments, 0.2 ml fractions were counted in toluene-Omnifluor plus TX-100 plus water (6:3:1).

RESULTS

Cells

As mentioned in the literature review, the physiological state of a cell affects its polysomes. One measure of the physiological state is the generation or doubling time of the cell. Throughout this study records were kept of MOPC-21 cell density and time of measurement. Conventional reasoning about generation time was translated into a simple FORTRAN program and measurements representing the span of this study were converted into generation times, a running calculation of mean generation times, and a corresponding running calculation of standard deviations as shown in Table 1. It is apparent there was variation in the generation times of the cells. It is doubtful that the variation is solely due to counting inaccuracies. Some of the variation reflects differences in the length of time cells were incubated before being counted (that is, cells may have been in stationary phase for different time intervals). Other variation may reflect the fact that toward the latter part of this study, tissue cultures were started from slower growing MOPC-21 ascites cells which probably took time to become adapted to the culture conditions. Finally, diverse factors such as variation in incubation room temperature and in partial pressure of O_2 (due to frequently leaky roller bottle caps and to larger volumes of media per bottle toward the latter part of this study) may have contributed

Table 1. Generation times of MOPC-21 tissue culture cells

generation time (hrs)	mean	standard deviation
2.1400714E+01	2.1400714E+01	.0
1.4430133E+01	1.7915424E+01	4.9289450E+00
1.3403770E+01	1.6411539E+01	4.3511212E+00
1.3958827E+01	1.5798361E+01	3.7583844E+00
1.4029844E+01	1.5444658E+01	3.3495701E+00
1.1187763E+01	1.4735175E+01	3.4635081E+00
1.2763397E+01	1.4453491E+01	3.2483335E+00
1.4592128E+01	1.4470821E+01	3.0078181E+00
1.6326490E+01	1.4677006E+01	2.8807483E+00
1.2325945E+01	1.4441900E+01	2.8159156E+00
1.5549587E+01	1.4542599E+01	2.6922082E+00
1.4673267E+01	1.4553488E+01	2.5671970E+00
1.4620533E+01	1.4558645E+01	2.4570743E+00
1.2485578E+01	1.4410569E+01	2.4259686E+00
1.7619271E+01	1.4624483E+01	2.4799147E+00
1.3959394E+01	1.4582946E+01	2.4015793E+00
1.3554933E+01	1.4528945E+01	2.3359543E+00
1.0223493E+01	1.4289753E+01	2.4830486E+00
1.5648184E+01	1.4361250E+01	2.4331304E+00
1.4596829E+01	1.4373029E+01	2.3688210E+00
1.2571491E+01	1.4287241E+01	2.3420710E+00
1.3684049E+01	1.4259823E+01	2.2892423E+00
1.5560254E+01	1.4316364E+01	2.2529862E+00
1.2992846E+01	1.4261217E+01	2.2199642E+00
1.6602426E+01	1.4354866E+01	2.2230942E+00
1.3407685E+01	1.4318436E+01	2.1860850E+00
1.8546383E+01	1.4475026E+01	2.2928625E+00
1.6728404E+01	1.4555504E+01	2.2899462E+00
1.5046791E+01	1.4572445E+01	2.2505323E+00
1.6446851E+01	1.4634925E+01	2.2377125E+00
1.4801216E+01	1.4640289E+01	2.2003040E+00
1.3493889E+01	1.4604464E+01	2.1739906E+00
1.4130827E+01	1.4590112E+01	2.1413403E+00
1.6794612E+01	1.4655950E+01	2.1422709E+00
2.1920533E+01	1.4862538E+01	2.4418421E+00
3.2643782E+01	1.5356461E+01	3.8175964E+00
1.4516811E+01	1.5333768E+01	3.7668295E+00
2.3387386E+01	1.5545705E+01	3.9385759E+00
1.6547470E+01	1.5571392E+01	3.8897161E+00
1.8774490E+01	1.5651469E+01	3.8727821E+00
1.6302963E+01	1.5667359E+01	3.8254193E+00
2.1221106E+01	1.5793591E+01	3.8744410E+00
2.2500000E+01	1.5955415E+01	3.9620654E+00
2.0139057E+01	1.6051634E+01	3.9673935E+00
1.3702368E+01	1.5999428E+01	3.9376597E+00
1.2883963E+01	1.5931701E+01	3.9206641E+00
1.4135244E+01	1.5893478E+01	3.8866575E+00
1.1025305E+01	1.5792058E+01	3.9087634E+00
1.5947453E+01	1.5795229E+01	3.8678966E+00
1.7454987E+01	1.5828424E+01	3.8354140E+00
2.2584036E+01	1.5960887E+01	3.9129351E+00
2.4471368E+01	1.6124550E+01	4.0501476E+00
2.8206936E+01	1.6352520E+01	4.3408129E+00
1.5895596E+01	1.6343966E+01	4.3001263E+00
1.6516237E+01	1.6347098E+01	4.2601875E+00
1.7600791E+01	1.6369485E+01	4.2246040E+00
3.9746909E+01	1.6779616E+01	5.2073377E+00
1.2375148E+01	1.6703676E+01	5.1937570E+00
1.7866974E+01	1.6723393E+01	5.1511155E+00
1.5521133E+01	1.6703356E+01	5.1095342E+00
1.6146458E+01	1.6694226E+01	5.0672775E+00
1.6900324E+01	1.6697550E+01	5.0256390E+00
1.5719559E+01	1.6683027E+01	4.9864674E+00
1.7556436E+01	1.6695639E+01	4.9479414E+00
2.5499802E+01	1.6831137E+01	5.0291241E+00
1.7277454E+01	1.6837900E+01	4.9905910E+00
2.6272514E+01	1.6976715E+01	5.0449947E+00
1.8943541E+01	1.7007609E+01	5.0525257E+00
1.1708793E+01	1.6930815E+01	5.0556429E+00
1.0877348E+01	1.6844337E+01	5.0707583E+00
2.0184889E+01	1.6891387E+01	5.0499940E+00
2.2850171E+01	1.6974286E+01	5.0634041E+00
2.4641244E+01	1.7079313E+01	5.1075643E+00
3.3608292E+01	1.7302678E+01	5.4241905E+00
1.7078795E+01	1.7299693E+01	5.3874779E+00
1.9147737E+01	1.7324009E+01	5.3556379E+00
1.8092714E+01	1.7333992E+01	5.3210079E+00
1.6468266E+01	1.7322893E+01	5.2872517E+00
1.8765503E+01	1.7341154E+01	5.2567565E+00
2.0891540E+01	1.7385534E+01	5.2374501E+00
1.4907236E+01	1.7354938E+01	5.2118926E+00
1.6041545E+01	1.7338421E+01	5.1816908E+00
1.3374867E+01	1.7291161E+01	5.1683067E+00
1.3958669E+01	1.7251483E+01	5.1493606E+00
1.4613874E+01	1.7220458E+01	5.1271718E+00

to variations in the observed cell generation time. The mean of all analyzed MOPC-21 cell generation times was 17 hours, and the standard deviation was 5 hours.

The data which follow were relatively consistent and made a coherent description of certain properties of MOPC-21 cells, even with the apparent variation in cell generation time, and thus should be pertinent to the isolation of free and membrane-bound polysomes from MOPC-21 cells regardless of their physiological state.

Separation of Free and Membrane-Bound Polysomes by Differential Centrifugation

Cells were lysed by Dounce homogenization and a PNSW (post-nuclear supernatant plus wash) was prepared as described in Materials and Methods and centrifuged at 27,000 x g (max) for 5 minutes. Unless otherwise noted, the percentage of membrane-associated polysomes was calculated as

$$100 \times \frac{A_{260} \text{ units}^* \text{ in pellet}}{A_{260} \text{ units in supernatant} + A_{260} \text{ units in pellet}}$$

or if the polysomes were labeled,

$$100 \times \frac{\text{CPM in pellet}}{\text{CPM in supernatant} + \text{CPM in pellet}}$$

The pellet contained membrane-associated polysomes and the supernatant free polysomes (Kimmel, 1969). However, a major concern was how efficient a separation of membrane-bound from free polysomes had been achieved.

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*A_{260} units = absorbance at 260 nm minus absorbance at 320 nm.

Figure 2 shows two sets of data. The first set of data was gathered early in this study and is summarized by the regression lines. A greater concentration of A_{260} units/ml of sample centrifuged resulted in a greater amount of A_{260} units found in the pellet. Regression was done by translating conventional thoughts about least squares analysis (as discussed in Kreyszig, 1970, sections 17.1-17.5) into a FORTRAN program to analyze the data points. Early in the study a buffer lacking sucrose (RSB) was used to lyse the cells, while shortly thereafter and for the remainder of the study, 0.25 M sucrose was included with the RSB isolation buffer. Since this may have made a difference, two lines were calculated, one for all points and one for those measurements taken when 0.25 M sucrose was included with the buffer.

The second set of data shown in Figure 2 and Table 2 shows the mean values for percentage membrane-associated polysomes versus sample volume, bracketed by their corresponding standard deviations,

Table 2. The effect of sample volume on the percentage of membrane-associated polysomes obtained by differential centrifugation at 27,000 x g (max) for 5 minutes

volume of sample (ml)	mean percent membrane-associated polysomes	standard deviation	number of measurements
0 - 1.5	32.8	5.4	16
1.5 - 2.5	31.1	3.64	16
2.5 - 3.5	29.1	3.9	11
3.5 - 4.5	29.5	4.1	6
4.5 - 5.5	26.6	4.1	13

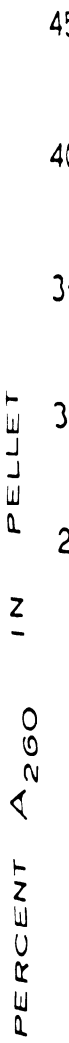

Figure 2. Variations in the percentage of membrane-associated polysomes isolated in the presence of RSB (O) and RSB(such) (●). The regression lines summarize variations which accompany changes in A_{260} units/ml of sample for all points (---) and for polysomes isolated in the presence of RSB(such) (——). The connected points (+——+) summarize data presented in Table 2 on the average variation which accompanies a change in sample volume.

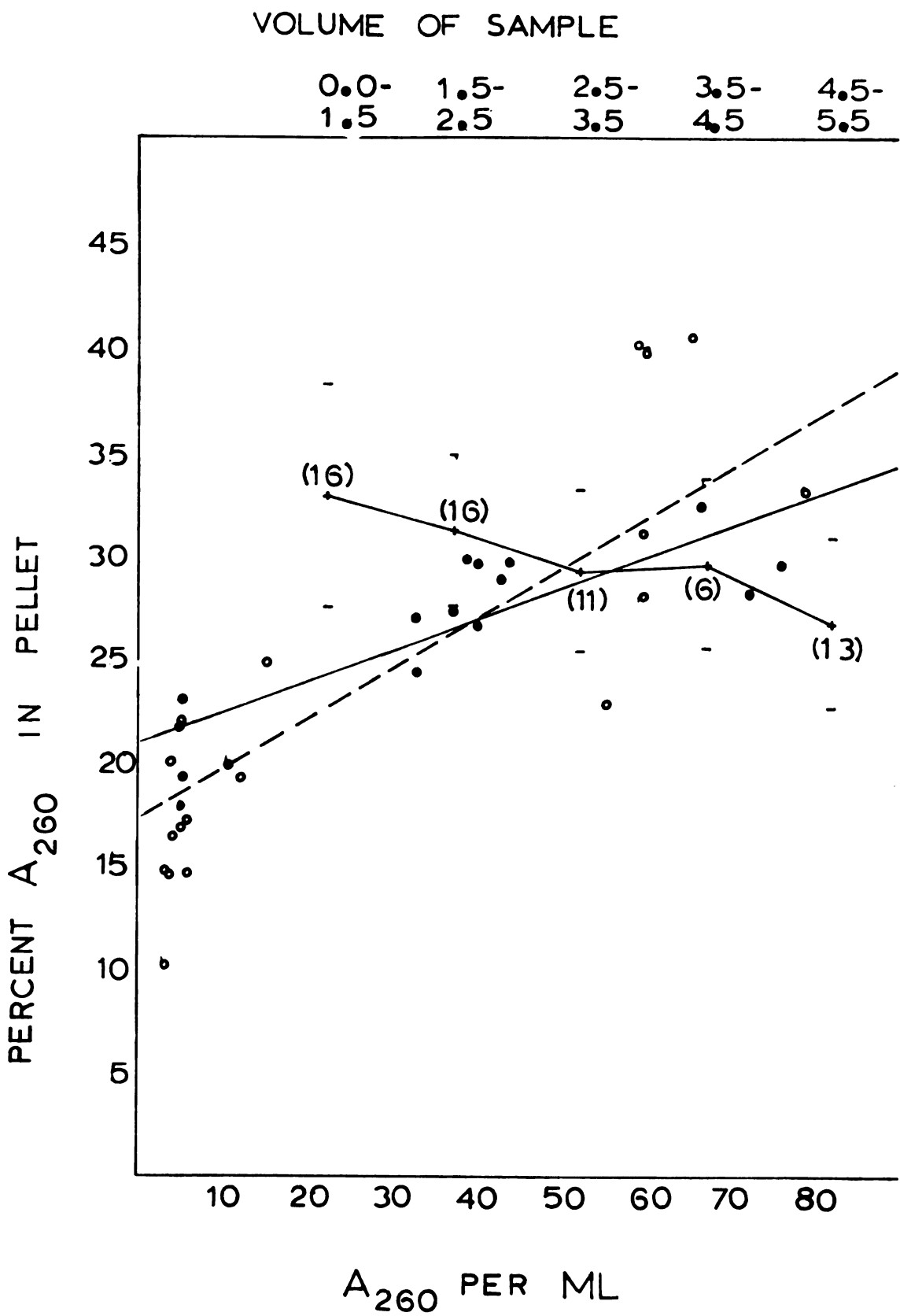
VOLUME OF SAMPLE
0.0-1.5
1.5-2.5
2.5-3.5
3.5-4.5
4.5-5.5
PERCENT A_{260} IN PELLET
45
40
35
30
25
20
15
10
5
(16)
(16)
(11)
(6)
(13)
10
20
30
40
50
60
70
80
A_{260} PER ML

Figure 2

with the total number of points used for the calculation in parentheses. Measurements were made throughout the latter part of this study. It is apparent that as the sample volume increased, the percentage of membrane-associated polysomes decreased! A t test was performed to determine whether the results obtained with the largest sample volume (4.5 to 5.5 ml) differed significantly from the results obtained with the smallest sample volume (0.0 to 1.5 ml). According to the t test, the possibility that there was *no* difference could be rejected at a significance level of 5%.

Explanations were sought for the variations in the percentages of membrane-associated polysomes obtained by differential centrifugation at 27,000 x g for 5 minutes. These variations were not reported by other investigators, although many did wash the crude 27,000 x g pellet before using it as a source of membrane-associated polysomes.

RSB(such) was the buffer routinely used in the isolation of membrane-associated polysomes. The low salt concentration of the buffer presented the possibility that some form of ionic interaction resulting in aggregation could occur at higher sample concentrations. To test this possibility membrane-associated polysomes were isolated in the presence of different concentrations of cations: 25 mM KCl, 160 mM NaCl, 2 mM lysine (pH 7.3), 20 mM lysine (pH 7.1), 6 mM Mg^{++}, and a dialyzed 0.5 M KCl wash fraction (which may or may not have been cationic). No appreciable effect was observed, although 160 mM NaCl and 20 mM lysine did slightly reduce the concentration of membrane-associated polysomes obtained from concentrated samples.

When ^{3}H-labeled free polysomes (PMS,S3 as prepared in Materials and Methods) were added to the PNSW before centrifugation, 160 mM NaCl did prevent sedimentation of some of the polysomes as compared with a control performed with low salt. Nevertheless, this study was completed using standard RSB(such) with its low salt concentration, primarily because the sucrose gradient profile of non-detergent treated MP1 showed evidence of being contaminated with free polysomes even when MP1 was isolated in the presence of 160 mM NaCl (profiles not shown). In summary, ionic interactions may be a factor, but they are not the sole or even major source of the variations in the amount of polysomes found in the pellet.

To test whether differences among cell types could be detected with differential centrifugation, PNSW fractions and membrane-associated polysomes from three different cell types were prepared as previously described. The results are shown in Table 3. The results indicate that differential centrifugation for 5 minutes at 27,000 x g (max), even with all its variability, was sufficient to detect differences among cell lines. As compared to the MOPC-21 cell line (data summarized in Figure 2), the S49.1 cell line had a generally greater percentage of membrane-associated polysomes, the XC.1 cell line had approximately the same percentage of membrane-associated polysomes, and the S180 cell line contained fewer membrane-associated polysomes.

What is the source of the variations observed in percentages of membrane-associated polysomes? What can be done to obtain a well defined, pure fraction of membrane-associated polysomes? These

Table 3. The percentage of membrane-associated polysomes in various cell lines

cell line	sample volume	A_{260} per ml of sample	percent membrane-associated polysomes
S49.1	2.0	5.36	58.8
	1.0	44.1	47.5
	1.0	24.5	50.0
	1.0	9.8	45.2
	1.0	4.9	42.8
XC.1	1.0	25.0	34.9
	5.0	5.0	25.0
	1.0	26.6	26.7
	1.0	13.3	25.8
	1.0	5.3	13.5
	5.0	5.3	17.9
S180	2.0	29.8	18.0
	2.0	14.9	17.8
	2.0	5.1	15.6
	1.0	66.8	19.2
	1.0	26.7	17.8
	1.0	6.7	18.3
	5.0	6.7	11.6
	5.0	26.1	11.9

questions are answered in the discussion with the help of the following pertinent data.

A PNSW was prepared as described in Materials and Methods. After centrifugation for 5 minutes at 27,000 x g (max) the pellet, MP1, was resuspended in RSB(such). Equal aliquots were taken and one aliquot was treated for 5 minutes with 0.5% DOC TX-100 to solubilize membranes. Each aliquot was analyzed by linear sucrose gradient centrifugation. Figure 3A is a representative profile of an MP1 fraction which has not been treated with detergent, and Figure 3B is the profile of the detergent treated replicate MP1 fraction. It is evident that a polysome profile appears in the non-detergent treated sample with a large peak of putative membrane-aggregate (non-microsomal) at the bottom of the gradient. A fraction of the sample also pelleted. The polysome profile apparently reflects the presence of free polysomes. The large peak disappears on exposure to DOC TX-100.

Table 4 shows the results of six differential centrifugations of a *non*-detergent treated PNSW which had first been column chromatographed on Sepharose 6B as described in Materials and Methods. The polysomes had been labeled for 22 hours with 1.0 μC of ^{3}H-uridine per ml. Notice that material, albeit less material, continues to pellet after the first spin which supposedly removed the membrane-bound polysomes. Similar percentages of pelleted material on second and third centrifugations were observed in other experiments, even in one in which the Sepharose chromatographed PNSW had been treated with either 0.5 or 1.0% DOC TX-100. This indicates that a fraction of free polysomes has pelleted.

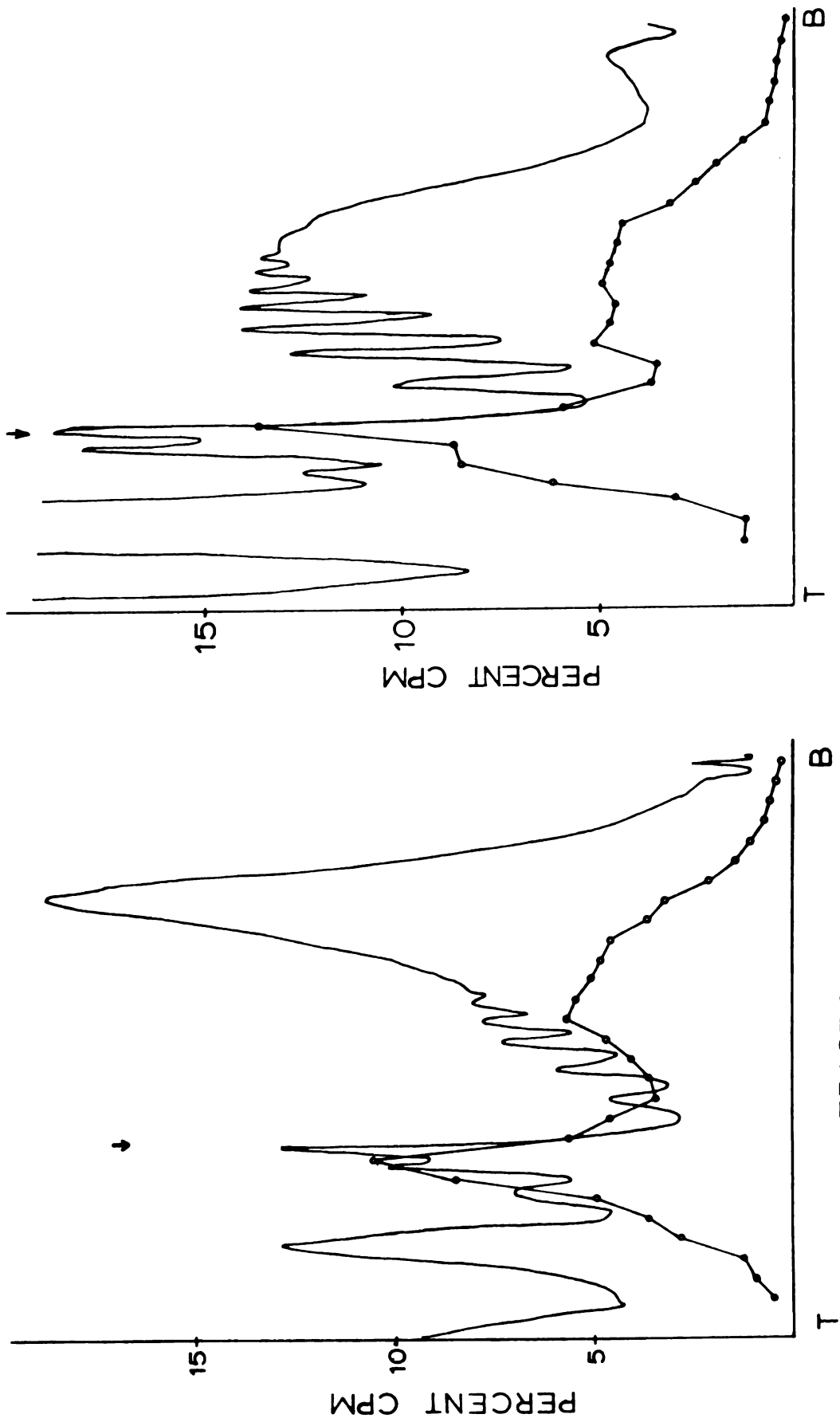

Figure 3. Sucrose gradient profiles of membrane-associated polysomes pelleted by differential centrifugation at 27,000 x g (max) for 5 minutes (MP1). A. Non-detergent treated. B. Treated with 0.5% DOC TX-100. A_{254} (——). Arrow indicates monosomes (80S).

Table 4. Removal of microsomes and free polysomes by repeated differential centrifugation

centrifugation	radioactivity in pellet	percent radioactivity in pellet
first	591,224	14.9
second	202,289	7.1
third	189,820	7.3
fourth*	32,972	4.6
fifth	17,980	3.3
sixth	14,702	3.2

*Supernatant from third centrifugation was stored at -80°C before experiment continued with fourth centrifugation of thawed material.

Table 5 is a compilation of data from two experiments in which ^{3}H-uridine labeled polysomes (PNSW) were exposed to 10 units of T1 RNase per ml for 10 minutes at 37°C before differential centrifugation. Label for experiment 1 was added at 0.5 μC ^{3}H-uridine/ml for 12 hours while label for experiment 2 was added at the same concentration but for 45.5 hours. In each experiment, at the smaller volume, RNase treatment reduced the amount of isotope in the pellet to approximately the amount deposited in the pellet at the higher volume. This implies the need for polysomal integrity in order for the usual variation in percentage membrane-associated polysomes to occur. That is, free polysomes seem to pellet, not random aggregates of ribosomes. Each experiment has internal consistency, and each resembles the other; however, it should be noted that the

Table 5. The effect of RNase on the quantity of material pelleted by differential centrifugation

volume	A_{260} per ml	sample	CPM	CPM per A_{260}	% CPM in pellet	% A_{260} in pellet	treatment
1.0	24.9	supt*	1,152,730	62,676	21.1	26.2	none
		pellet	307,330	47,137			
1.0	24.9	supt	1,186,417	58,358	13.5	19.9	T1 RNase**
		pellet	185,231	36,788			
5.0	5.0	supt	1,771,893	90,773	10.1	16.5	none
		pellet	197,911	51,272			
5.0	5.0	supt	1,457,786	73,182	6.9	16.6	T1 RNase
		pellet	108,150	27,242			
1.0	3.4	supt	220,963	83,596	17.9	22.1	none
		pellet	48,236	64,314			
1.0	3.4	supt	206,999	84,836	10.1	22.5	T1 RNase
		pellet	23,234	32,723			
1.0	25.0	supt	1,619,996	117,937	31.3	34.4	none
		pellet	727,272	102,275			
1.0	25.0	supt	1,799,066	117,989	19.3	30.3	T1 RNase
		pellet	400,128	54,876			
5.7	4.4	supt	2,248,400	126,577	15.5	21.0	none
		pellet	396,693	87,306			
5.0	3.9	supt	1,644,500	107,732	7.1	18.0	T1 RNase
		pellet	120,235	37,261			

*supt = supernatant

**10 units T1 RNase/ml, incubate for 10 minutes at 37°C

percentage of membrane-associated polysomes differs between experiments. This is one case in which the physiology of the cells, as discussed in the first part of the Results section, might have made a difference in observed percentage of membrane-associated polysomes.

It is possible that not all membrane-bound polysomes are pelleted during differential centrifugation of a larger sample volume. If this is so, then one would expect a second extensive application of differential centrifugation (for example, 30 minutes instead of 5 minutes) to pellet more membrane-associated polysomes from the supernatant of a previously centrifuged 5 ml sample than from the supernatant of a previously centrifuged 1 ml sample. The results of Table 6 were consistent with this possibility, but the results

Table 6. A test for unpelleted microsomal material by extensive centrifugation

sample	volume of sample (ml)	A_{260} per ml	% A_{260} pelleted
Initial isolation (27,000 x g for 5 min)			
A	1.0	25.0	37.2
B	1.0	50.0	36.4
C	5.0	5.0	24.7
D	5.0	10.0	25.7
Second centrifugation (27,000 x g for 30 min)			
A	4.8	2.7	12.4
B	4.8	5.4	13.5
C	4.6	3.5	15.6
D	4.6	7.0	16.4

were not unequivocal so a second experiment was performed with labeled polysomes.

The results of the second experiment are presented in Table 7. Only a very slight difference is seen in the percentage of radioactivity pelleting in the second differential centrifugation step. This indicates that not many, if any, membrane-associated polysomes are left in the sample with the larger volume after the first differential centrifugation, or at least not many more than are left in the sample with the smaller volume. There is, however, a marked difference in the quantity of absorbance units pelleted in the second differential centrifugation in contrast to the results noted with the radioactivity. The supernatant of the sample with the large volume

Table 7. A test for unpelleted microsomal material by extensive centrifugation

sample	volume of sample (ml)	A_{260} per ml	CPM in pellet	% CPM in pellet	% A_{260} in pellet
Initial isolation of 27K x g pellet 27K x g for 5 minutes					
A	1.0	25.0	300,038	38.5	35.8
B	5.0	5.0	200,390	18.1	27.2
C	1.0	10.3	134,461	29.5	32.3
Second 27K x g centrifugation 27K x g for 30 minutes					
A	4.5	2.6	72,281	11.8	8.9
B	4.5	3.8	103,286	12.5	13.0
C	4.5	1.1	28,411	10.7	8.9

in the first differential centrifugation pelleted the most absorbance units in the second differential centrifugation. Since the extra absorbance was not associated with the polysomal radioactivity, it represented miscellaneous non-microsomal membrane fragments. The data in Table 8 support this interpretation. Cells were incubated for 29 hours with 0.5 μC of ^{3}H-choline/ml to label membrane. A PNSW

Table 8. The percentage of membrane material pelleted by repeated differential centrifugation

centrifugation	fraction	CPM*	CPM per A_{260}	% CPM in pellet	A_{260} in pellet
initial	supt	119,248	9,755	47.0	16.6
	pellet	105,777	43,422		
second	supt	70,920	-**	20.1	-
	pellet	17,851	-		
third	supt	54,581	6,606	8.3	-
	pellet	4,951	-		

*cells labeled with ^{3}H-methyl choline for 29 hours

**not determined

was prepared, chromatographed on Sepharose 6B, and then subjected to three consecutive differential centrifugations at 27,000 x g (max). The sample volume was about 3.0 ml. It is apparent that a considerable amoiunt of membrane which did not pellet in the first differential centrifugation was able to pellet in the second differential centrifugation and, further, a significant amount of membrane was available to be pelleted in the third differential centrifugation.

Free polysomes were detected in the 27,000 x g pellet by washing MP1 fractions which had been isolated from samples with large and small volumes. The results are shown in Table 9. Supernatants from the washes of pellets from smaller volume samples contained considerably more A_{260} units than supernatants from the washes of pellets from larger volume samples. In relation to polysomal

Table 9. The percentage of membrane-associated polysomes that will pellet after resuspension

sample	volume of sample	A_{260} per ml	% A_{260} in pellet	% A_{260}* repelleted
A	1.0	47.9	38.8	65.7
B	1.0	25.0	38.5	62.0
C	5.0	5.0	29.7	75.9
D	5.0	5.0	29.9	80.3

*repelleted = resuspended pellet in 1.5 ml and centrifuged for 5 minutes at 27,000 x g (max)

material, the interpretation given is that more free polysomes pelleted in the smaller volume than in the larger volume during the initial centrifugation.

Isolation of Membrane-Bound Polysomes by Partition Separation in an Aqueous Polymer Two-Phase System in Combination with Differential Centrifugation

The data from the previous section indicated that a single differential centrifugation of a PNSW would not adequately separate membrane-bound polysomes from free polysomes. Free polysomes were apparently contaminated with negligible amounts of membrane-bound

polysomes after one differential centrifugation; thus following two additional differential centrifugations, the supernatant (PMS3) was used as a source of free polysomes.

Membrane-associated polysomes obtained after one differential centrifugation needed to be further processed in order to separate membrane-bound from contaminating free polysomes. The approach taken was to combine differential centrifugation with partition separation. Albertsson (1959) reported that the partition behavior of a particle in a dextran-methylcellulose aqueous two-phase polymer system depended primarily on the surface area of the particle.

The partition coefficient in a liquid two-phase system is defined as:

$$K = \frac{\text{concentration of substance in top phase}}{\text{concentration of substance in bottom phase}}$$

Polysome fractions labeled with ^{3}H-uridine were prepared and then partitioned once in a D68-MC4000 aqueous two-phase polymer system as described in Materials and Methods. The partition coefficient for the crude pellet of membrane-associated polysomes MP1 was 0.043. The partition coefficient for the free polysomes PMS,S3 was 0.179. The partition coefficient for an unlabeled PMS was 0.353. These values agree fairly well with expected values put forth by Albertsson (1960, p. 178). The values support what is readily ascertained visually--that membrane-bound polysomes partition into the bottom phase.

Membrane-associated polysomes (MP1) labeled with ^{3}H-uridine were processed by a combination of differential centrifugation and partition separation. The final pellet (MP2XP) was resuspended in

RSB(such) and analyzed on linear sucrose gradients. Figure 4 shows a profile of non-detergent treated MP2XP. The polysome profile which could be seen in non-detergent treated MP1 (Figure 3A) is not evident. Figure 4 also shows the profile of a replicate aliquot of MP2XP treated with 0.5% DOC TX-100 (the profile is labeled " + DOC"). Figure 5B is a plot of CPM per fraction and Figure 5A is a similar plot of percent radioactivity for both the non-detergent and detergent treated MP2XP. Notice that the large peak at the bottom of the gradient shown in Figure 4 does not have a correspondingly large amount of radioisotope. The exact nature of this peak remains something of an enigma.

Further characterization of polysome fractions was sought by following enzyme markers throughout the isolation procedure. Free and membrane-bound polysomes were isolated as described in Materials and Methods with two modifications: (1) the PMS was chromatographed on Sepharoxe 6B before further differential centrifugation, and (2) membrane-bound polysomes were not partitioned a second time with the top phase of a blank; instead they were recovered by differential centrifugation and partitioned a second time in a completely fresh system as at first. Various parameters of the isolation procedure are presented in Table 10. The final pelleted microsomal fraction has been designated MP6. The membrane fractions have typically lower A_{260}/A_{280} ratios. The Sepharose chromatographed PMS has a typically higher A_{260}/A_{280} ratio. The yields shown for recovery of A_{260} units from the Sepharose column are representative; that is, when this column was used an average of about 47% of the

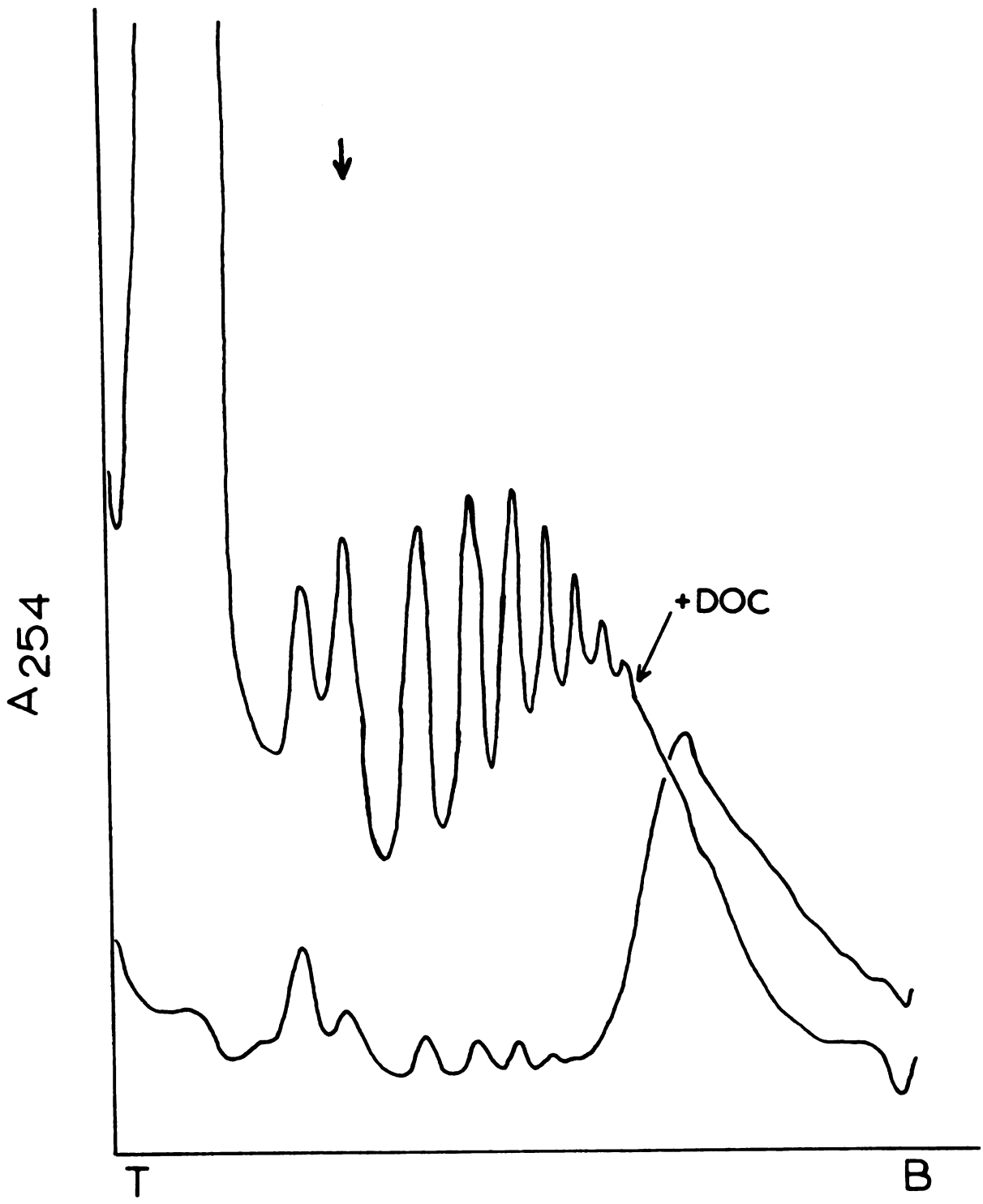

Figure 4. Sucrose gradient profiles of membrane-bound polysomes partitioned in a dextran-methylcellulose aqueous polymer two-phase system. Arrow indicates monosomes (80S). A_{254} (——).

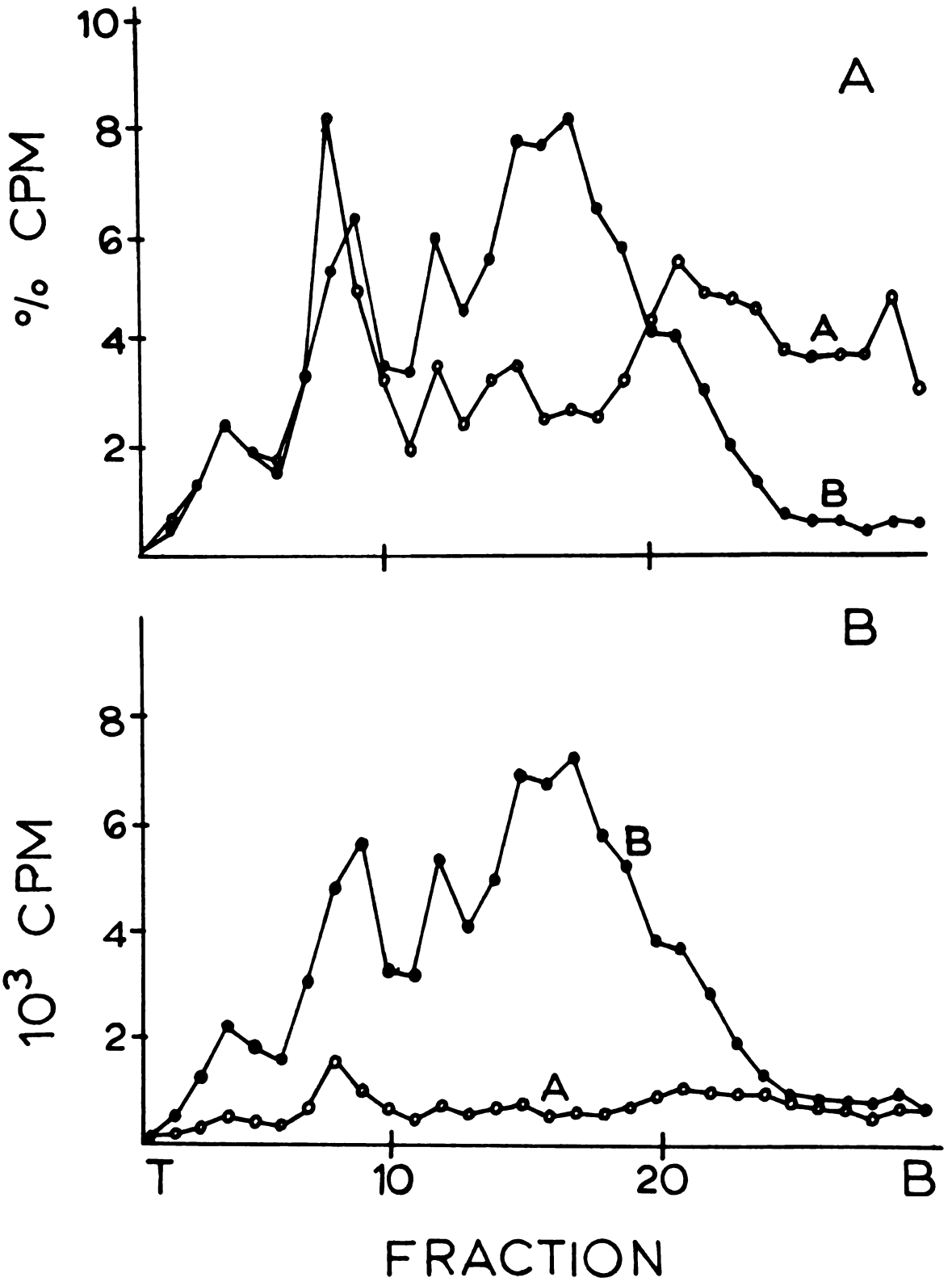

Figure 5. Radioactivity profiles of the gradients shown in Figure 4. A. Percent CPM per fraction. B. Absolute CPM per fraction. Non-detergent treated (O——O). + detergent (●——●).

Table 10. Summary of enzyme marker data on polysome fractions

parameter	PNSW	PMS,S1	MP6
A_{260}/A_{280}	1.648	1.765	1.466
% yield A_{260}*		46.7	32.2
units G-6-Pase**	22.7	7.9 (76.7)*	7.0 (111.0)
	33.6	3.3 (13.3)	3.2 (52.5)
units 5'-mononuc	130.7	16.4 (26.3)	15.1 (30.2)
	218.7	54.9 (63.9)	12.4 (20.8)
	212.4	95.4 (77.6)	8.4 (17.7)

* $\frac{\text{PMS,S1}}{\text{PMS}} \times 100 =$ % yield PMS,S1, $\frac{\text{MP6}}{\text{MP1}} \times 100 =$ % yield MP6

** one unit = one microgram phosphate released per minute

A_{260} units (PMS,S1) of a PMS could be recovered. The final method adopted for partition separation (using the top phase of a blank) gave slightly higher yields, 40 to 50%, of membrane-bound polysomes (MP6) than the yields of about 30 to 36% recorded in Table 10.

One microgram of phosphate released per minute was defined as a unit. Table 10 also presents a summary of the glucose-6-phosphatase activity found in the polysome fractions. It appears that the reaction was not optimized (data not shown), and therefore was not sensitive enough to serve as a probe for microsomal membrane. Nevertheless, the results suggest a slight enrichment for microsomal membrane in the partitioned fraction. It also seems that some enzymic activity remains in the PMS after the first differential

centrifugation. This activity is probably associated with small fragments of membrane not associated with polysomes. Table 10 further shows the activity of 5'-mononucleotidase found in the polysome fractions. An adequate amount of activity was obtained with this enzyme. Again, some enzyme activity is still retained by the PMS after one differential centrifugation. This is consistent with the data presented in Table 8 and could be due to small fragments of plasma membrane which will not pellet under the conditions employed. The activity was reduced in the partitioned fraction. This may indicate that some of the plasma membrane fragments do not partition with the microsomes during the isolation procedure. Alternative reasons for reduction in activity such as competing enzyme reactions or inactivation due to harsh treatment were not investigated.

The sucrose gradient profiles of non-detergent treated MP1 and MP2XP (partitioned MP1) differed, as has already been mentioned. To examine the difference between these fractions from another perspective, MP1 and MP2XP were either exposed to RNase or to 0.5 M KCl and then pelleted by differential centrifugation. Untreated fractions served as controls. The results are shown in Table 11. Consistently more A_{260} units remained in the supernatants of crude (MP1) fractions than in the supernatants of partitioned (MP2XP) fractions. This indicated that the partitioned fractions had been separated from free polysomes, which presumably contaminated the crude fractions.

Another approach to determine whether free polysomes contaminated the MP2XP fraction was to add ^{3}H-uridine labeled free polysomes to a PNSW and to proceed through the fractionation scheme and

Table 11. Differences between partitioned and crude membrane-associated material following treatment with RNase or high salt

A_{260} per ml	isolation procedure	fraction	% A_{260}	treatment
3.75	27,000 x g	supt	22.4	none**
		pellet	77.6	
3.41	partition	supt	2.4	none
		pellet	97.6	
3.75	27,000 x g	supt	36.6	RNase A***
		pellet	63.4	
3.41	partition	supt	20.2	RNase A
		pellet	79.8	
3.75	27,000 x g	supt	30.8	0.5 M KCl**
		pellet	69.2	
3.41	partition	supt	9.6	0.5 M KCl
		pellet	90.4	
2.94	partition	supt	12.9	none
		pellet	87.1	
2.94	partition	supt	16.6	RNase A
		pellet	83.4	
2.94	partition	supt	17.6	T1 RNase****
		pellet	82.4	
2.94	partition	supt	12.6	0.5 M KCl
		pellet	87.4	

* all samples 1.0 ml volume

** 5 min at 4°C

*** RNase A 1 min at 37°C, 1 microgram/ml

**** T1 RNase 1 min at 37°C, 10 units/ml

determine radioactivity at each step. The free polysomes (PMS,S3) were highly labeled making it possible to add only two A_{260} units (less than 2% of the total). Thus, the environment of the PNSW remained essentially the same after the addition of the labeled free polysomes. Table 12 gives the percentage counts found with each fraction. The volume for the first differential centrifugation was 2.9 ml, there were 46.1 A_{260} units/ml, and the percentage membrane-associated polysomes was 23%. Only 0.4% of the counts remained

Table 12. Separation of membrane-bound polysomes from added labeled free polysomes by a combination of differential centrifugation and partition separation

Fraction:	PNSW	PMS	MP1	MP2XP	Loose
CPM in fraction:	333,790	282,003	30,195	1,246	11,676
Percent CPM:	100	84.5	9.1	0.4	3.5

associated with the membrane-bound polysomes. The expected percentage based on the yield of MP2XP from MP1 was 2.3%. The percentage CPM of MP2XP plus Loose do not add up to that found in the MP1 since MP2XP and the loose fraction were isolated from only a fraction of the total MP1. The percentage of CPM in this fraction was 6.0%. A combination of differential centrifugation and partition separation was sufficient to allow only a negligible quantity of free polysomes to contaminate the membrane-bound polysomes.

Figure 6 shows profiles of the various fractions. The profiles of MP1 are shown in Figures 3A and 3B. The percentage CPM of labeled free polysomes per fraction has been plotted with certain absorbance profiles. It is obvious that a labeled free polysome profile is not present in the MP2XP fraction, but it is present in all the other fractions. Notice also that the large peak at the bottom of the gradient of non-detergent treated MP2XP is not enriched for labeled free polysomes, thus tending to preclude it from being implicated as an aggregate involving free polysomes.

It is interesting to note that the detergent treated MP2XP fraction seems to be somewhat degraded (enriched for smaller polysomes, monosomes, and subunits) in contrast with all the other fractions. This same occurrence has been noted in other experiments. This implies that membrane-bound polysomes can be degraded (or appear degraded) while other polysome populations in the cell are not. An artifact, if it exists, would have to be one that could exclusively cause degradation of membrane-bound polysomes. The detergent or the aqueous polymer could be the source of such an artifact. Nuclease action of the polymer (that is, the polymer being a nuclease or containing a nuclease) is ruled out because the loose polysomes have an undegraded profile and were also extensively exposed to the polymer. The possibility that Mg^{++} precipitation artifically produced a good profile was ruled out by precipitating polysomes in the presence of a small amount of RNase. The profile of the precipitated polysomes showed complete degradation (profile not shown). The possibility that the detergent contained a nuclease was ruled out because other detergent treated fractions were not

Figure 6. Separation of membrane-bound polysomes from added labeled free polysomes by a combination of differential centrifugation and partition separation.

A. A. PNSW. B. PNSW + detergent. C. PMS

B. A. MP2XP. B. MP2XP + detergent

C. Loose

(Figure 3 shows the MP1 profiles)

A_{254} (——). Percent CPM (O—O)

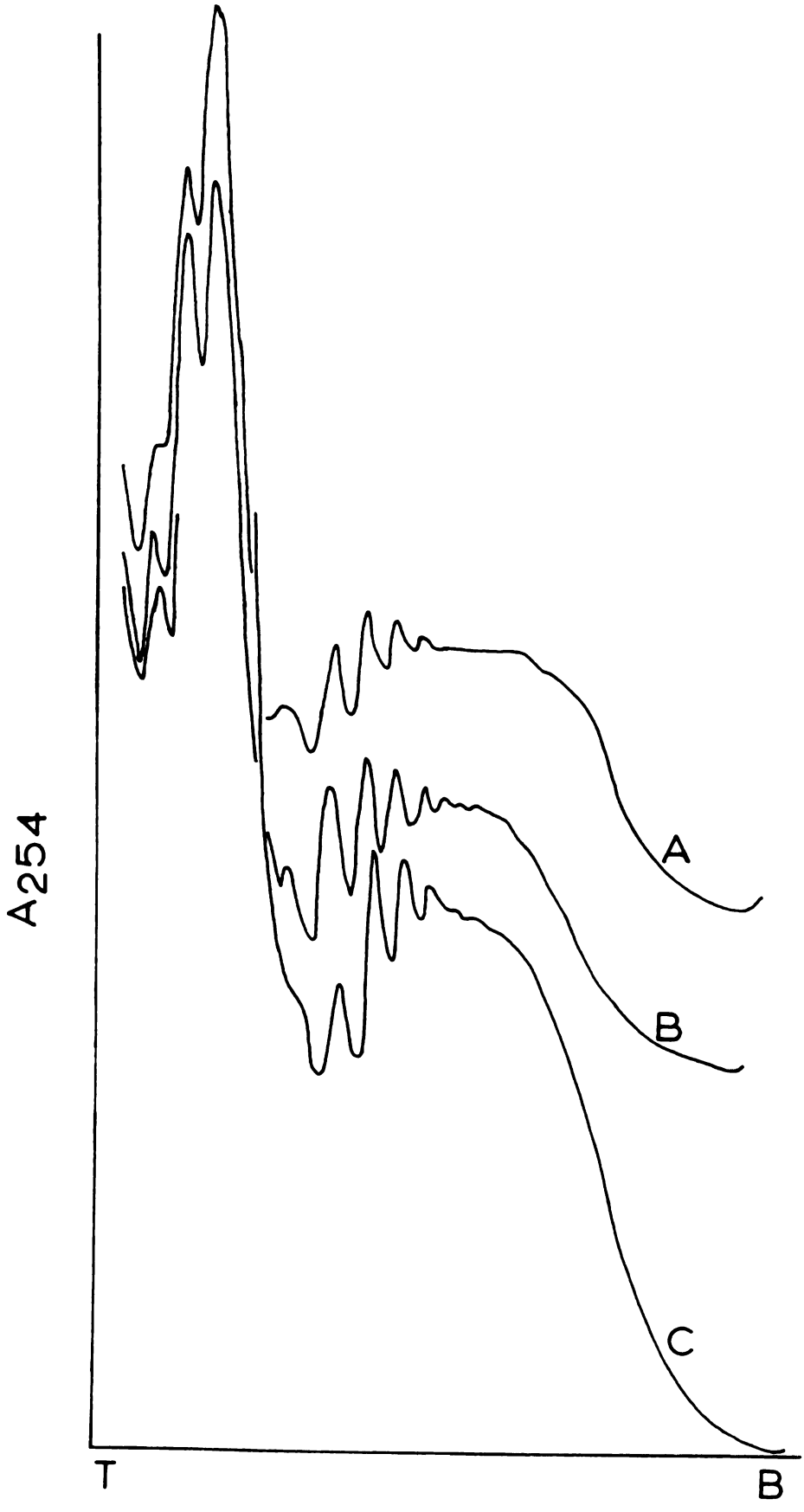

Figure 6A

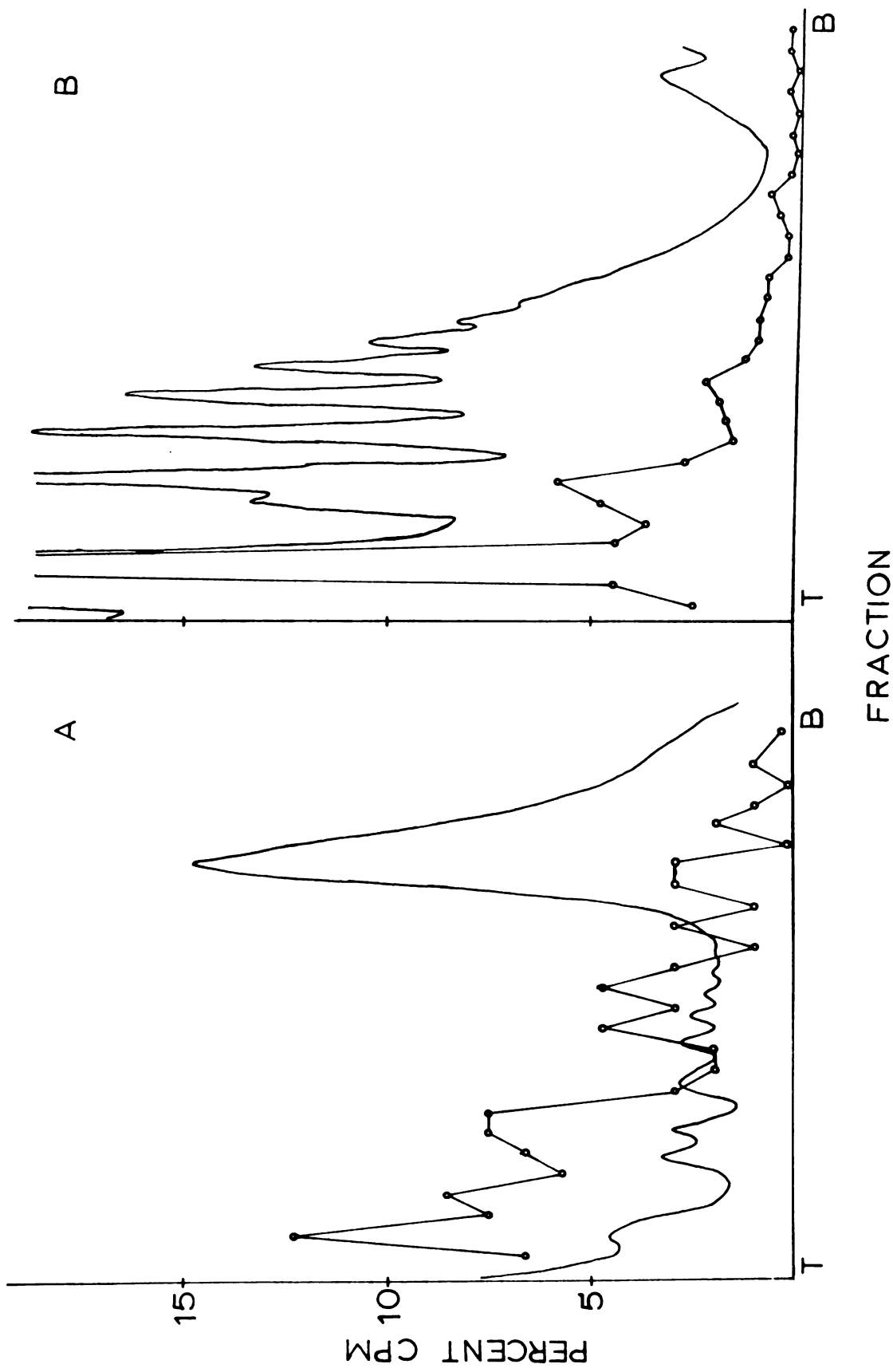

Figure 6B

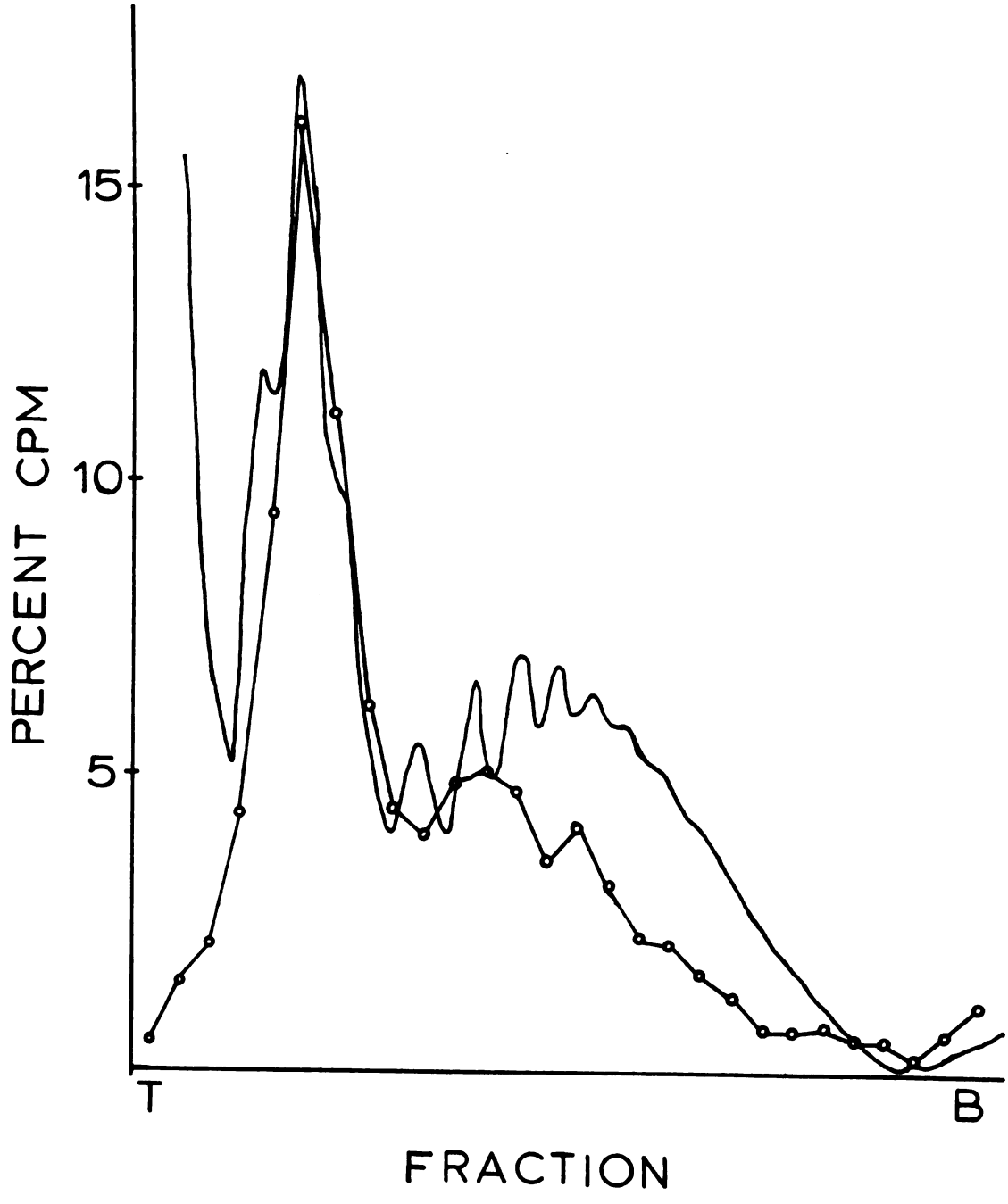

Figure 6C

degraded. Detergent activation of a membrane nuclease was ruled out since the detergent treated MP1 fraction was not degraded.

The possibility that homogenization caused some degradation could not be eliminated. The pellet is resuspended and homogenized with a small homogenizer several times during the course of the isolation procedure. Another possibility which could not be eliminated was the chance that the forces involved in partition separation between phases were sufficient to shear polysomes from microsomal membrane thus leaving the membrane enriched for small polysomal fragments. However, artifacts caused by homogenization or partition separation were tentatively ruled out by replicate treatment of a PNSW obtained from ascites cells freshly started in tissue culture. The profiles of the near-ascites MP2XP fraction, detergent and non-detergent treated, are shown in Figure 7. The detergent treatment reveals undegraded polysomes.

Cell-Free System for Protein Synthesis

It was established that relatively pure, undegraded fractions of free and membrane-bound polysomes could be routinely obtained. Membrane-bound polysomes are reported to be enriched for myeloma message (see Literature Review). To show that a polysome fraction contains a particular messenger, translation of the polysomes in a cell-free system and demonstration that the particular polypeptide has been synthesized are necessary. Accordingly, a cell-free system for protein synthesis was developed as described in Materials and Methods. This system was primarily used with polysomes that had been Mg^{++} precipitated. The system was dependent on an energy source

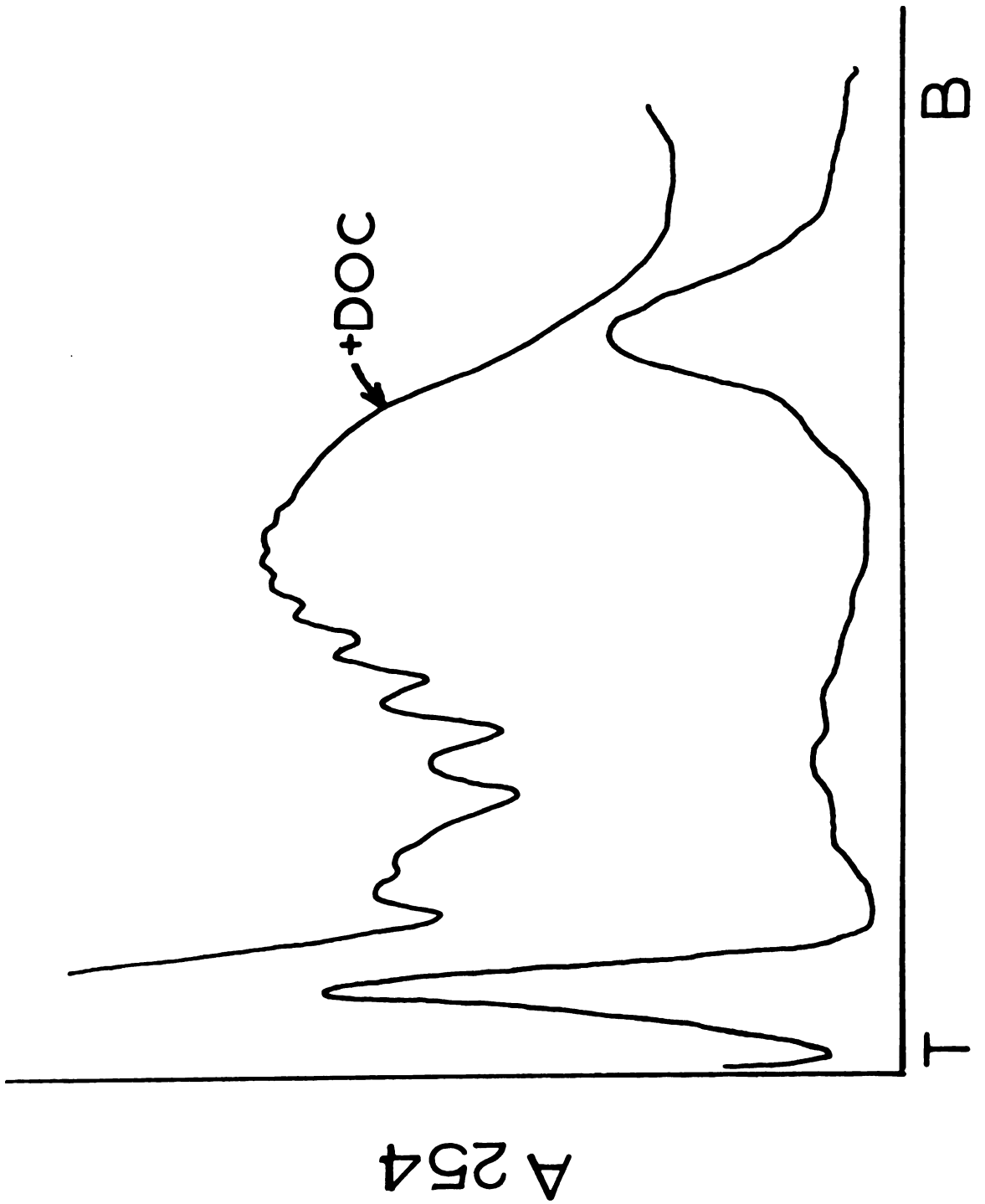

Figure 7. Sucrose gradient profiles of partitioned membrane-bound polysomes obtained from ascites cells freshly started in tissue culture. A_{254} (——).

and was inhibited by cycloheximide (even as low as 0.1 mg/ml). A high speed supernatant contained both stimulatory and inhibitory factors. Figures 8A,B and C show the Mg^{++}, K^{+}, and pH optimums respectively, as described in Materials and Methods. These optimums were obtained with polysome fraction PMS,S3, not Mg^{++} precipitated polysomes. The optimum curves each have fairly wide ranges. The K^{+} optimum was consistently erratic. Two kinetics curves are also presented in Figure 8. Figure 8D was obtained with Mg^{++} precipitated polysomes, and Figure 8E with a PMS. Unfortunately, the fractions of Figure 8D were *not hot* TCA precipitated (they were TCA precipitated without heating) and so the counts are somewhat exaggerated due to the presence of tRNA charged with radioactive amino acids. The kinetics curves indicate that polypeptide synthesis was essentially complete in 10 to 15 minutes. The optimums were performed with free polysomes in which synthesis was stimulated approximately 10-fold over background. MP1 fraction polysomes under the same conditions were consistently stimulated only 5-fold over background. MP2XP were stimulated only 3-fold over background.

Assay for Myeloma Polypeptide Synthesized in the Cell-Free System for Protein Synthesis

Polypeptides synthesized in the cell-free system by different fractions of polysomes were chromatographed over anti-myeloma and normal rabbit gamma globulin affinity columns. The results are shown in Table 13. The partitioned membrane-bound polysomes consistently lacked the ability to synthesize myeloma polypeptides, though theoretically these membrane-bound polysomes should be enriched for myeloma messenger.

Figure 8. Parameters of a cell-free system for protein synthesis. A. Mg^{++} optimum. B. K^{+} optimum. C. pH optimum. D. Kinetics for Mg^{++} precipitated PMS. E. Kinetics for PNS. CPM (O—O).

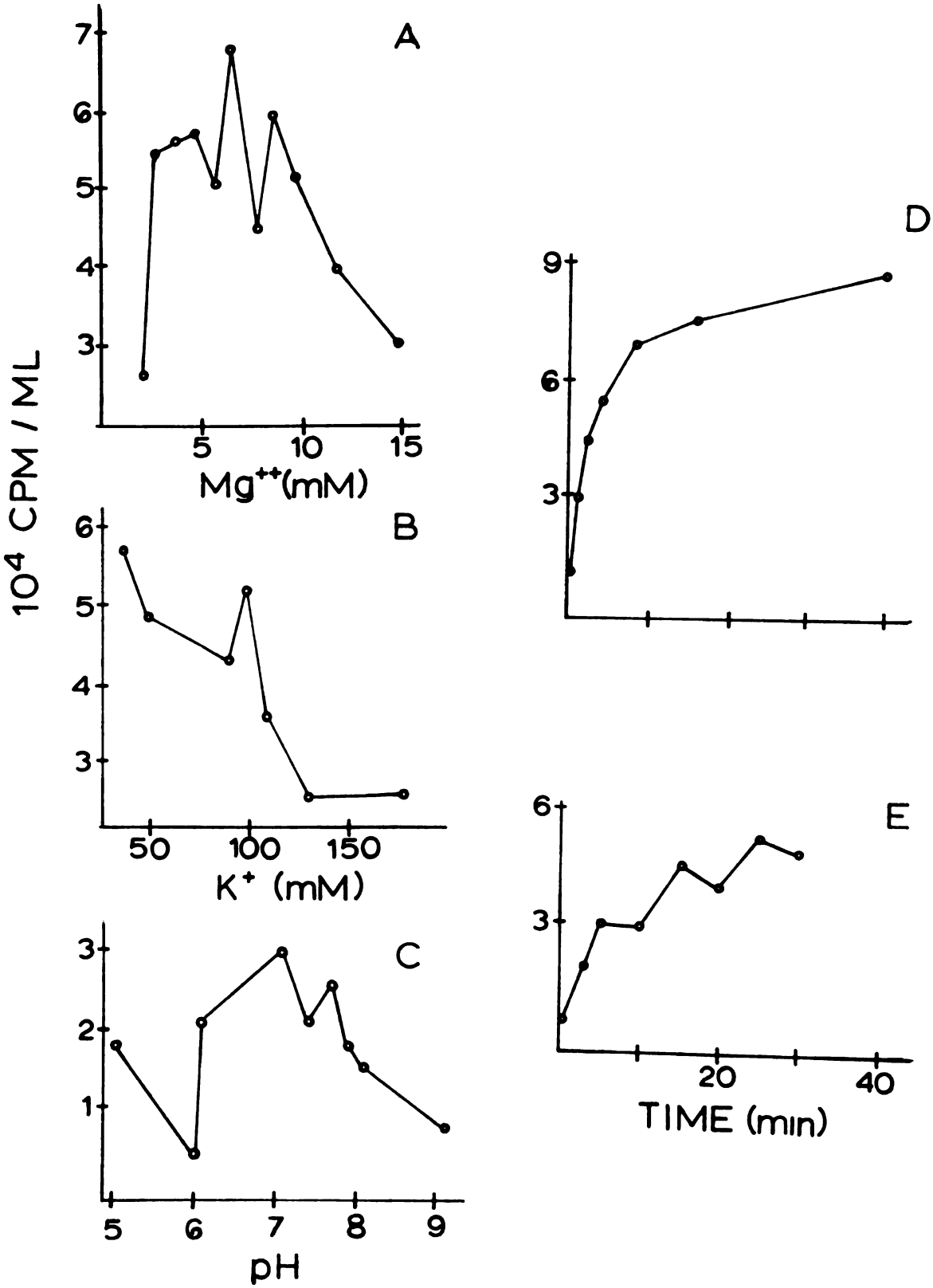

Figure 8

Table 13. Affinity chromatography of polypeptides produced in a cell-free system for protein synthesis

	Polysome Fraction				Control
expt.	free	27,000 x g membrane-associated	partition separated membrane-bound	loose	^{14}C-labeled myeloma protein
1	13.4*	**-	0	-	75.9
2	0	24.6	0	-	75.6
3	0	0	0	-	73.0
3a	17.1				
4	0	14.0	0	-	87.0
5	0	8.7	1.6	13.0	95.3
6	21.8	9.8	5.4	-	105.0
7	-	-	-	0	79.4

*The data are expressed as the percentage of CPM held by the anti-MOPC column minus the percentage of CPM held by the NRGG column. In the event that equal percentages of CPM were held by the columns, or if the NRGG column held more CPM than the anti-myeloma protein column, a zero was recorded.

** - indicates that the fraction was not tested.

DISCUSSION

In order to determine mechanisms that regulate the segregation of messenger RNA's between free and membrane-bound polysomes, it is important to obtain purified fractions of these two classes of polysomes. Membrane-bound polysomes should be enriched for immunoglobulin mRNA (Blobel and Dobberstein, 1975; Cioli and Lennox, 1973). Purified fractions would provide components which would allow the needed resolution for kinetic studies, and chemical and biological characterization, including insight into translational control mechanisms of which one step is the segregation of free and membrane-bound polysomes (for review see Rolleston, 1974; also see Mechler and Vassalli, 1975, I, II, III; Blobel and Dobberstein, 1975, I, II; Shires *et al.*, 1975).

The methods currently used to isolate membrane-bound polysomes include differential centrifugation (Kimmel, 1969; Harrison *et al.*, 1974), centrifugation of a post-nuclear supernatant through a cushion of approximately 30% sucrose (Rosbash and Penman, 1971; Adams and Cory, 1975), and isopycnic banding of microsomes in a discontinuous gradient (Blobel and Dobberstein, 1975, I; Mechler and Vassalli, 1975, I; Cioli and Lennox, 1973).

The source of free polysomes is either the supernatant of the differential centrifugation or the pellet or band in a discontinuous sucrose gradient depending on the construction of the gradient.

The efficiency of separation of free from membrane-bound polysomes in discontinuous gradients depends on the physiological state of the cell (Cioli and Lennox, 1973; Blobel and Dobberstein, 1975, I). Centrifugation must take place at ultracentrifugal speeds and the pellet of free polysomes which results from some methods is very difficult to resuspend (personal communication from Dr. Ronald J. Patterson).

Differential centrifugation (including centrifugation through a cushion of sucrose) as practiced to date has apparently not yielded a pure--completely separated from free polysomes--fraction of membrane-bound polysomes except under fortunate conditions. These conditions seem to be little understood by most investigators, but when contamination, as judged by a sucrose density gradient profile of non-detergent treated microsomal pellet, seems to be negligible (no profile in the polysome region of the gradient), they accept their good fortune and use the microsomal pellet as a source of "pure" membrane-bound polysomes (personal observation; Mechler and Vassalli, 1975).

Differential centrifugation, however, would be the most convenient method to obtain pure fractions. Thus, from the standpoint of the laboratory worker's time and effort, and from consideration of the temperature labile nature of many biological "factors", it would be the most desirable technique to characterize and develop as a reliable, efficient method for separation of free and membrane-bound polysomes.

Table 14 is a mathematical statement of the nature of differential centrifugation. Many simplifying assumptions have been made,

Table 14. Generalizations about differential centrifugation

(1) $xS = \frac{dr/dt}{\omega^2 r}$ This is the definition of the Svedberg coefficient measured in Svedberg units, S.

by rearrangement and integration we get (2)

(2) $$t_2 - t_1 = \frac{\ln(r_{max}/r_{min})}{(xS)\ \omega^2}$$

$t_2 - t_1$ = length of time needed to go from r_{min} to r_{max}

ω = radians/sec

x = the number of Svedberg units (S)

(3) $t_2 - t_1$ we will call just t

$S = 1 \times 10^{-13}$ sec

$$t(hrs) = \frac{K/S}{x}$$

$$K/S = \frac{\ln\ (r_{max}/r_{min})}{\omega^2} \times \frac{10^{13}}{3600}$$

(4) All differential centrifugation was done at 27,000 x g (max), which in the SS 34 rotor is equivalent to 15,000 rpm.

$$rpm = \frac{2\ \pi \text{ radians}}{60 \text{ sec}} = 0.10472 \text{ rad/sec}$$

$$K/S = \frac{\ln\ (r_{max}/r_{min})}{[(15{,}000)(0.10472)]^2} \times \frac{10^{13}}{3600}$$

$$K/S = \ln\ (r_{max}/r_{min}) \times 1125.8$$

Table 14 (continued)

(5) In the SS 34 rotor, r_{max} = 4.25 inches

r_{min} (in inches):	4.15	4.05	3.81	3.56
equivalent ml of sample in a 15 ml Corex tube:		1		5

Minutes for a particle of x Svedberg units to travel from r_{min} to r_{max}:

	r_{min}			
x	4.15	4.05	3.81	3.56
80	20.0	40.3	91.8	150.0
160	10.0	20.1	45.9	74.9
240	6.8	13.4	30.6	49.9
480	3.3	6.7	15.3	25.2
960	1.7	3.4	7.7	12.5
4000	0.4	0.8	1.8	3.0

perhaps the most important of which is that the S value is known. This states that if the S value of a molecule or particle is known, then the time needed for it to traverse any distance in a centrifuge tube, including traversal of the maximum distance to pellet, can be theoretically determined. Figure 9 illustrates the differential centrifugation statement for the situation encountered in this thesis. The distance from the center of the rotor to the end of the centrifuge, r_{max}, was 4.25 inches. For a 1 ml sample r_{min} was 4.05 inches and for a 5 ml sample it was 3.56 inches. It is apparent that there

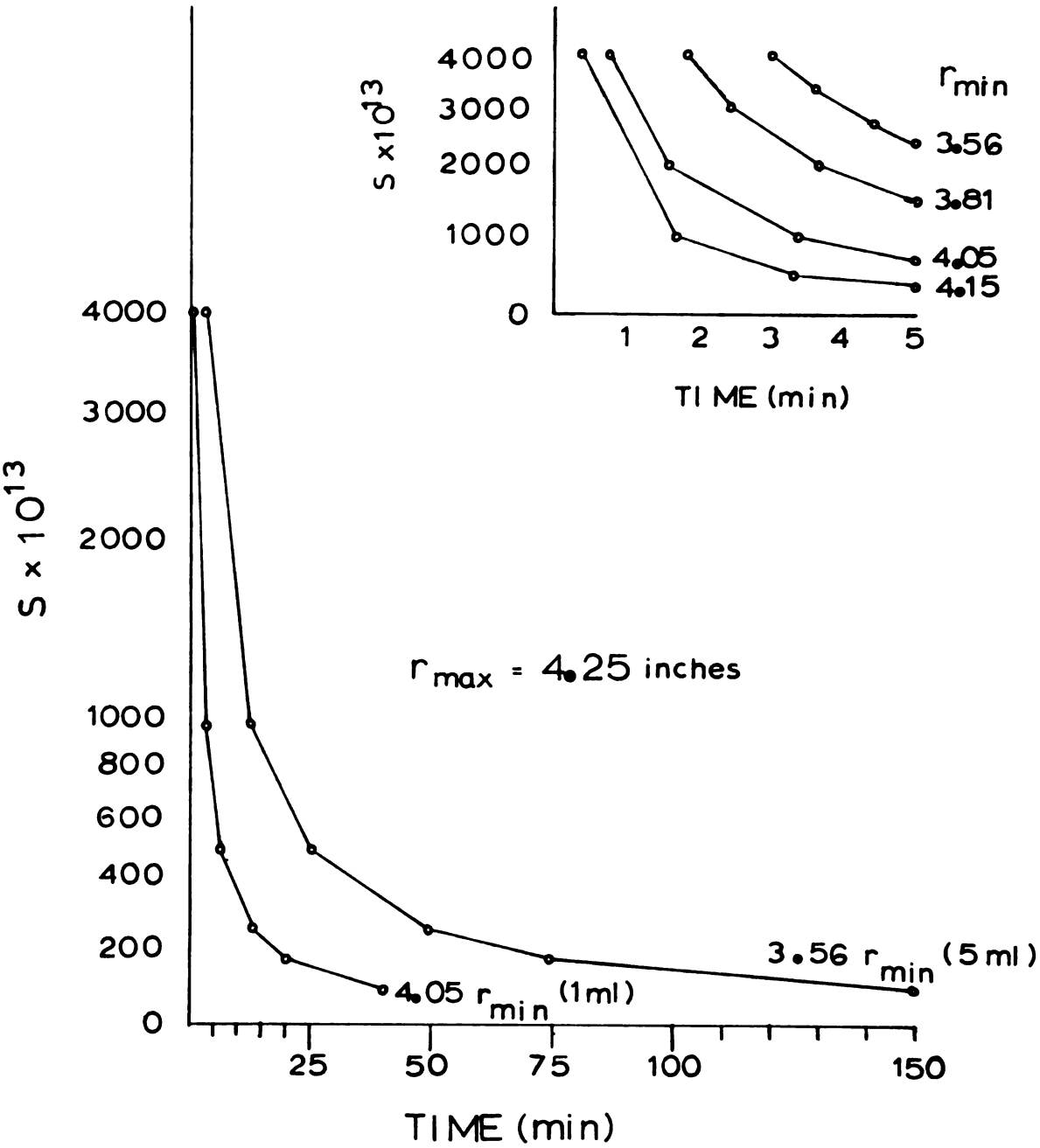

Figure 9. Time needed to sediment from r_{min} to r_{max} by differential centrifugation at 27,000 x g (max).

is quite a difference between the samples in the sizes of particles (S value) which will sediment from r_{min} to r_{max} in r_{max} in 5 minutes.

The equation may also be expressed as a curve as shown in Figure 10. Some of the data in this study may be understandable in terms of the curve in Figure 10. Figure 2 and Table 2 present data which indicate that as the concentration of absorbance units (free and membrane-bound polysomes for simplicity) increases, the observed percentage of membrane-associated polysomes (polysomes in the pellet) will increase. The data also show that as the volume of the sample increases, the observed percentage of membrane-associated polysomes decreases. These changes correlated with similar changes in ^{3}H-uridine labeled polysomes as seen in Table 5 and other experiments. Table 5 also shows data representative of experiments in which identical changes occurred in samples from two different cell cultures that probably differed in physiological state. Finally, the same type of changes in observed percentage membrane-associated polysomes occurred in three other cell types. This is an indication that what is being observed is a physical phenomenon of differential centrifugation and not some peculiar characteristic of MOPC-21 cells.

Figures 3A and 3B are profiles of non-detergent and detergent treated MP1 (crude 27,000 x g pellet), respectively. Here there is an indication of the cause of variation in observed A_{260} and radioactivity in the pellets. The pellet should contain only membrane-bound polysomes; there should not be a profile in the polysome region of the non-detergent treated sample, yet there is a profile. The percentage CPM shown with the profile is of purified free polysomes which were added to the PNSW before differential centrifugation.

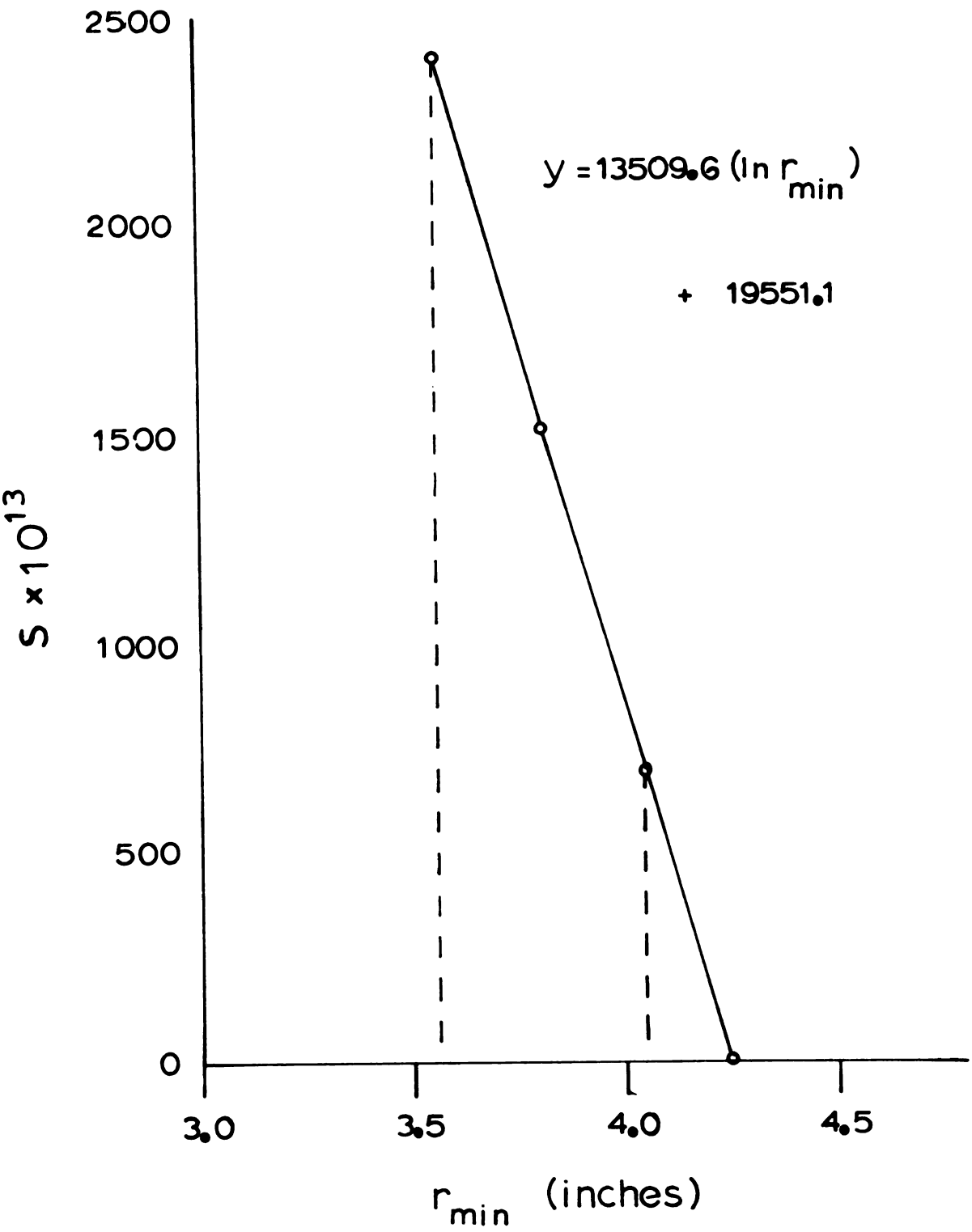

Figure 10. The minimum S value of particles pelleting from r_{min} to r_{max} in 5 minutes of centrifugation at 27,000 x g (max).

It is obvious that free polysomes were pelleted. Table 4 shows another experiment in which a Sepharose 6B chromatographed, ^{3}H-uridine labeled, PNSW was subjected to consecutive differential centrifugations. Assuming that all the microsomes were pelleted after the first centrifugation, free polysomes definitely pelleted. The assumption that all the microsomes pelleted is supported by the data in Table 7. A 1 ml and a 5 ml sample were differentially centrifuged. The typical difference in observed membrane-associated polysomes occurred. The 1 ml sample seemingly had a much higher percentage. The supernatant of each sample was brought to the same volume and spun again for a relatively long period. This long centrifugation is certainly sufficient to pellet microsomes which have S values of 1000 or greater, large in comparison with polysomes (Rosbash and Penman, 1971a). If the difference could be attributed to microsomes not pelleting in the 5 ml sample, then a similar large difference should appear in the pellets from the longer spin. Much more material should pellet from the original 5 ml sample. This did not occur; instead, almost identical CPM pelleted from the samples. Moreover, a moderate increase in A_{260} pelleted could reasonably be attributed to non-polysome associated membrane (Table 8).

Another approach to determine whether free polysomes had pelleted was to wash the pellets from an initial centrifugation of a 1 and 5 ml sample. One would expect more to be washed from the pellet of the 1 ml sample and this was indeed the case. All the evidence indicated that free polysomes were pelleting. The curve shown in Figure 10 explains the evidence. The area beneath the curve is proportional to the quantity of polysomes of given S values

which will *not* pellet in 5 minutes at 27,000 x g (max) from r_{min} to r_{max}. It is apparent that much more material will not pellet in 5 ml than will not pellet in 1 ml. In fact, if one makes the reasonable assumption that free polysomes are essentially 700S or less (Breillatt and Dickman, 1966), then the chance that a free polysome will pellet if it is outside that one ml closest to the bottom of the 15 ml Corex centrifuge tube is negligible. Compare a one ml sample and a 5 ml sample, each containing the same total number of A_{260} units (same amount of PNSW). Four-fifths of the free polysomes that will pellet in the one ml sample will *not* pellet in the 5 ml sample; they are outside the critical 1 ml range. This four-fifths amount is empirically equal to approximately 6% of the *total* A_{260} according to the data in Table 2 (that is, 6% is the difference between the amounts pelleting in the 1 ml and 5 ml samples).

$$(4/5)y = 0.06x$$

y = free polysomes that will pellet in a 1 ml sample
x = total polysomes in PNSW

Solving, we see that y = 7.5% or that 7.5% of the total polysomes in the bottom 1 ml can be expected to pellet in differential centrifugation even though by all other criteria these polysomes would be considered free polysomes. The expected amount agrees well with consistently observed amounts of free polysomes (as measured by A_{260} differences) which pelleted during the course of this study.

There are three solutions to the problem of obtaining pure fractions of free and membrane-associated polysomes. First, an alternative technique may be employed. Second, a combination of

differential centrifugation with another method to improve the purity of the fractions may be attempted. This was chosen for this study. The last choice is to manipulate the conditions of differential centrifugation in a manner that will result in pure fractions. The way to go about this is suggested by the curve in Figure 10. If a gradient of free polysomes of 700S or less pellets is 1 ml, then if the pellet is resuspended in 5 ml and centrifuged a second time, the percentage of free polysomes that will *not* pellet will be equal to at least 4/5 the amount of what initially pelleted. That is, at least a 5-fold reduction in the amount of free polysomes contaminating the membrane-bound polysomes will result. Resuspension of the pellet and differential centrifugation for a third time would yield at least a 25-fold reduction in contamination, and the pellet should be suitable for use as a source of membrane-bound polysomes. For this technique to work, large aggregates must not form when free polysomes are subjected to differential centrifugation and pellet. Preliminary indications are that aggregation of free polysomes would not pose a problem, at least for MOPC-21 cells.

Free polysomes could be obtained in high purity (relative to microsomal contamination) simply by two more differential centrifugations of the initial supernatant, the technique employed in this study. Thought should be given to the concentration of polysomes per ml. Figure 11A shows profiles of MPl obtained with a 5.0 ml sample volume and 37 A_{260}/ml. Please compare Figure 11A with Figure 3A and note the absence of free polysomes in the non-detergent treated sample profile in Figure 11A.

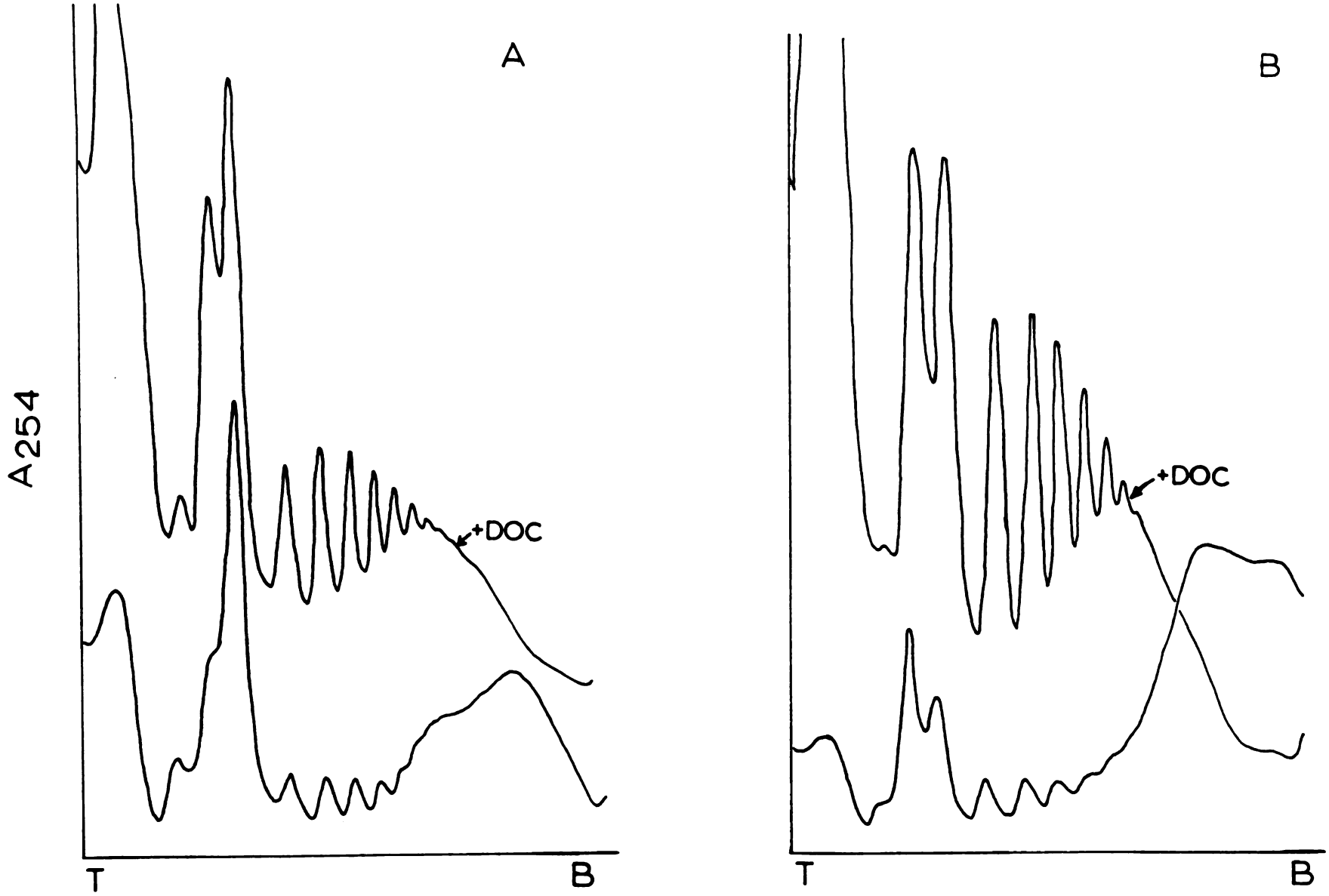

Figure 11. Sucrose gradient profiles of MP1. A. MP1. B. MP1 after partition separation in the aqueous polymer two-phase system (MP2XP). A_{254} (——).

However, Figure 11A shows membrane-bound polysomes that are still probably contaminated with some free polysomes. The MP1 of Figure 11A was further processed by partition separation in a dextran-methylcellulose aqueous polymer two-phase system. The result, MP2XP, is shown in Figure 11B. There is a large drop in monosomes in Figure 11B (non-detergent treated MP2XP) as compared with Figure 11A (non-detergent treated MP1). This was a consistent feature of partitioned membrane-bound polysomes.

Partition separation is less likely than differential centrifugation to fail to discriminate between aggregates of free polysomes and microsomes. The partition coefficient K is the concentration of the substance in the top phase divided by the concentration of the substance in the bottom phase. It has been found to be highly dependent on the surface area of the partitioned molecules and particles (Albertsson, 1959). Its effectiveness is based on the fact that RNP particles having diameters of 100-175 Å should have a different K value than the vesicles with diameters of 500-5000 Å. Also, according to Albertsson (1960) surface properties are predicted to play a role in the distribution. Two opposing tendencies govern the distribution behavior of a particle. Brownian motion tends to distribute the particles randomly while interfacial forces tend to distribute the particles unevenly so that they collect in the phase in which they have the lowest energy (Albertsson, 1960, p. 95).

The separation of phases is also governed by two opposing tendencies. The tendency to mix rather than separate into phases is furthered by the gain in entropy on mixing which is related to the number of molecules. The tendency to form phases is encouraged by the interaction energy between molecules which increases with the size of the

molecule since it is the sum of the interaction between each small segment of the molecules. For very large molecules the interaction energy per mole dominates over the entropy of mixing per mole and determines the result of mixing two polymers (for example, phase separation occurs if the interaction is "repulsive" in character) (Albertsson, 1960, p. 19). A system of two polymers is a multicomponent, not ternary, system because of different molecular weight species composing each polymer. However, some predictions made for the ternary system are still applicable (Albertsson, 1960, p. 25--if you plan to use an aqueous polymer two-phase system, this book is highly recommended as a source for insight into what is still largely an empirical process).

Membrane-associated polysomes (MP1) labeled with ^{3}H-uridine were processed by a combination of differential centrifugation and partition separation. The profiles are shown in Figures 4 and 5. Notice that there is not a corresponding increase in radioactivity in the large peak at the bottom of the gradient of non-detergent treated MP2XP profiled in Figure 4, although some radioactivity does appear in the peak. The radioactivity which is present in the peak may be due to nonspecific trapping or adsorption, but other, non-artifactual, possibilities exist which have not been ruled out.

^{3}H-uridine labeled free polysomes were added to a PNSW which was then processed through to MP2XP. Each fraction was monitored for the amount of free polysomes entering it. Table 12 gives the results in tabular form. Less than 1% of the initial counts ended up with the partitioned membrane-bound polysomes. All fractions other than MP2XP contained radioactive profiles indicative of significant contamination with labeled free polysomes (Figures 3 and 6).

Notice that the large A_{254} peaks at the bottom of the gradients of non-detergent treated MP1 and MP2XP are not correspondingly enriched for radioactivity. This precludes the peak from being an aggregate of free polysomes. In Figure 5 the profile of radioactivity indicates that the peak is also not an extensive aggregate of rough microsomes; however, some involvement of microsomal polysomes has not been ruled out. By comparison with the profiles of detergent treated fractions, it is apparent that the peak is solubilized by detergent. The peak does not appear in the non-detergent treated PNSW, another membrane containing fraction. Taken together, this information indicates that the peak is probably an aggregate of non-microsomal membrane which is formed when the membrane is pelleted by differential centrifugation. The sucrose gradients are 15 x 45% (w/v). Forty-five percent (w/v) sucrose is equivalent to about 39% (w/w). That would place the large peak in the vicinity of 37% (w/w) sucrose, which is equivalent to about 1.16 gm/cc in density. Bosmann *et al.* (1968), Ray (1969), Caliguiri and Tam (1970), and Rosbash and Penman (1971) all found membrane in the cell which would band at about this density. The nature of the membrane in each case may not have been exactly the same as the putative membrane considered in this study, but the banding of membrane at a similar density does tend to affirm the membranous nature of the large peak. The large peak in non-detergent treated MP1 and MP2XP fractions thus seems to contain only a small amount of RNA, is solubilized by detergent, and bands at a density characteristic of membrane. However, when an MP1 fraction was treated for 30 minutes at 37°C with 10 units of RNase T1/ml, the peak

disappeared. It is tentatively assumed that the RNase was contaminated with other enzyme activities, although other interpretations are possible.

Polysome fractions were also assayed for glucose-6-phosphatase and 5'-mononucleotidase activity to test for the presence of microsomal membrane and plasma membrane, respectively. It is known that the distribution of enzyme activities may vary with cell type (Bosmann, 1968). In rat liver, smooth membrane can have moderate 5'-mononucleotidase *and* glucose-6-phosphatase activity (Ray, 1969), while in HeLa cells 5'-mononucleotidase is detected only in the plasma membrane (Bosmann, 1968). One must therefore be cautious in interpreting the enzyme data gathered in this study. The data in Table 10 tentatively indicate no particular enrichment for G-6-phosphatase activity in the partitioned microsomal pellet (MP6). 5'-Mononucleotidase activity seems to be selectively removed from the microsomal fraction which could be interpreted as a purification of microsomes from contaminating plasma membrane.

Table 11 presents a comparison of partitioned membrane-bound polysomes, MP2XP, and crude membrane-associated polysomes, MP1. The membrane fractions were treated with RNase or 0.5 M KCl and then washed by differential centrifugation. Untreated, washed fractions served as controls. In every case more material was released into the supernatant by the crude MP1 fraction than by the partitioned MP2XP fraction. We believe the *extra* material to mainly consist of contaminating free polysomes, not the loose polysomes referenced in the Literature Review. The material released from the partitioned membrane *was* probably loose material. The results were

consistent with those of Mechler and Vassalli (1975), who found that 0.5 M KCl detached only 10% of the ribosomal particles from MOPC-21 microsomal membrane.

In summary, partitioned membrane-bound polysomes have been shown to differ from crude MP1 polysomes by a number of different criteria, all of which favor the idea that crude MP1 polysomes are contaminated with free polysomes which can be successfully removed by a combination of differential centrifugation and partition separation in a dextran-methylcellulose aqueous polymer two-phase system.

An anomaly was occasionally observed. The partitioned polysomes showed a degraded profile while the other polysome fractions were undegraded. This is documented in the Results section. Perhaps some physiological effect caused only one polysome population to appear degraded in the cell. Alternatively, perhaps some physiological effect caused the membrane-bound polysomes to become susceptible to shear by Dounce homogenization or by the forces acting in partition separation.

Theoretically, a pure fraction of membrane-bound polysomes should have been enriched for myeloma messenger. A cell-free system for protein synthesis was constructed to translate polysomal message. Optimization was performed with Sepharose 6B chromatographed free polysomes. The optimum curves are shown in Figure 8. The pH and the Mg^{++} optimums had wide ranges. The K^{+} optimum was not only wide, but was also erratic. The reason for this is not clear.

Sepharose 6B was used early in this study to separate small molecules, including RNase, from polysomes. With an exclusion limit

of 10^5 - 1 x 10^6 daltons, small molecules such as RNase and tRNA were included thus allowing their separation from free and membrane-associated polysomes. Polysomes could then be stored at -80°C for up to six months without degradation and were more responsive than unchromatographed preparations to added components (especially high speed supernatant) in a cell-free system for protein synthesis. Later in this study, however, Mg^{++} precipitation was used to isolate polysomes from other cellular material. One advantage of this expedient technique over chromatography on Sepharose was that when the precipitate was centrifuged and resuspended, the polysomes were usually concentrated, while elution from the Sepharose column diluted the polysomes.

Figures 8D and 8E are kinetics curves made with a PNS and a Mg^{++} precipitated PMS3. Mg^{++} precipitation apparently did not alter the kinetics of the reaction. The reaction was essentially complete in approximately 10 to 15 minutes. Initiation probably does not take place in this system due to contamination by the nuclease inhibitor heparin which is routinely included in isolation buffers to protect mRNA from ribonuclease. For some reason membrane-associated polysomes are not being translated as efficiently as free polysomes. This may possibly be due to the detergent concentration at which these membrane-associated polysomes are Mg^{++} precipitated.

Polypeptides synthesized in the cell-free system for protein synthesis were chromatographed over normal rabbit gamma globulin and anti-myeloma affinity columns. The least myeloma protein was detected in the fraction that should have contained the most. There are not enough data and too low a binding efficiency to the column

to allow speculation on this matter as yet. Control specificity for myeloma protein was excellent. The anti-myeloma protein was made against whole myeloma protein and as such nascent polypeptide may not have presented suitable antigenic determinants to the antibody to allow for efficient binding. Also, the newly made polypeptide was undoubtedly in the form of heavy and light chain or, at any rate, not in the form of a whole antibody molecule. Heavy and light chains have been shown to exhibit only low levels of binding to anti-myeloma columns (Eschenfeldt, M.S. thesis, 1975, p. 63). The next step, in view of the excellent specificity that the anti-myeloma column has for myeloma protein, seems to be the construction of an anti-heavy chain and an anti-light chain affinity column. Perhaps the cell-free product will bind with greater efficiency to such columns.

SUMMARY

Membrane-bound polyribosomes were separated from free polyribosomes by a combination of (1) differential centrifugation and (2) partition separation in an aqueous two-phase polymer system. Conditions were defined in which an adequate separation could be obtained by differential centrifugation alone. The polysome profiles were related to the physiological state of the cells. The polysomes were active in a cell-free system for protein synthesis. However, the polypeptide products could not be unequivocally shown to be myeloma polypeptide with a myeloma-anti-myeloma affinity column system.

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