NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

THE MOLECULAR BIOLOGY OF LYMPHOCYTE ACTIVATION; a study of RNA metabolism in resting and mitogen stimulated lymphocytes.

by

Mary F. Mitchell

A Thesis submitted to the School of Graduate Studies of University of Ottawa, as partial fulfilment for the M.Sc. degree in Biology.

Ottawa, Ontario, 1979

The University of Ottawa requires the signatures of all persons using or photocopying this publication. Please sign below, and give address and date.

To my parents,

Angus and Hannah; who first pushed me in this direction.

ACKNOWLEDGEMENTS

Words cannot express my gratitude to, and affection for, Prof. J. Gordin Kaplan. His infectious enthusiasm inspired this work and his advice and insight kept me going in the right direction. Most of all, I am grateful for the friendship and support he has given me; he made what I was doing seem worthwhile.

I would also like to express appreciation of the help, constructive criticism and friendship offered to me by Dr. Enzo Bard.

I am grateful to Rod L'Anglais for his technical assistance with some of the lengthy experiments and to Jaques Helie, Paul Brunon and George Ben-Tchautchaudzé for helping me produce the figures for this thesis.

Last, but not least, I am grateful to all the folk in our laboratory for providing me with a riendly, supportive atmosphere to work in.

ABSTRACT

An improved method affording clean separation of cytoplasm from nuclei was developed and used to study the kinetics of appearance of [^{3}H]uridine-labelled, polyadenylated and non-polyadenylated RNA in the cytoplasm of human lymphocytes stimulated with the mitogen concanavalin A (Con A). While the earliest observable increase in labelled total cell RNA occurred after 10 hours exposure to mitogen, I was able to detect significant increases in uridine-labelled non-polyadenylated RNA in the cytoplasm of lymphocytes, which had been exposed to mitogen for 6 hours; these cells had been pulsed with [^{3}H]uridine for two hours prior to harvest. At least 13 hours exposure to Con A was required before uridine-labelled polyadenylated RNA appeared in the cytoplasm of stimulated cells and it did not appear in resting cells. The lag between addition of isotope and appearance of labelled RNA in cytoplasm was inversely related to the duration of incubation of cells with mitogen. The bulk of the non-polyadenylated RNA transported into the cytoplasm of resting cells was low molecular weight RNA, smaller than 6S (LMW-RNA); LMW-RNA accumulated in the cytoplasm of stimulated cells significantly earlier than the remainder of the non-

polyadenylated RNA. Cells pre-incubated with [^{3}H]uridine before stimulation with Con A showed an increase in uridine-labelled cytoplasmic non-polyadenylated RNA after only 3 hours exposure to mitogen. When adenine was used as label a significant increase in labelled cytoplasmic polyadenylated RNA was detected less than 5 hours after exposure to mitogen.

Other investigators have reported that an increased rate of transcription occurs soon after mitogenic stimulation. My data suggest that mitogen acts primarily to accelerate the processing, especially polyadenylation of pre-mRNA, and transport of RNA transcripts.

A concentration (0.05ug/ml) of actinomycin D which is known to inhibit specifically the transcription of nucleolar RNA in a variety of cell types was found to affect the transcription of all RNA classes in human lymphocytes; in some experiments specific inhibition of nucleolar RNA synthesis was observed at 0.005ug of actinomycin D per millilitre.

In contrast with a publication by Kay(1971) all RNA synthesis was found to be sensitive to 10^{-6} M ouabain.

RESUME

J'ai utilisé une méthode améliorée, permettant la séparation des noyaux du cytoplasme, pour étudier la cinétique de l'apparition du RNA polyadénylé et non polyadénylé dans le cytoplasme des lymphocytes humains stimulés par un mitogène, la concanavaline A (Con A). Alors qu'une exposition de 10 heures était nécessaire a la Con A pour voir apparaître une première augmentation perceptible du RNA cellulaire total marqué, je pouvais déceler des augmentations significatives des quantités de RNA non polyadénylé marqué a l'uridine dans le cytoplasme des lymphocytes exposés au mitogène durant 6 heures; ces cellules ont subi un marquage avec la [^{3}H]uridine pendant 2 heures avant la récolte. Il fallait au moins 13 heures d'exposition à la Con A avant de voir apparaître le RNA polyadénylé marqué dans les extraits cytoplasmiques des cellules stimulées et il n'apparaissait pas dans les cellules au repos. L'intervalle entre l'addition de l'isotope et l'apparition du RNA marqué dans le cytoplasme était en relation inverse avec la durée de l'incubation des cellules avec le mitogène. La plupart du RNA non polyadénylé qui était transporté dans le cytoplasme des lymphocytes au repas était du RNA de bas poids moléculaire,

plus petit que 6S (LMW-RNA). LMW-RNA s'accumulé dans le cytoplasme des cellules stimulées plus tôt que le reste du RNA non polyadénylé. Les cellules qui ont subi un marquage avec la [^{3}H]uridine avant stimulation avec Con A démontrait une augmentation du RNA non polyadenylé dans le cytoplasme en moins de 5 heures d'exposition au mitogène.

D'autres investigateurs ont avancé que la transcription du RNA est accelérê tot après stimulation par un mitogène. Mes résultats suggèrent que les premiers effets du mitogène seraient donc d'augmenter la fabrication et le transport du RNA, particulièrement la polyadénylation du précurseur-mRNA, plutôt que d'accroître la production des transcriptions géniques.

Une concentration de 0.05 ug/ml d'actinomycine D, reconnue pour inhiber spécifiquement la transcription du RNA nucléolaire dans divers types de cellules, affectait la transcription du RNA polyadénylé dans les lymphocytes humains; dans plusieurs expériences, j'ai observé une inhibition spécifique de la synthese du RNA nucleolaire a une concentration d'actinomycin D de 0.005ug/ml.

Contrairement à une publication de Kay (1971) j'ai observé que toute la synthese du RNA est inhibée par 10^{-4} M ouabain.

TABLE OF CONTENTS

List of Figures

List of Tables

Chapter I

INTRODUCTION

1.1 STATEMENT OF THESIS

The study of the control of gene expression in animal cells is facilitated by selecting a system in which cells in a resting state can be stimulated to become active and undergo mitosis. Many diverse systems are utilised ranging from contact inhibited culture cells to regenerating liver cells.

The lymphocyte is the cell type responsible for many, if not all, of the specific immune responses of vertebrates. When human peripheral blood or mouse splenic lymphocytes are cultured in vitro, most do not enlarge and undergo mitosis; cultures of these so called small or resting lymphocytes may be maintained for several days. If antigens or appropriate concentrations of mitogens such as PHA or Con A are added to the cultured resting lymphocytes they undergo a series of biochemical and cytological events eventually culminating in mitosis; this is termed blastogenesis and is generally accepted to be a model of the in vitro proliferation process associated with the immune response. Thus, while many systems are available for studying molecular events accompanying the transition from resting non-mitotic to

actively dividing cells, the cultured lymphocyte is exceptional in having direct physiological significance.

The transition from quiescence to proliferation is accompanied by a variety of biochemical events, at least some of which must be due to changes in the mode of utilisation of the cells' genetic information. The purpose of this thesis is to resolve some of the paradoxes present in the literature, on the changes in RNA metabolism that occur following mitogenic stimulation of human lymphocytes, and to present a perspective of the early effects of mitogens on RNA transcription and processing.

1.2 THE LYMPHOCYTE POPULATION IN VIVO

THe lymphocyte population can be divided into two general categories, B and T, on the basis of differences in ontogeny, cell surface markers and function (Owen and Ritler, 1969). At what point during ontogeny the stem cell becomes commited to the T or B cell pathway is not yet clear. Thymus derived T lymphocytes are responsible for cellular immunity and also function as helper cells in the antibody response to many antigens. Bone marrow derived B lymphocytes are responsible for humoral immunity, functioning predominantly as precursers of antibody secreting cells.

Populations of B and T lymphocytes consist of thousands of different clones; each member of a clone bears at its surface a characteristic receptor for an antigen which, it is supposed, provoked the proliferation of the progenitor lymphocyte that originally displayed this receptor.

1.3 LYMPHOCYTE DIFFERENTIATION IN CULTURE

Cultured resting lymphocytes may be stimulated by antigens or by plant mitogens such as PHA, PWM or Con A; the latter agents are sometimes called polyclonal mitogens since they affect many clones of cells.

Antigenic stimulation of cultured lymphocytes requires that the cells be from animals who have been pre-sensitised with the antigen. The antigenic response as measured by thymidine incorporation peaks 5-8 days after addition of antigen to the sensitised culture.

The response of the culture to polyclonal mitogens is several orders of magnitude higher than it is to antigens and prior sensitisation is not required. Thymidine incorporation also peaks earlier, 2-4 days after addition of mitogen to the culture.

The mode of action of plant mitogens is not well understood. All are capable, to varying degrees, of agglutinating cells and a crucial first step in the activation of lymphocytes by a mitogen is the binding of that mitogen to the lymphocyte cell surface. B and T

lymphocytes have different cell surface properties and vary in their response to each mitogen; PHA and Con A stimulate mostly T cells while PWM stimulates both B and T cells. There is also an optimum amount of mitogen binding for blast transformation which is less than the number of molecules binding at full saturation (Anderson, Sjoberg and Muller, 1972).

1.4 EVENTS FOLLOWING MITOGENIC STIMULATION OF CULTURED LYMPHOCYTES

In animal cells it is becoming clear that the plasma membrane plays a vital role in initially recognising signals that will bring about cell activation at both a metabolic and a genetic level. Many of the early events in lymphocyte activation are associated with the plasma membrane. These include the appearance of new Na-K-ATPase sites at the cell membrane causing increased Na^+ and K^+ fluxes and increased ouabain sensitive ATPase activity (Quastel and Kaplan, 1970). Increased fluxes of Ca^{2+} (Alford, 1970), glucose (Averdunk, 1972), uridine (Peters and Hausen, 1971) and amino acids (Mendelsohn, Skinner and Kornfeld, 1971) have also been observed. There is some evidence for stimulation of membrane bound adenyl cyclase within 10min of adding mitogen and both cAMP and cGMP levels are thought to increase in stimulated cells (Hadden et al 1972).

Other events associated with activation of lymphocytes with mitogens include changes in the metabolic rate of the cell (Roos and Loos, 1970) and an increased rate of acetylation and phosphorylation of histones (Cooper, 1972).

An increase in protein synthesis occurs soon after mitogenic stimulation (Levy and Rosenberg, 1973). Some of this early de novo protein synthesis may be in part responsible for the increases observed in DNA polymerase and RNA polymerase activities (Loeb, 1974). A logical supposition, made by many of the workers in this field, is that increase in protein synthesis is preceded and accompanied by increased transcriptional activity; in fact, an impressive body of literature reports increased incorporation of [^{3}H]uridine into RNA less than one hour after stimulation by mitogen (e.g. Cooper and Rubin, 1965; Kay and Cooper, 1969; Pago and Allfrey, 1966). This literature has been reviewed (Kaplan Mitchell and Bard, 1978). However some investigators, including myself, find that one of the principal early effects of mitogen is to increase the rate of processing and transport of all classes of RNA and that there is little or no increase in the overall rate of transcription in the first 10 hours following mitogenic stimulation (Mitchell et al, 1978; Hauser et al, 1976). Various perspectives on RNA metabolism in stimulated lymphocyte will be discussed in detail later in this chapter.

An increased rate of DNA synthesis is measurable after about 24 hours contact with mitogen; this synthesis is asynchronous and peaks after 2-4 days. While actively synthesising DNA the small lymphocytes complete the sequence known as blast transformation or blastogenesis and may proceed through mitosis. The blast cell is 15-30 micrometres in diameter, that is 2-3 times the size of the small lymphocyte. The size increase is mostly accounted for by an increased cytoplasmic volume rich in organelles. In the nucleus the densely packed heterochromatin typical of the small lymphocyte decondenses; the ratio of euchromatin to heterochromatin changes from 0.56 to 10.8 in the transformation from small lymphocyte to blast cell (Cooper, 1972).

1.5 LEVELS OF REGULATION OF GENE EXPRESSION

Several recent reviews have discussed the regulation of gene expression at various levels and in a variety of cell types (e.g. Perry, 1976; Revel and Groner, 1978). For the sake of clarity we can distinguish three levels of regulation which could cause increased utilisation of the genetic material:

1. Transcriptional. This may include increased synthesis and/or activation of pre-existing RNA polymerase and/or activation of the DNA template. Thus qualitative and quantitative changes in the DNA sequences being transcribed are possible.

2. Post-transcriptional. The primary gene transcripts, in the case of all kinds of RNA, must be converted to mature functional molecules by a variety of modifications and cleavages; this is called processing. Regulation may be mediated by altering the efficiency of processing of RNA primary transcripts to mature cytoplasmic molecules or by regulating the translocation from nucleus to cytoplasm of mature or semi-mature molecules. The rate of turnover, that is, the half life , of cytoplasmic RNA may also be considered as a post-transcriptional level of regulation.

Final cytoplasmic pool levels of any species of RNA depend not so much on the rate of its synthesis but on the balance of this process and the subsequent events of processing (including transport) and turnover.

3. Translational. Forms of regulation that have been proposed to act at this level include the availability of initiation factors for protein synthesis and activation of "masked" mRNA present in the cytoplasm as messenger-ribonucleoprotein particles. Control may also be mediated at a post translational level by modification of proteins.

Obviously a much longer review than can be presented here would be required to discuss all of these possibilities. I will concentrate on ways in which altered RNA transcription and processing affect final cytoplasmic levels of RNA species.

1.6 PROCESSING OF RNA

Processing includes methylation and other modifications of bases and sugars, terminal addition of nucleotides to the 3'OH and/or the 5'P ends of the molecules, cleavage of (non-conserved) sequences and splicing and juxtaposition of originally distant sequences, following cutting out of an intermediate part.

tRNA is transcribed by polymerase III as precursor molecules (most likely monomeric) that are on average 20 nucleotides longer than the mature product. tRNA methylases are mainly located in the nucleus but soluble cytoplasmic fractions contain nucleases able to cleave the extra sequences from pre-tRNA; pre-tRNA is found in the cytoplasm after short labelling pulses. Throughout the life of tRNA molecules the three terminal residues at the 5' end, CCA, turn-over quite rapidly. The synthesis and processing of tRNA has been reviewed by Smith (1976).

rRNA is transcribed in the nucleolus as a precursor molecule of 4.5×10^6 daltons (45S pre rRNA) which contains

the sequences of 18S, 5.8S and 28S rRNA in this order from the 5' end. Successive endonucleolytic cleavages yield a series of intermediate products. In the case of mouse L-cells in culture these products are 41S, 36S and 32S; the rate limiting step of this process is the transformation of the 32S intermediate into mature 28S rRNA.

Most methylation takes place on the nascent RNA chain; most of the ribosomal structural proteins also become associated at this time. Methylation seems to play a role in the selection of conserved sequences - only the sequences corresponding to future mature products are methylated. Processing occurs mostly in the nucleolus and appears to be synchronised with the duration of the cell cycle in growing cells. Resting cells exhibit the phenomenon of "wastage" (see below) in which a fraction of precursor molecules is completely degraded instead of becoming functional. rRNA processing is discussed in a review by Perry et al (1976).

mRNA is transcribed as large precursor molecules which form part of the so called heterogenous nuclear RNA (hnRNA). While this statement seems quite safe at this time there was considerable controversy, until quite recently, about both the nature and biological significance of hnRNA. (Williamson, 1977).

hnRNA contains molecules of a wide variety of sizes. Some of the smaller molecules overlap in size with the upper end

of the mRNA population, but on average hnRNA molecules have 4 to 5 times the number OF bases as the average mRNA molecule.

Studies by hybridisation saturation of mammalian cells growing exponentially in culture, indicate that about 20% of hnRNA molecules contain sequences homologous to mRNA. This is in agreement with results measuring sequence complexity; hnPNA is 5-10 times more complex than mRNA.

The initial transcript of pre-mRNA undergoes several modifications during the maturation process, most of them in the nucleus:

1. cleavage of non-conserved sequences;

2. modification of the 5`P end by attachment of methyl guanosine linked by a 5`-5` pyrophosphate bridge ("capping"), methylation of two or three nucleotides from the capped end and methylations of some internal adenosines.

3. addition of a stretch of polyadenylic acid (polyA) to its 3`OH end.

These modifications do not necessarily follow each other in the above order. Very recently Darnell (1979) has shown that capping occurs almost as soon as transcription is initiated in adenovirus and polyadenylation is the next

event in subsequent processing. He has postulated that polyadenylation may even be a trigger for subsequent processing. While this work is exciting, care must be taken when extrapolating viral data to mammalian cells. It should also be noted that some mRNAs are not polyadenylated and some polyadenylation takes place after transport to the cytoplasm.

In 1977 a complete revision of theories on RNA processing became necessary when reports on mRNA produced during infection of cells with adenovirus (Dunn and Hassel, 1977; Berget, Moore and Sharp, 1978) or SV40 (Lavi, Groner and Weizman, 1978) indicated that a mature mRNA could be composed of fragments transcribed from different, indeed widely separate, parts of the viral genome. Almost simultaneously, it was found that the gene for β-globin chain in the rabbit contains two inserts interrupting the coding region, one of which has about 600 base pairs (Jeffreys and Flavell, 1977). Similar inserts have subsequently been found in almost every mammalian gene that has been looked at. These include the genes for ovalbumin (Breathnach, Mandel and Chambon, 1977), ovamucoid (Stein et al, 1978), and immunoglobulins (Nóbumichi and Tonegawa, 1978). These inserts, (called introns by Gilbert, 1978), appear to be transcribed into the precursor molecules and later excised (Roop et al, 1978). The topic of split genes and RNA splicing has recently been reviewed by Crick (1979).

The biological significance of these alterations is not fully understood. There is some evidence that the 5' cap may funtion in ribosomal binding by the mRNA (Perry and Kelley, 1976). The role of polyadenylation is more obscure; there are some indications that it can have a stabilising effect on mRNAs (Marbaux, Huez and Soreq, 1977). As for the distribution of sequences of the same mRNA in different portions of the genome, not only might it provide genetic flexibility, but it may facilitate in some way the regulation of mRNA production.

1.7 CHANGES IN RNA METABOLISM ASSOCIATED WITH THE ACTIVATION OF QUIESCENT CELLS

Mitogen stimulated lymphocytes are not the only cell system that has been used to study the molecular events that accompany the transition from resting to actively dividing cells. The growth of primary cell cultures and some established cell lines may be density dependent. At high cell densities growth is usually arrested at a point in the cell cycle just before the onset of DNA synthesis; the inhibition can often be reversed by adding agents such as fresh serum to the growth medium. Another system is regenerating rat liver. When two lobes of the liver of the adult rat are removed, the cells of the remaining lobe are aroused to renewed cell division. Egg activation is another system; some studies on the activation of sea urchin eggs are discussed in chapter 4.

In all of these systems the transition from quiescence to proliferation is accompanied by many biochemical events and investigators have tried to establish what changes occur in the mode of utilisation of the genetic information of the cell so as to provide changed cellular activities. According to its physiological state the cell makes a qualitative and/or quantitative selection of genes to be transcribed and/or hnRNA sequences to be processed and transported to the cytoplasm as functional mRNA. The mechanism by which the transcripts are selected is not well established. Quantitative changes may be mediated by activation of RNA polymerase, or activation of the DNA template by detachment of repressors or conformational changes, possibly due to rearangements of nuclear proteins. A combination of the different processing steps is probably involved in selecting transcripts for export from nucleus to cytoplasm.

In the systems I will concentrate on here, serum stimulated fibroblasts and mitogen-stimulated lymphocytes, the task of finding qualitative differences in functional mRNA is formidable. It is probable that new mRNAs (if they exist) will account for only a minimal fraction of the total steady state population, and if the search is not for a specific mRNA, the measurement of increased amounts of transcripts will be beyond the limits of detection of present methods.

The steady state distribution of hnRNA does not represent that of the mRNA for which it is precursor; as described, the relative abundance of an mRNA is regulated by the rates of transcription, processing and turnover. For this reason it may be more valid to study cytoplasmic populations of RNA than to study whole cell or nuclear populations.

It should finally be mentioned that free ribonucleoprotein particles (mRNP) are of widespread occurrence, but so far there is no hard evidence as to their significance.

I shall now review relevant data obtained from cultured mammalian fibroblasts arrested in growth either due to serum deprivation or to contact inhibition. This system has been widely used as a model for study of regulation of gene expression. In many ways it is similar to the lymphocyte system but there is need for caution in extrapolating data from one system to another. The first quantitative measurement in this system were made nine years ago by Stanners and Becker (1970). 3T6 growing cells contain per unit of DNA 1.8 times more rRNA and tRNA and 2-3 times more polyadenylated RNA than do resting cells (Johnson et al, 1974).

Before considering the multitude of studies showing how these changes might be achieved let us consider some of the hazards in the interpretation of data reflecting incorporation of radioactive nucleotides into RNA. In all

cells many classes of RNA are constantly being synthesised and degraded at varying rates so that incorporation of isotope into RNA is a very complex measurement; many investigators choose to ignore this. It is also very important to consider the importance of nucleoside triphosphate pool sizes in relating nucleotide incorporation to macromolecular synthesis.

It has been reported that rRNA is stable in growing mouse fibroblasts (Abelson et al, 1974), though perhaps this is true only for established cell lines and not for primary cultures (Liebhaber, Wolf and Schlessinger, 1978), while it turns over with a half life of about 60h in resting cells. tRNA turns over with a half life of about 60h in growing cells as compared with a half life of about 36h in resting cells (Abelson et al, 1974). No appreciable differences in the stability of the total population of polyadenylated RNAs have been detected between resting and growing cells. These differences in stability are not sufficient to account for the quantitative differences in RNA content.

In fibroblasts, there seem to be separate control systems for monitoring the cellular content of rRNA, tRNA and mRNA. The mobilisation of polymerase 1 appears to be the principal controlling factor in rRNA formation (Johnson et al, 1976); the total activity of polymerase 1 in whole nuclei is linked to the growth rate of the cell. rRNA content in the cell

can be increased in the absence of DNA duplication (Mauck and Green, 1974); inhibition of DNA synthesis in ghost monolayers prepared from serum stimulated cells does not affect the increase in RNA synthesis up to 34h in culture.

The synthesis of tRNA seems to be regulated like that of rRNA but in a much narrower range. Immediately after serum stimulation the rate of synthesis of pre-tRNA rises together with that of pre-rRNA but later it fails to increase in the same proportion (Mauck and Green, 1974); after DNA duplication the rate rises again. Some mechanism must exist which coordinates the amounts of tRNA and rRNA in the cell, because the ratio rRNA/tRNA for a particular cell line is constant, independent of whether it is growing or arrested. Coordination of amount does not, in this case, mean coordination of synthesis, because the turnover rates are different.

The control mechanism(s) monitoring the amount of polyadenylated mRNA produced seems to operate at a post-transcriptional leval. The rate of synthesis of hnRNA does not change during the transition from resting to growing conditions (Johnson et al, 1974; Mauck and Green, 1973); the amount of hnRNA transcribed is proportional to the content of DNA in the cell. HnRNA production increases between 10 and 20 hours after serum stimulation, paralleling DNA duplication ; if DNA synthesis is prevented

there is no increase in hnRNA production (Mauck and Green, 1973). Despite the fact that synthesis of hnRNA does not increase after serum stimulation, and that the proportion of polyadenylated to non-polyadenylated hnRNA and the total nuclear polyA content remain the same (Johnson et al, 1975), the content of newly synthesised polyadenylated mRNA in the cytoplasm increases two fold before replication of DNA takes place (Johnson et al, 1974). This parallels the observed increase in protein synthesis. Non polyadenylated mRNA seems to increase in a coordinated manner(Levis, McReynolds and Penman, 1976). There is one report (Rudland, 1974) that no appreciable change in the amount and rate of synthesis of cytoplasmic polyadenylated mRNA takes place after serum stimulation. However, it seems the author did not distinguish between total cell polyadenylated RNA and mRNA; he did not distinguish nuclear from cytoplasmic fractions. Thus his interpretation of the data leaves something to be desired.

The main control mechanisn responsible for the increase in cytoplasmic polyadenylated mRNA seems to be an increased efficiency of hnRNA processing and transport to the cytoplasm (Johnson et al, 1974; 1975; Mauck and Green, 1973).

One group of researchers compared RNA metabolism in a number of fibroblast cell lines. (Johnson et al, 1976). The principal correlation they found is that the more

rapidly the cells grow, the higher is the ratio of mRNA:rRNA.

There is some controversy as to what mechanism is responsible for the increase in the rate of protein synthesis that occurs soon after serum stimulation. It has been claimed that the appearance of newly synthesised mRNA in cytoplasm is solely responsible for that increase (Johnson et al, 1974;1975). Evidence supporting this hypothesis comes from work on established cell lines of mouse fibroblasts growing on monolayers and on Chinese hamster ovary cells growing in suspension, including measurements of cytoplasmic polyadenylated RNA in the sub-ribosomal and polysomal fractions and the demonstration that the degree of loading of mRNA with ribosomes is the same in resting and growing cells.

On the other hand several investigators (Rudland, 1974; Bandman and Gurney, 1973; Meedel and Levine, 1976) studying mouse and human fibroblasts growing in monolayers concluded that the increase in new transcripts in the cytoplasm cannot, by itself, account for the increase in protein synthesis. They postulate a mechanism of recruitment of stored free mRNA into polysomes soon after stimulation; evidence comes from similar measurements of the distribution of polyadenylated mRNA in the cytoplasm and experiments of recruitment of "free" mRNA into polysomes in the presence of

cycloheximide which inhibits elongation. Johnson and Meister (1977) showed that cycloheximide causes a disproportionate accumulation of polyadenylated mRNA especially in serum stimulated cells and recommend caution in interpreting results obtained in presence of the drug. Meedel and Levine (1976) point out that 3T3 and 3T6 lines are not normal cells in the sense that one has been classified as transformed and the other causes tumors when injected into mice. The contradictory results could also be explained by different authors' use of different labelling times (pulse as opposed to steady state). I think the existance of translational control after serum stimulation remains an open question.

The sequence complexity of cytoplasmic polyadenylated RNA has been measured by hybridisation, in resting and in serum stimulated fibroblasts (Williams and Penman, 1975; Getz et al, 1976). Very few differences were found; in both cases the mRNA present corresponds to 9000-11000 different genes. If there is any expression of new genes after serum stimulation it represents an increase in complexity of not more than 5%. The significance of this number, however, is very difficult to interpret since technical considerations, among them difficulties in resolving complex kinetic curves, preclude much confidence in the values reported. Furthermore, These experiments cannot distinguish between qualitative differences and extreme quantitative differences.

Thus, it is clear that the transition from resting to growing state after serum stimulation is not accompanied by large scale activation of new genes. The regulatory mechanisms seem to be at the post-transcriptional level and may involve changes in transport and turnover.

1.8 RNA METABOLISM IN CULTURED LYMPHOCYTES

Cooper and colleagues have extensively studied the changes in rRNA metabolism that accompany lymphocyte transformation. They observed increased labelling of acid precipitable RNA 30min or 1hour after adding PHA to human peripheral blood lymphocytes (Kay and Cooper, 1969). The early increase was mainly in 4S and polydisperse material, the types of RNA which turn over most rapidly in resting lymphocytes. Abolition of RNA synthesis did not inhibit the initial increase in protein synthesis suggesting this could be attributed to increased utilisation of pre-existing ribosomes. The accumulation of new ribosomes is, however, essential for normal growth and division of lymphocytes. (Kay, Leventhal and Cooper, 1969). In work that has been reviewed (Cooper, 1972) Cooper and colleagues have shown there is a 10-20 fold increase in the rate of accumulation of labelled rRNA in the cytoplasm in the first 20 hours of mitogenic stimulation. They report increases in the rate of synthesis of 45S RNA within 1hour of stimulation and an increase in the rate of processing of the 45S species to

mature cytoplasmic rRNA. Cooper also found that in resting lymphocytes the 28S:18S labelling ratio showed a marked deficiency of label in the 18S RNA suggesting that at least 50% of the newly synthesised 18S RNA molecules were immediately degraded in the nucleus never reaching the cytoplasm. A similar number of 28S molecules appeared to meet the same fate after a delay period in the nuclear pool. This "wastage" was abolished within 6 hours of PHA stimulation but returned after 20-40 hours of incubation with PHA. Inhibition of protein synthesis with cycloheximide prevented the reduction of RNA "wastage" but did not affect the increased production of 45S RNA. This led Cooper to conclude that the two events were independent, preservation of the newly processed molecules being mediated by some protective protein.

Kay also reported that the labelling of cytoplasmic RNA is stimulated more than the labelling of whole cell or nuclear RNA soon after stimulation (Kay, 1968). This is evidence I cite in support of my hypothesis that processing and transport of RNA rather the rate of transcription is accelerated by mitogen. Kay found that the cytoplasmic RNA labelled during the first two hours of incubation with PHA sedimented mainly at 4S.

The effect of mitogenic stimulation on polyadenylated RNA metabolism is less well documented. An obvious hypothesis

is that the transition of lymphocytes from resting to growing state involves widespread gene activation. An attempt has been made to detect new species of mRNA in stimulated cells using the technique of RNA:DNA hybridisation but the sensitivity of the technique used was limited; it is not surprising that no new mRNAs were detected (Berke, Sarid and Feldman, 1971). With the techniques now available for the production of cDNA from the messenger population and subsequent accurate hybridisation analysis, it may seem surprising that these experiments have not been re-attempted. The problem is that it is nearly impossible to purify intact polysomes and hence polysomal mRNA from lymphocytes; this is discussed in appendix B to this thesis. The first data on polyadenylated RNA metabolism in lymphocytes was obtained by Rosenfeld and colleagues (1972) who reported that the addition of PHA to human peripheral blood lymphocytes increased polyadenylated RNA synthesis 40% within 2 hours and 100-300% within 12 hours, the percent poly(A) content remaining constant. The increase they measured in labelled polyadenylated RNA was the same whether they labelled with uridine or adenine; this led them to suggest that an increased rate of polyadenylation is not one of the early effects of PHA. These data, which have never been repeated, are in conflict with the data presented in this thesis.

Cooper and colleagues have also reported specific early changes in the metabolism of polyadenylated RNA. They found increased production of polyadenylated RNA after 20 hours stimulation of human peripheral blood lymphocytes with PHA. They found a greater increase in [^{3}H]-adenosine labelled cytoplasmic polyadenylated RNA than was observed in total cell lysates (Cooper,1974) and hypothesised that mitogenic stimulation accelerated the rate of processing and transport of newly synthesised polyadenylated RNA. They did not discuss the possibility that part or all of the increase could be due to polyadenylation of pre-existing transcripts rather than to a total de novo messenger synthesis, a viable interpretation since they used an adenosine label. Cooper presented further evidence that the rate of processing and transport of polyadenylated RNA was accelerated in cells stimulated for 20 hours with PHA using a pulse chase technique with labelled adenosine; he measured label in cytoplasmic and in whole cell polyadenylated RNA at different times after chase and found that in cytoplasmic extracts from resting cells the increase in radioactivity was slower presumably due to faster exhaustion of the pool of labelled nuclear transcripts in the stimulated lymphocytes. Cooper also compared the size distribution of polyadenylated RNA in resting and growing cells after a three hour labelling period, and found a size shift towards smaller molecules in stimulated cells. As normal mRNA

processing involves a size reduction this data suggests an increased rate of processing in stimulated cells (Cooper, 1974). The data in this thesis is compatible with this data of Coopers.

The data in this thesis is also supported by work done simultaneously by Schaefer and colleagues (Hauser et al, 1976; Land and Schaefer, 1977) who also find no increase in the rate of transcription during the first 6 hours following addition of mitogen. They report an increased rate of RNA polyadenylation during this period, together with an accelerated transport of poly(A) sequences from the nucleus to the cytoplasm. This work which is pertinant to my interpretation of my data is thoroughly reviewed in chapter 4.

Berger and Cooper (1978) have studied the relationship between nuclear and cytoplasmic polyadenylated RNA in resting human peripheral blood lymphocytes and found over 90% of the non-polyadenylated RNA to be degraded. They present some data to suggest there are two distinct processing pathways for mRNA precursor and non-precursor polyadenylated hnRNA. On their own admission, however, the interpretation of much of their data depends on the reliability of half-life determination for polyadenylated hnRNA made utilising actinomycin D. This drug is thought by many investigators to have other than transcriptional

effects (e.g. Sholtissek, 1972) and I have found the drug to have particularly peculiar effects in lymphocytes (Mitchell et al,1978); this is discussed in appendix I.

Berger and Cooper (1975) have also reported the existence of two classes, very short lived and relatively stable, of polyadenylated cytoplasmic RNA in resting human peripheral blood lymphocytes. This study did not involve the use of drugs such as actinomycin D but there are several other difficulties in their interpretation of the data.

In a study using PHA stimulated pig blood lymphocytes, Jagus-Smith and Kay (1976) have determined mRNA concentrations by the extent of binding of RNA to [^{3}H]poly(U). This gives a much better estimate of the number of messenger molecules than does the more popular technique of binding labelled RNA to unlabeled poly(U). They found that, assuming the size of poly(A) sequences remains the same after stimulation, there are twice as many mRNA molecules per ribosome 20h after PHA stimulation and approximately four times more mRNA per cell. As before they found an early (less than 4 hours) increase in protein synthesis but no increase in polyadenylated RNA levels until 12 hours after stimulation.

The conflicting data I have summarised led to the work presented here. In this thesis I have attempted to clarify the extent to which de novo transcription, as opposed to

increased transcript conservation, is responsible for the increased cytoplasmic pools of RNA molecules observed after stimulation of human peripheral blood lymphocytes with Con A as well as to establish the role of processing and transport of RNA at various stages of the activation process.

Chapter II

MATERIALS AND METHODS

2.1 CULTURE OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES

2.1.1 Preparation of Cells

60ml blood from normal donors was collected by venipuncture into a syringe containing heparin (100U/ml of blood).

The blood was diluted to a total volume of 100ml with RPMI 1640 culture medium (Flow Labs) and layered in a 25ml aliquot onto 15ml of Ficoll Hypaque (Pharmacia). After centrifugation for 35min at 400g at 18-20°C lymphocytes banded on top of the ficoll while red ceells sedimented through the ficoll. The lymphocyte band was removed from the gradient with a pasteur pipette and the cells washed with two volumes RPMI.

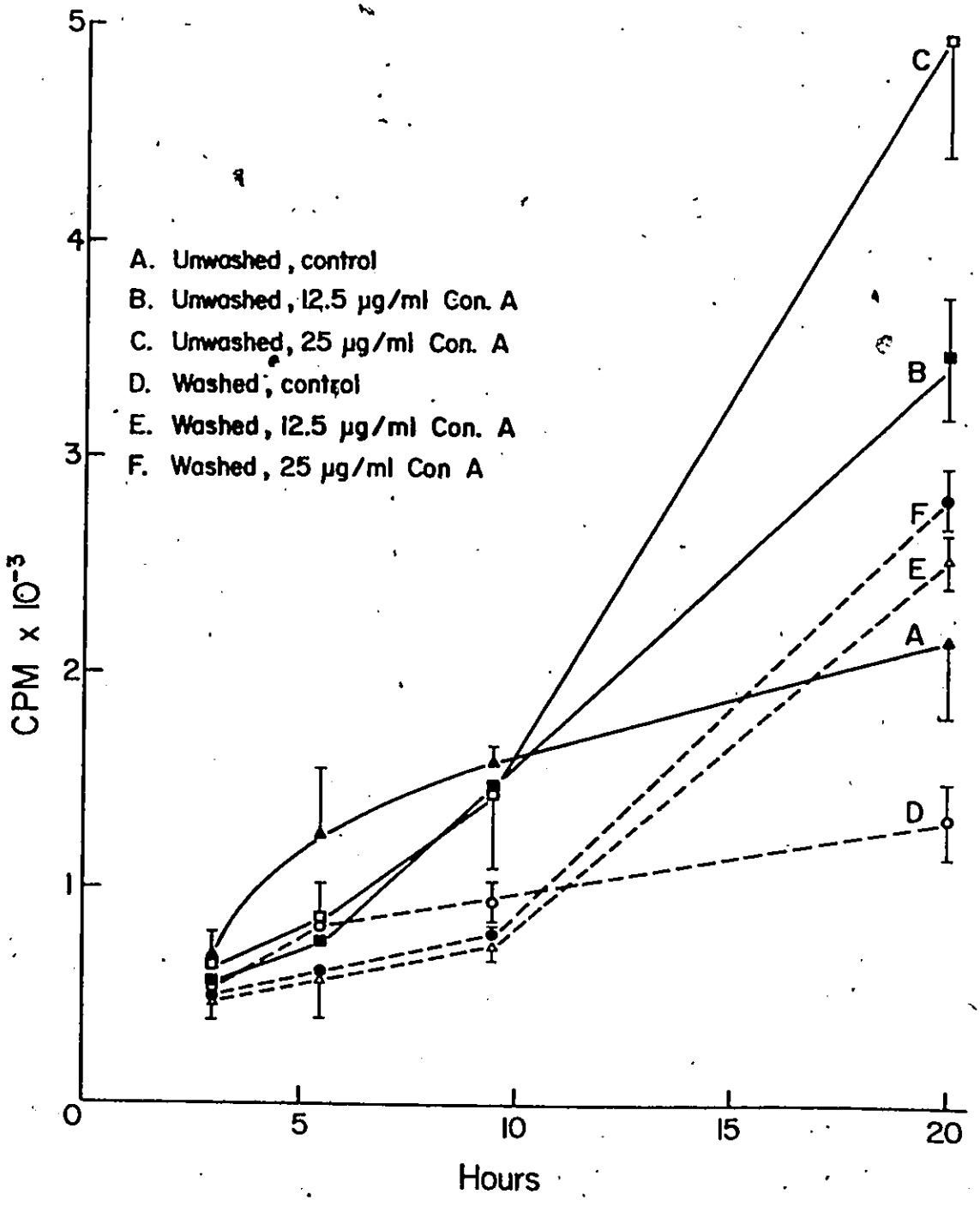
60ml of blood yields 10-15 x 10^7 lymphocytes, at least 95% pure and 95% viable as assayed by trypan blue dye exclusion.

Lymphocytes prepared by this method responded best to mitogen after being incubated in culture medium for 12-24 hours at 37° C; the cells probably make some recovery from the trauma of isolation and adjust to culture conditions during this period.

It is not possible to store cells under refrigeration. The amount of [^{3}H]uridine incorporation in 30hour Con A stimulated cells dropped by a factor of three when the cells were stored in at 4°C for 1day prior to stimulation.

Ficoll-Hypaque, itself, has mitogenic properties. Figure 2.1 shows the response of cells prepared on Ficoll-Hypaque as described above, and with the final washing step omitted, to several concentrations of Con A. Unwashed cells, both resting and Con A stimulated, incorporated [^{3}H]uridine to a higher level than the washed cells. This is probably partly due to the mitogenic effect of the small amount of Ficoll-Hypaque present in the unwashed cultures but is also probably due, in part, to the unwashed cells being in better condition because of their being subjected to cenrifugation only once. This experiment was done on microplates. It is very difficult to remove cells adhering to the plastic surface of microplates because of the automated harvesting procedure utilised. Con A tends to cause human peripheral blood lymphocytes to adhere to plastic and this probably accounts for the consistantly low radioactivity in the Con A cultures during the first 6-10 hours of incubation. I suggest that microplates are not suitable for studying events during the first 20 hours of stimulation of human lymphocytes with Con A because of this problem; it is possible to remove most adherent cells from flasks by disturbing the layer with a pasteur pipette and rinsing the flask well.

Fig.2.1. Response of cells prepared on Ficoll-Hypaque, and on Ficoll-Hypaque with the final washing step omitted, to Con A. Ordinate represents [^{3}H]uridine counts per minute x 10^{-3}. Abscissa represents hours of incubation with Con A. Each point represents mean counts per minute incorporated into 4 175ul cultures. Bars represent standard error in 4 duplicate assays. 3uCi [^{3}H]uridine per millilitre was added to cultures from 0 time.


A. Unwashed, control
B. Unwashed, 12.5 µg/ml Con. A
C. Unwashed, 25 µg/ml Con. A
D. Washed, control
E. Washed, 12.5 µg/ml Con. A
F. Washed, 25 µg/ml Con A
CPM x 10⁻³
Hours
0
1
2
3
4
5
10
15
20
A
B
C
D
E
F

2.1.2 Culture Conditions

Cells were cultured in Corning culture flasks, or in microplates (Falcon), in medium RPMI 1640 supplemented with 10% autologous serum and penicillin (110/ml)-streptomycin (100ug/ml) at a density of 10^6 cells per millilitre.

Cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37° C.

2.1.3 Mitogens

In one experiment, to determine choice of mitogen, 200ul cultures containing Con A (20ug/ml) (Calbiochem), PHA (25ug/ml) (DIFCO), PWM (25ul/ml) (GIBCO) or no mitogen were set up in microplates (Falcon). 7uCi/ml [^{3}H]uridine (NET-367, New England Nuclear) or 7uCi/ml [^{3}H]thymidine (NET-0277, New England Nuclear) was added before harvest at 24, 36, 48 or 71 hours.

Con A was added to a concentration of 20ul/ml in all other experiments.

2.1.4 Sterile Conditions

To reduce the risk of contamination of cultures by microorganisms and to inactivate ribonuclease from all materials, normal precautions were taken. When appropriate, manipulations were carried out in a laminar flow hood (Conn. Environmental Research and Design Corp.). All solutions and

glassware were sterilised by autoclaving which also inactivates ribonuclease, or by filtration (Millipore).

2.2 ISOLATION OF RNA

2.2.1 Separation of Nuclear and Cytoplasmic Fractions

Cells were harvested by centrifugation and washed once in isotonic saline. The plasma membranes were disrupted by suspension for 13min at room temperature in homogenisation buffer (10mM HEPES, 50mM KCl, 5mM $Mg(CH_3COOH)$; pH 7.8) with 0.005% Triton X-100, 5ug polyvinylsulphate per millilitre (Sigma) and 100ug spermine per millilitre (Calbiochem). Nuclei were separated from cytoplasm by cenrifugation in a HB4 rotor and Sorvall RC2-B centrifuge at 2000rpm for 3min at 0°C.

2.2.2 Extraction of RNA

An equal volume of SDS buffer (0.01M Tris-HCl, 0.005M EDTA, 0.1M NaCl, 0.5% SDS; pH7.4) was added to each sample of cytoplasm which was then extracted twice with 1:1 phenol chloroform. Two volumes of ethanol were added to the aqueous phase and RNA was precipitated overnight at -20°C. The precipitate was redissolved in SDS buffer.

2.2.3 Separation of polyadenylated RNA

Polyadenylated RNA was isolated by affinity chromatography on oligo dT cellulose (Collaborative Research

Ltd.). 250mg oligo dT cellulose was swollen for 90 hours in 1M NaCl. This solution was poured into an upright pasteur pipette with a glass wool plug in the narrow end. The column was washed well with high salt application buffer (0.4M NaCl, 20mM Tris-HCL, 0.1%SDS; pH 7.2) before an RNA sample dissolved in application buffer was applied. Under these conditions RNA containing poly(A) remained on the column due to hybridisation between poly(A) and oligo dT. Poly(A) was eluted from the column with low salt elution buffer (0.002M EDTA, 20mM TrisHCL, 0.1% SDS; pH 7.2). This eluant was adjusted to 0.4M salt and reapplied to the column. The second passage of polyadenylated RNA was precipitated with ethanol at -20° C; 10-40ug bovine serum albumin per millilitre was added as a carrier to facilitate precipitation.

The volumes of application and elution buffer necessary to clear the column were approximately 20ml and 25ml respectively. Since the RNA samples being fractionated were radioactive a few drops collected in a scintillation vial provided a simple assay to check if elution was complete.

Columns were characterised using ^{3}H-poly(A) (Miles Res.). 100% recovery of cpm applied to the column was normal and more than 98% of authentic poly(A) was retained on the column in application buffer.

Between batches columns were washed with 0.1M NaOH to remove any remaining RNA then saturated with 1M NaCl.

2.3 DETERMINATION OF RADIOACTIVITY IN RNA

2.3.1 Preparation of RNA for scintillation counting

RNA molecules larger than 15 nucleotides were precipitated by treating samples containing RNA dissolved in SDS buffer with 5% trichloracetic acid for 30min at 0°C. Samples were then passed through GF/C filters (Whatman) and each filter was washed well with 5% trichloracetic acid.

Radioactivity of polyadenylated RNA was determined by the method of Sheldon, Jurale and Kates (1972). GF/C filters were saturated with poly(U) (1mg/ml in H_2O) (Miles Res.) allowed to dry and subjected to UV irradiation to crosslink the poly(U) to the filter. Samples were dissolved in Kates buffer (120mM NaCl, 10mM Tris-HCl; pH7.4) and allowed to hybridise to the filters under conditions as described by Sheldon et al (1972). The filters were then washed with Kates buffer and then trichloracetic acid.

Radioactivity of low molecular weight RNA was estimated by solubilising ethanol precipitated RNA in cold 1M NaCl; only the molecules smaller than 6S dissolve (personal communication;E.Bard). Non-soluble material was pelleted and acid precipitable material in the supernatant was filtered as described above.

All preparations were conducted in duplicate or triplicate.

2.3.2 Scintillation counting

Filters were allowed to dry before submerging in 10ml Scintilene (Fisher) in plastic scintillation vials (CanLab). Each vial was counted for 2-20mins on a Beckman liquid scintillation counter.

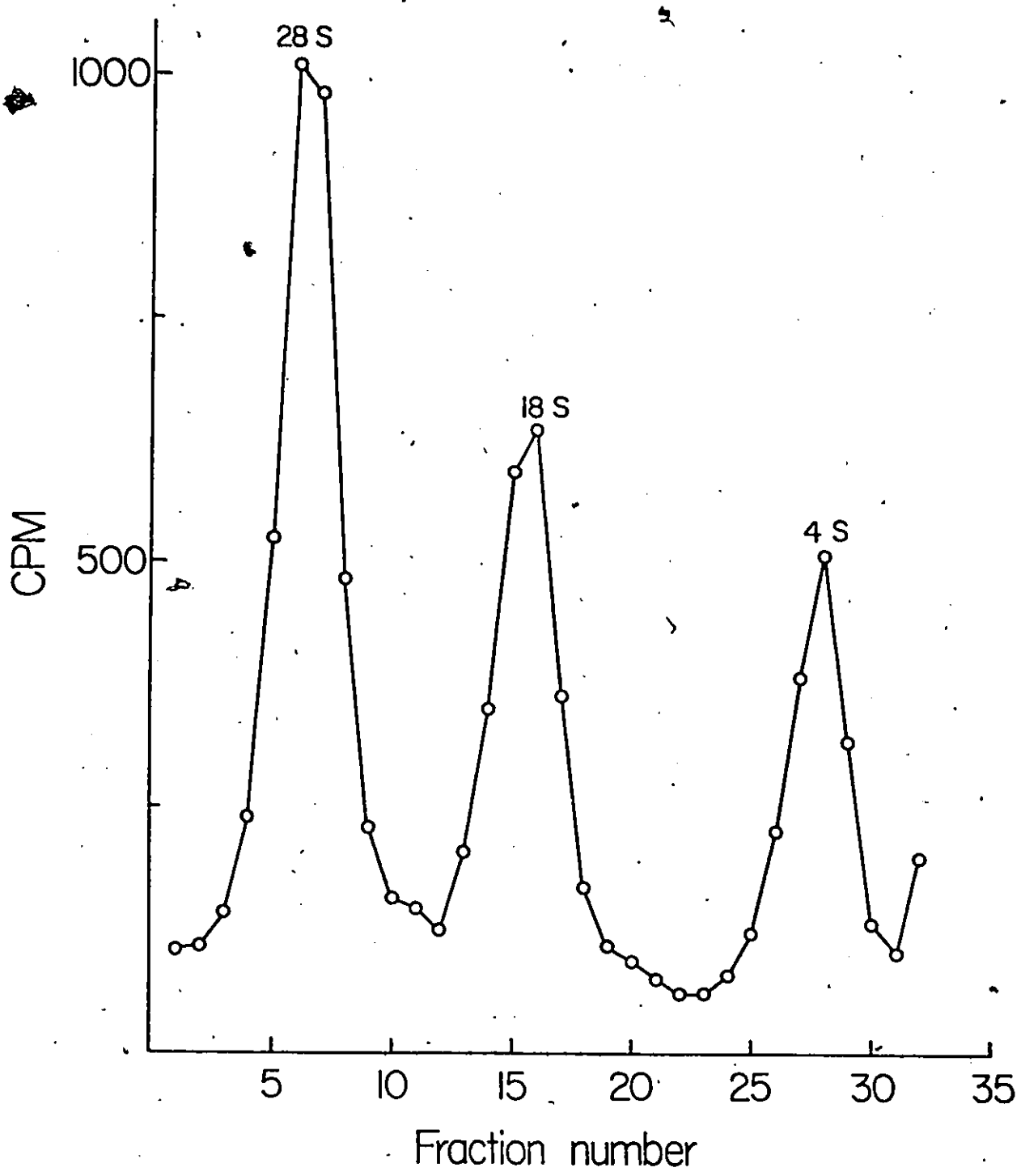
2.4 SEDIMENTATION ANALYSIS OF RNA

0.7ml samples of RNA were applied to 12ml sucrose gradients (5 to 25% in 0.01M Tris-HCl, 0.01M EDTA, 0.1M NaCl; pH 7.4). Sedimentation was carried out in a SW41 rotor and a Beckman L5-50 centrifuge at 40,000rpm for 6 hours at 4°C.

The gradients were harvested in 0.33ml aliquots and counted on a Beckman liquid scintillation counter after adding 1ml water + 10ml Hydromix (Fisher).

Figure 2.2 shows a typical sedimentation profile of whole cytoplasmic RNA. rRNA and tRNA appear intact but this, of course, does not exclude degradation of other species of higher molecular weight RNA.

Fig. 2.2 Sedimentation profile on 5-25% sucrose gradients of total cytoplasmic RNA from cells incubated for 37 hours with Con A and [^{3}H]uridine.


28 S
18 S
4 S
1000
500
CPM
5
10
15
20
25
30
35
Fraction number

2.5 TRANSPORT EXPERIMENTS

In the transport experiments 10uCi of [^{3}H]uridine (NET-367, New England Nuclear) or [^{3}H]adenine per millilitre was added at varying times before or after stimulation and to resting controls. Duplicate or single 10ml samples were taken immediatly and at subsequent time intervals. Total acid precipitable radioactivity, polyadenylated RNA radioactivity and radioactivity in low molecular weight RNA were determined in RNA extracted from cytoplasmic cell fractions as described above.

Before the first transport experiment a batch of cells labelled with ^{32}P was prepared: Human peripheral blood lymphocytes were incubated with Con A for 70 hours then incubated with 80uCi/ml ^{32}P for 4 hours. The cells were then harvested, washed, resuspended in 30ml medium RPMI 1640, divided into aliquots of 1ml in corex tubes and frozen. Samples taken during the first transport experiment were harvested straight into these tubes and manipulated as described. The ^{32}P radioactivity was constant for each RNA preparation showing that any loss of material during the RNA purification procedures is constant and may therefore be disregarded.

Chapter III

RESULTS

3.1 EFFECT OF PHA, CON A AND PWM ON LYMPHOCYTES

Figure 3.1 shows the incorporation of [^{3}H]uridine at various times stimulation with mitogens as described in Materials and Methods. Table 3.1 shows the incorporation of [^{3}H]thymidine after 40 hours contact with mitogens; this measurement was made to ensure that the mitogens were in fact stimulating the cultures to blastogenesis. All three mitogens significantly stimulated uridine incorporation. PHA had the greatest effect on uridine incorporation (Fig.3.1) but the stimulation index was lower than that for Con A (Table 3.1). Cell viability was assayed after after 48 hours incubation and 75% of the cells incubated with Con A were still viable. Viability with the other mitogens was somewhat lower; the viability of cells incubated with PHA was estimated to be 60% but these cells were difficult to count because of the agglutinating properties of PHA.

The difficulties in cell counting created by agglutination with PHA, together with the lower stimulation index and greater cell mortality, resulted in my selecting Con A as the most suitable mitogen for future experiments.

Fig. 3.1 Time course of response to mitogens. Human peripheral blood lymphocytes were cultured in microplates; 7uCi/ml [^{3}H]uridine was added for 2 hours prior to harvesting. Ordinate represents [^{3}H]uridine counts per minute. Abscissa represents time after addition of mitogen.

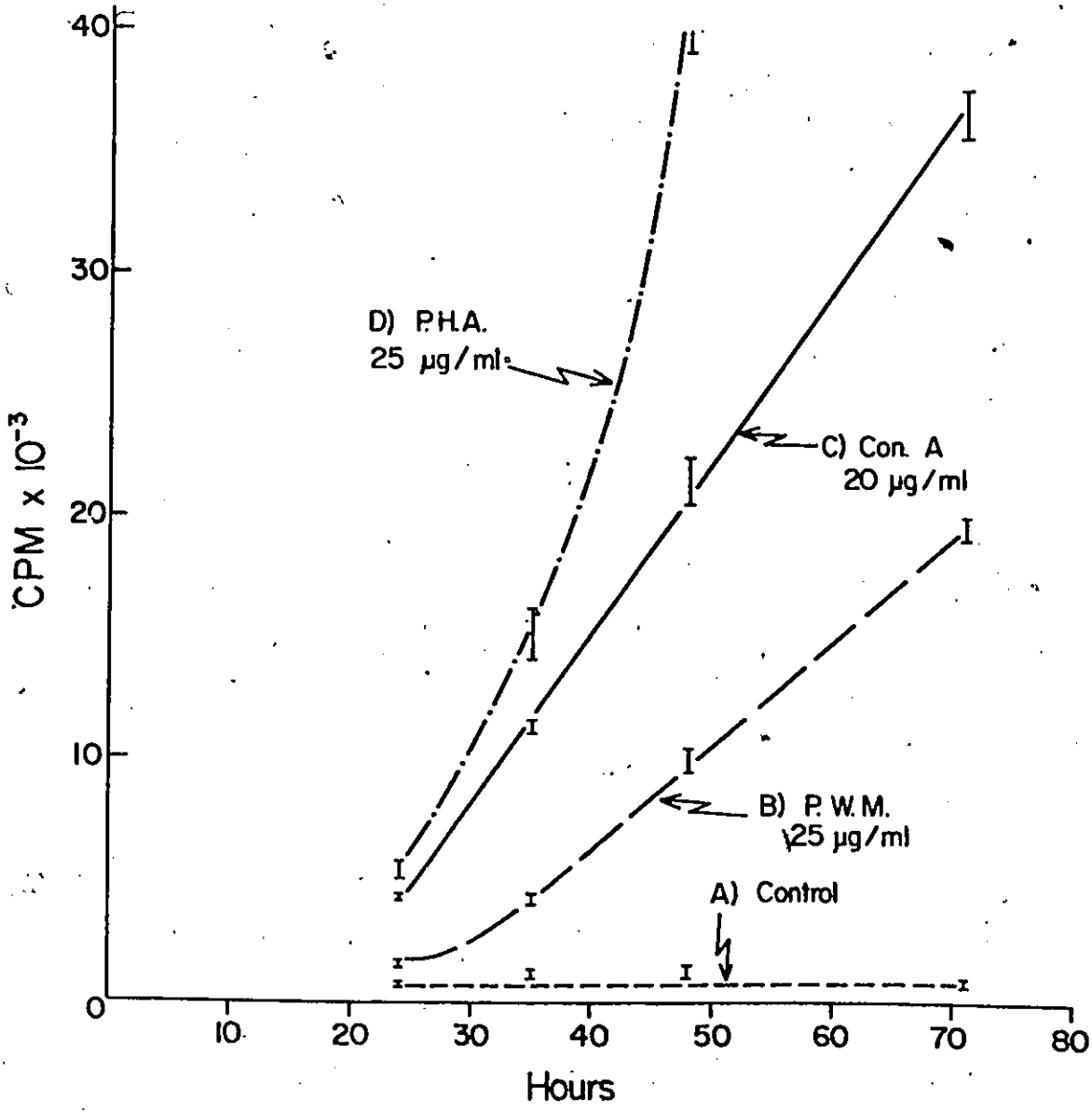

40
30
20
10
0
CPM x 10^{-3}
10
20
30
40
50
60
70
80
Hours
D) P.H.A.
25 µg/ml
C) Con. A
20 µg/ml
B) P. W. M.
25 µg/ml
A) Control

Table 3.1

Response of human peripheral blood lymphocytes to mitogens. Lymphocytes were cultured in microplates for 48 hours; 7uCi ^{3}H-thymidine per millilitre was added for two hours prior to harvest. The stimulation index represents the ratio of radioactivity in stimulated cells to that in resting cells.

Mitogen	Radioactivity incorporated (counts per minute)	Stimulation Index
Con A (20ug/ml)	43,300 +/- 2,600	42
PHA (25ul/ml)	29,700 +/- 1,300	28
PWM (25ul/ml)	11,500 +/- 1,400	11
Control	1,000 +/- 170	-

3.2 SEDIMENTATION PROFILES OF TOTAL CELL RNA AND TOTAL CELL POLYADENYLATED RNA

Lymphocytes were incubated for 45 hours with Con A. 3uCi/ml [^{3}H]uridine was added with the Con A or 3 hours before harvest. Cells were lysed in SDS buffer immediately after harvest. RNA was extracted, polyadenylated RNA separated and sedimentation analysis carried out as described in chapter 2.

Figure 3.2A shows the sedimentation profile of total RNA from cells incubated with Con A and [^{3}H]uridine for 45 hours. Figure 3.2B also shows the sedimentation profile from 45hour Con A stimulated cells but in this case the RNA was radioactively labelled for only the 3 hours prior to harvest. In figure 3.2B the 45S rRNA precursor constitutes a greater fraction of the labelled material; this is to be expected as it is a precursor to the mature rRNAs. The 28:18S rRNA ratio is also smaller in 3.2B than in 3.2A. This is consistent with the rate limiting step in RNA processing being the production of 28S rRNA. As mentioned in chapter 1, the conversion of the 32S intermediate into 28S rRNA is the rate limiting step in rRNA processing in mouse L cells.

Figure 3.2C shows the sedimentation profile of total polyadenylated RNA in cytoplasm of cells incubated with Con A and [^{3}H]uridine for 45 hours. Figure 3.2B is the same except the RNA was radioactively labelled only for 3 hours prior to harvest. Both these profiles show typical

heterogenous sedimentation of polyadenylated RNA. Both profiles peak near 18S; there is a slight shift towards molecules with higher sedimentation coefficients in the pulselabelled cells which is, of course, compatible with mRNA processing.

3.3 SEPARATION OF NUCLEAR AND CYTOPLASMIC FRACTIONS

Figure 3.3a shows a photomicrograph of a resting small human lymphocyte and figure 3.3b shows a clump of blast cells following 36 hours of incubation with Con A. Figure 3.3c shows the stimulated cells after 13min of incubation in homogenisation buffer; the loss of integrity of the cell membrane has caused loss of material to the surrounding medium but the nuclear membrane is intact. The fact that no precursor rRNA or hnRNA is observed in sedimentation profiles of cytoplasmic RNA also indicates there is no significant contamination of the cytoplasmic fraction with nuclear RNA. Figure 3.3d shows unwashed nuclei following resuspension after treatment in the homogenisation buffer; very little cytoplasm adhered and this was removed by a single wash.

This method provided a good yield of cytoplasm and of nuclei without significant cross contamination.

Fig. 3.2. (a) Sedimentation profile of total RNA from cells incubated with Con A and [^{3}H]uridine for 45 hours. Ordinate represents counts per minute in each gradient fraction. Abscissa represents fraction number. (b) As (a) but in this case the RNA was radioactively labelled only for 3 hours prior to harvest. (c) Sedimentation profile of total cytoplasmic polyadenylated RNA from cells incubated with Con A and [^{3}H]uridine for 45 hours. Coordinates as in (a). (d) As (c) but in this case the RNA was radioactively labelled only for 3 hours prior to harvest.

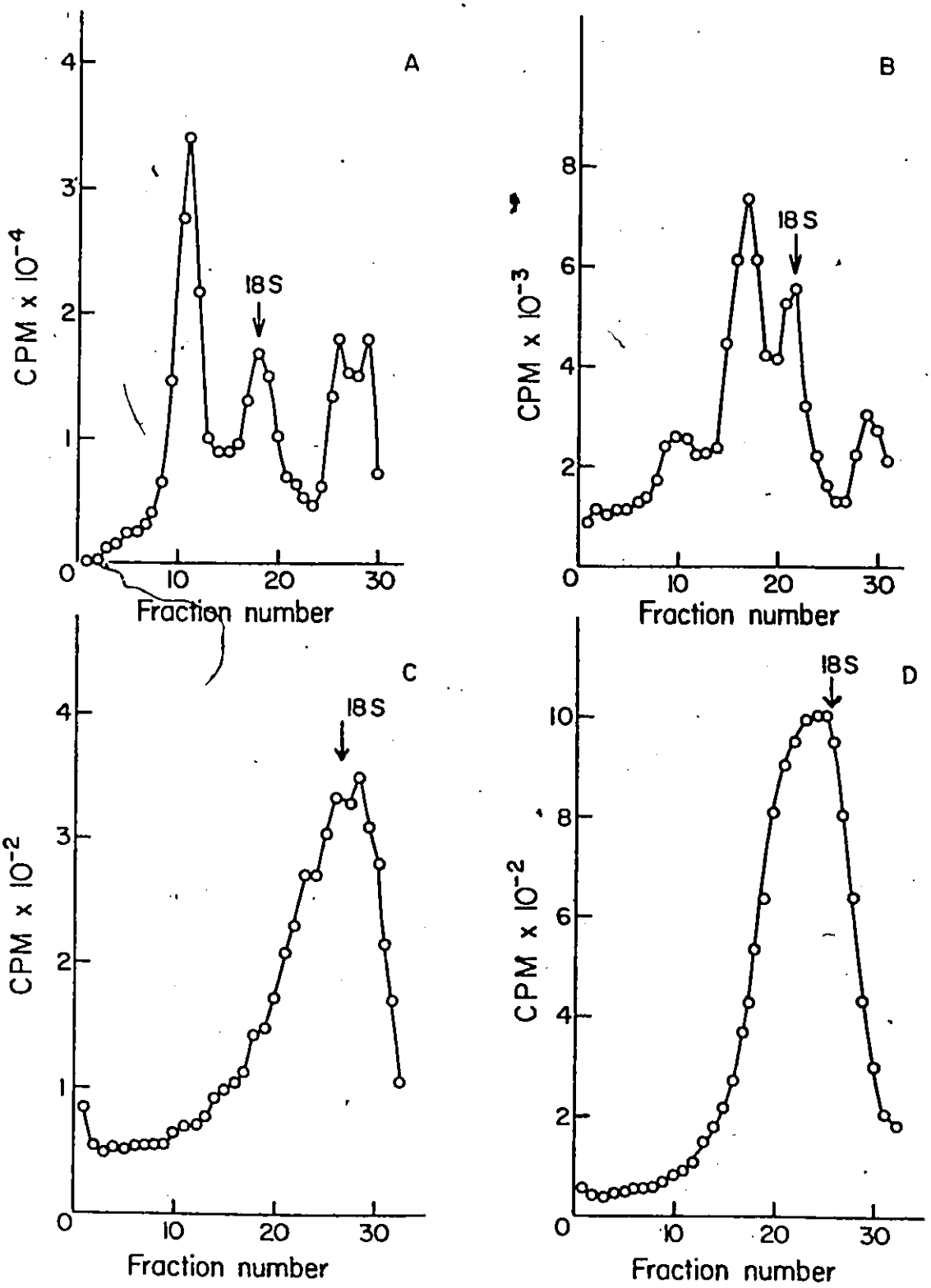
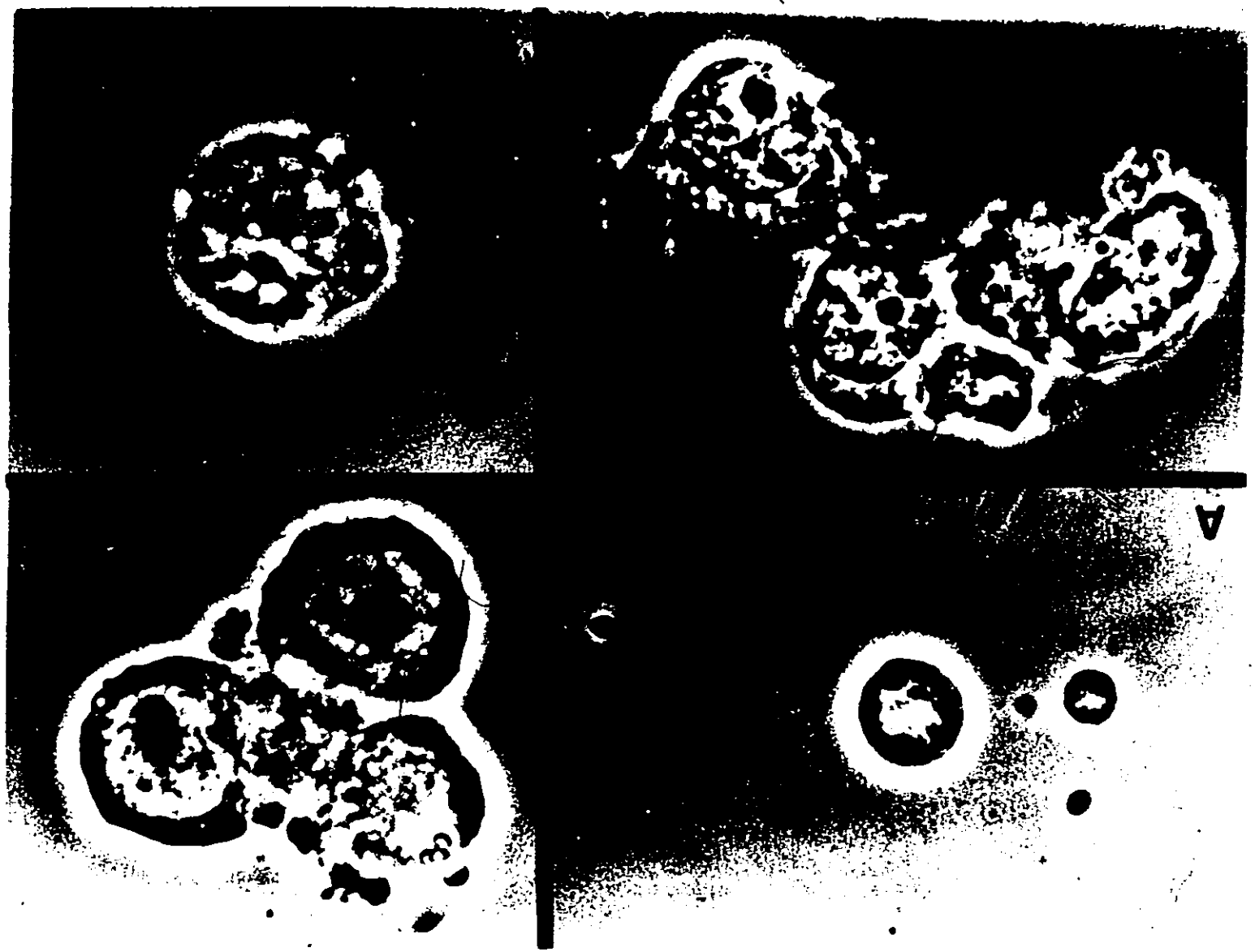

A
B
C
D
18S
18S
18S
18S
CPM x 10⁻⁴
CPM x 10⁻³
CPM x 10⁻²
CPM x 10⁻²
Fraction number
Fraction number
Fraction number
Fraction number
0
10
20
30
1
2
3
4
6
8

Fig. 3.3 (A) Resting small lymphocyte. (B) Blast cells following 36 hours of incubation with Con A. (C) Blast cells after 13 min. of incubation in homogenisation buffer. (D) Unwashed nuclei from blast cells after 13 min. of incubation in homogenisation buffer.

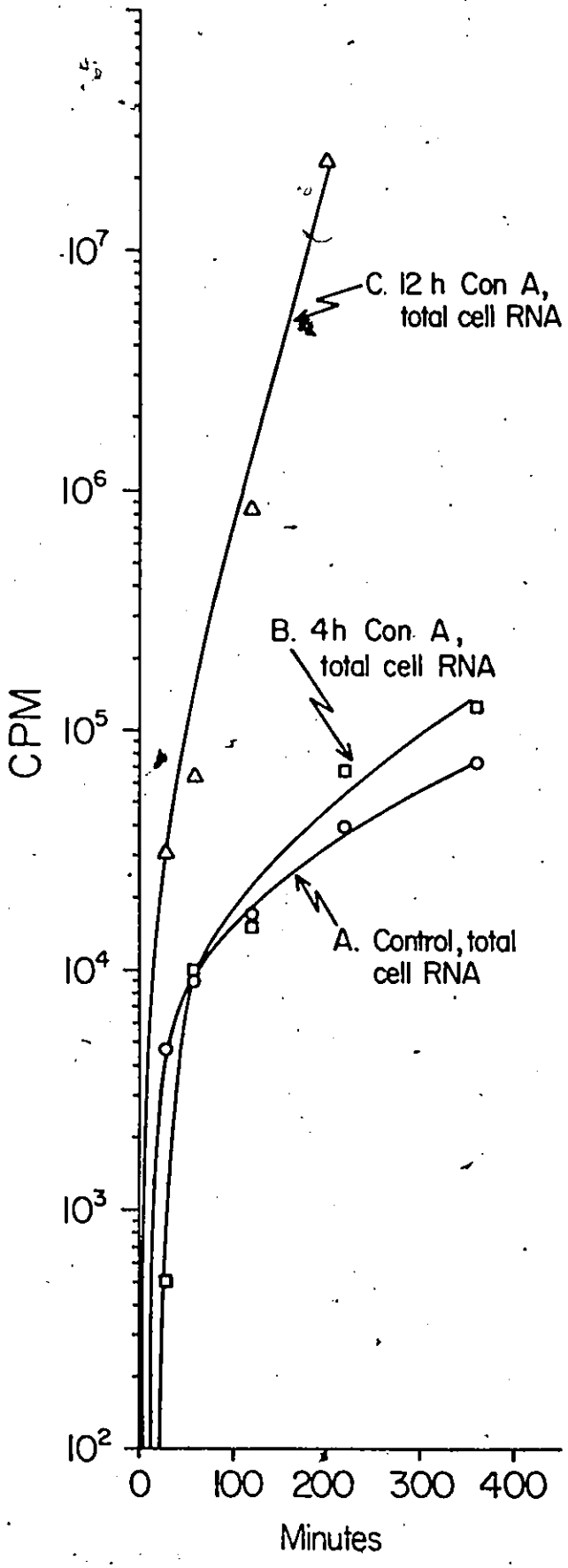

A

3.4 ACCUMULATION OF [^{3}H]URIDINE IN TOTAL CELL RNA FOLLOWING INCUBATION WITH CONA

Figure 3.4 shows the kinetics of appearance of label in whole cell RNA, ie, acid precipitable radioactivity measurable in cells lysed in SDS buffer without separation of the nuclear fraction. Curves A and B show the appearance of labelled RNA in cells which had been incubated for 4 hours either with or without Con A before the addition of [^{3}H]uridine. These curves show no significant difference. Curve C shows the incorporation of label into total RNA of cells stimulated for 12 hours before the addition of [^{3}H]uridine. After 30min, incorporation reached a level significantly higher than that of the unstimulated cells. It is interesting that lymphocytes taken from this donor, and also from the donor whose cells were used in the experiment of figure 3.5, invariably incorporated uridine to levels to levels 3-5 times those of most other donors. Stimulated lymphocytes from these two donors also characteristically incorporated thymidine into DNA to a level higher than that of the average healthy donor.

The data presented in figure 3.4 are typical of those obtained in three experiments with cells from different donors.

Fig. 3.4 Accumulation of [^{3}H]uridine in total cell RNA following 4 hours and 12 hours of incubation with Con A. Semilog graph permits expression of observed [^{3}H]uridine counts, minus background [^{3}H]uridine counts per minute measured at 0 time, on the ordinate. Abscissa represents time after addition of 10uCi [^{3}H]uridine per millilitre.


CPM
10^7
10^6
10^5
10^4
10^3
10^2
C. 12 h Con A, total cell RNA
B. 4h Con A, total cell RNA
A. Control, total cell RNA
0
100
200
300
400
Minutes

3.5 TRANSPORT OF NUCLEAR RNA INTO CYTOPLASM FOLLOWING STIMULATION

It is of interest to compare the kinetics of appearance of labelled cytoplasmic RNA with that of label in whole cell RNA.

A lymphocyte culture was stimulated for 36 hours with Con A as described in chapter 2. [^{3}H]uridine was added and at different times polyadenylated RNA and nonpolyadenylated RNA were assayed in cytoplasmic fractions.

Figure 3.5 shows the time course of appearance of label in these fractions; the semilog format was adopted solely to be able to include on the one figure five orders of magnitude of recorded counts. Curve A shows the appearance of non-polyadenylated RNA in the cytoplasm of stimulated cells; [^{3}H]uridine appeared in this fraction within 10min of its addition to the stimulated culture. In contrast, curve B shows the unstimulated controls, the first measurable appearance of non-polyadenylated RNA in the cytoplasm required 150min after pulsing and the counts were several orders of magnitude less than in the stimulated cells. Curve C shows the kinetics of accumulation of polyadenylated RNA in the cytoplasm of stimulated cells. It was first measurable in the cytoplasm 30min after addition of isotope and accumulated more slowly and to a considerably lower level than did the non-polyadenylated RNA. There was no significant radioactivity measurable in polyadenylated RNA

in the cytoplasm of the control cells. This experiment was performed four times with similar results.

To establish the shortest period of stimulation by mitogen which could cause significant transport of labelled RNA into cytoplasm, I performed experiments identical to that shown in figure 3.5 but decreased the period of contact with Con A.

Figure 3.6A shows that labelled non-polyadenylated RNA was first detectable by 30min following 12 hours of preincubation with mitogen. The kinetics of accumulation were essentially the same as that following 36 hours and occurred to about the same level. The longer lag before appearance of label in the 12 hours pre-incubation suggests that processing of the nuclear transcripts was slower than after 36 hours in presence of Con A. However, it must be borne in mind that these cells were obtained from different donors. Curve B shows that the appearance of polyadenylated RNA is also delayed by comparison with that after 36 hours contact with mitogen.

I next performed experiments in which [^{3}H]uridine was added after 4 hours of incubation with Con A and one of these is shown in figure 3.7. In this experiment non-polyadenylated RNA appeared in the cytoplasm of stimulated cells after 120min suggesting that an even greater time may be required for processing of nuclear RNA in this case.

Thus with the purified preparations of lymphocytes used in our laboratory, significant synthesis and transport of RNA were first noted in these experiments after a total of 6 hours of incubation in the presence of mitogen (see table 3.2). However, there was no measurable increase in polyadenylated RNA in the cytoplasm of stimulated cultures even after 400min of incubation with isotope. The combined data of the experiments shown in figures 3.6 and 3.7 show that the first appearance of polyadenylated RNA required 13 hours of incubation with the mitogen (see table 3.2). In no experiment did I ever observe significant polyadenylated RNA in the resting controls, irrespective of duration of incubation. It is unlikely that turnover of the mRNA would have much effect on detectable radioactivity. There is thus little production of cytoplasmic polyadenylated mRNA in resting human lymphocytes.

Fig. 3.5 Appearance of polyadenylated and non-polyadenylated RNA in cytoplasm following 36 hours of incubation with Con A. Coordinates as in Fig.3.4.

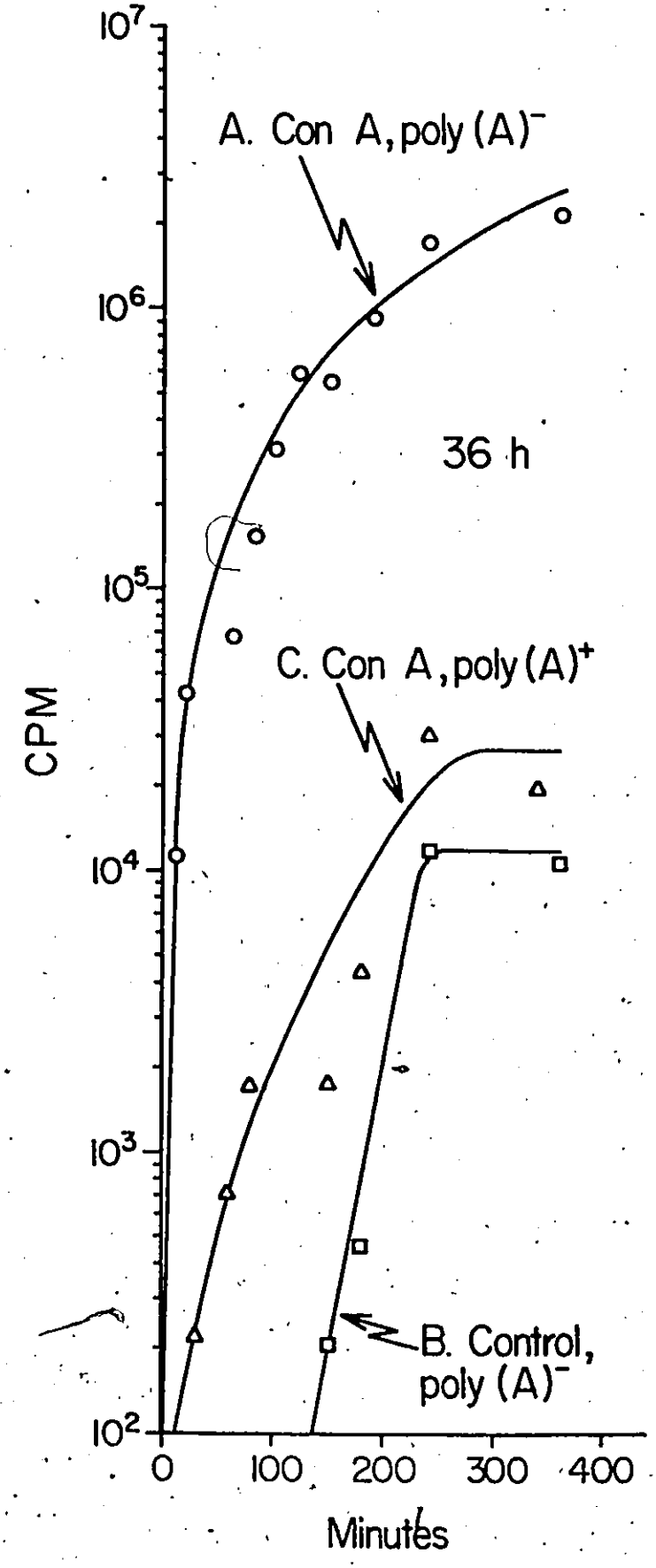

10^7
10^6
10^5
10^4
10^3
10^2
CPM
A. Con A, poly (A)⁻
36 h
C. Con A, poly (A)⁺
B. Control, poly (A)⁻
0
100
200
300
400
Minutes

Fig. 3.6 Appearance of polyadenylated and non-polyadenylated RNA in cytoplasm following 12 hours of incubation with Con A. Coordinates as in Fig.3.5.

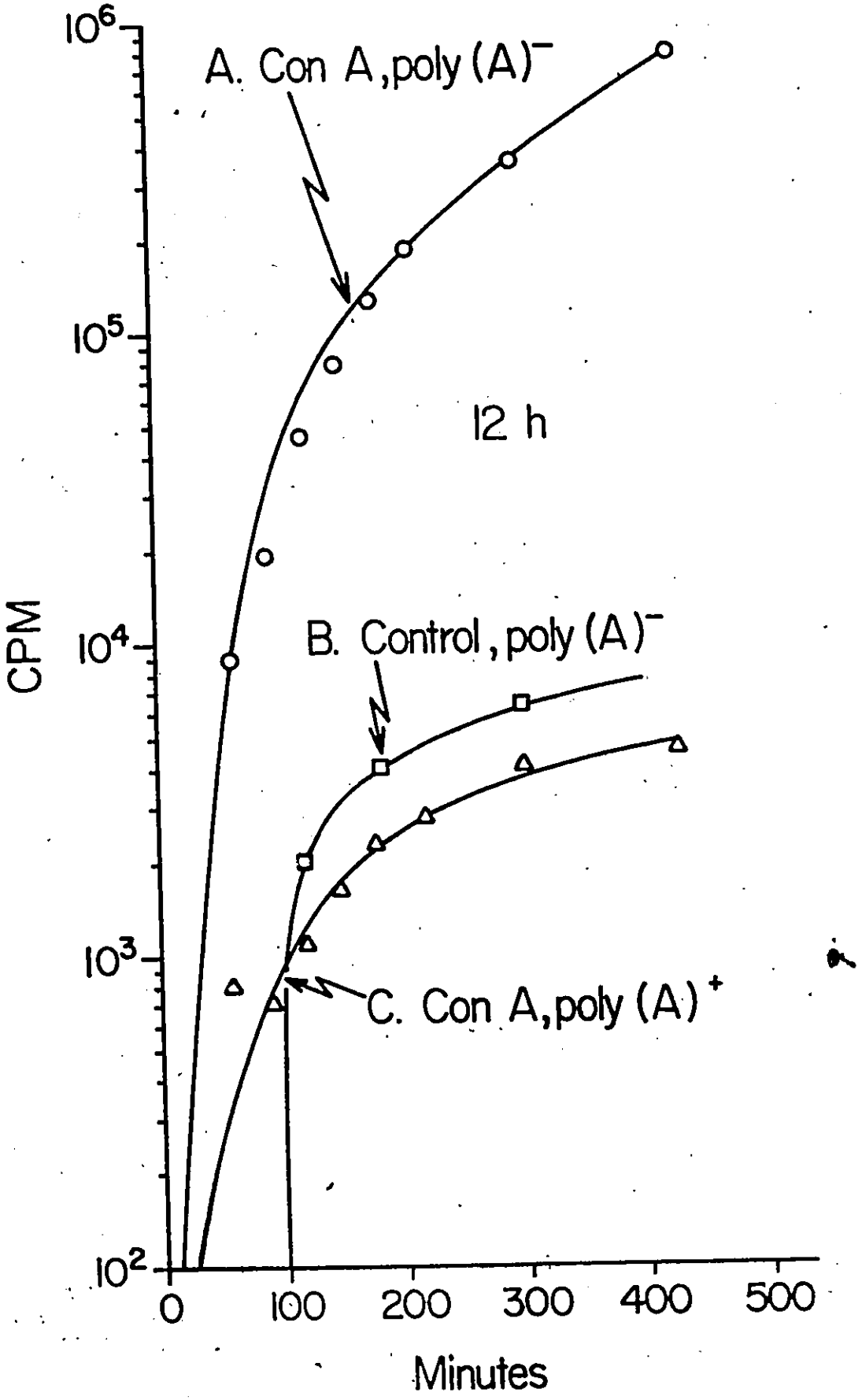
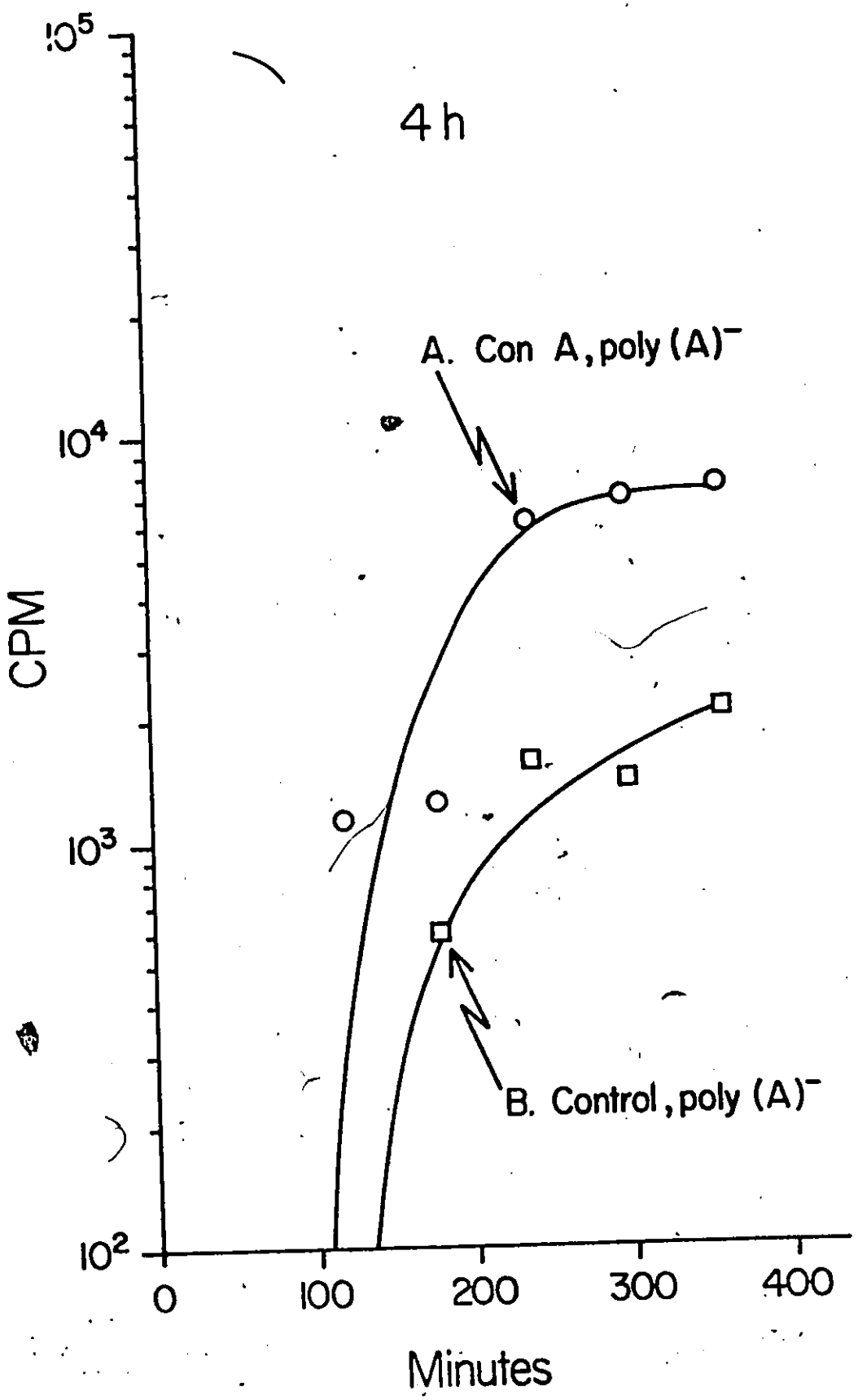

10^6
10^5
10^4
10^3
10^2
CPM
A. Con A, poly (A)$^-$
12 h
B. Control, poly (A)$^-$
C. Con A, poly (A)$^+$
0
100
200
300
400
500
Minutes

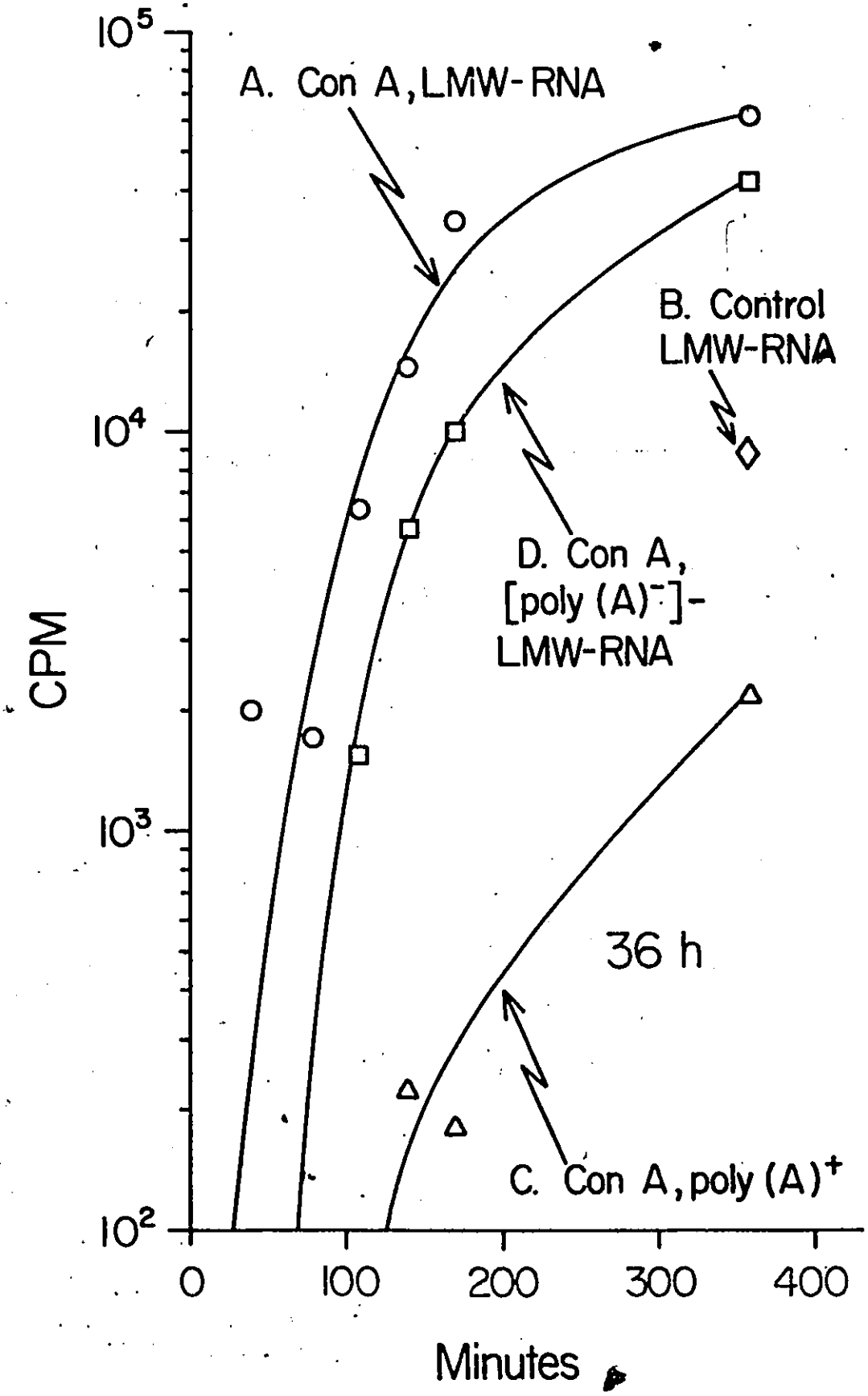
Fig. 3.7 Appearance of polyadenylated and non-polyadenylated RNA in cytoplasm following 4 hours of incubation with Con A. Coordinates as in Fig.3.5.


4 h
A. Con A, poly (A)⁻
B. Control, poly (A)⁻
CPM
Minutes
10⁵
10⁴
10³
10²
0
100
200
300
400

3.6 QUANTITATIVE SEPARATION OF NON-POLYADENYLATED CYTOPLASMIC RNA

Figure 3.8 shows the kinetics of appearance of uridine-labelled polyadenylated, low molecular weight (LMW) and non-polyadenylated - LMW RNA in cytoplasm following 36 hours of incubation with Con A. The latter category mainly represents rRNA. Curve A shows that LMW RNA appeared in the cytoplasm of cells stimulated with Con A for 36 hours and incubated for 40min with [^{3}H]uridine. Curve D shows the remainder of the non-polyadenylated RNA fraction consisting of RNA greater than 6S (presumably rRNA) did not begin to appear until 110min, after which the rate of accumulation of both non-polyadenylated RNA fractions was similar. These data suggest that the appearance of rRNA in the cytoplasm of stimulated cells occurs significantly later than that of tRNA. It is unlikely that a significant amount of the radioactivity appearing in LMW RNA is due to the conversion of [^{3}H]uridine to [^{3}H]cytidine coupled with the turnover of the terminal CCA residues on tRNA since we utilised a relatively short labelling period. The same conclusion can be drawn from the data of Kay and Cooper (1969).

Fig. 3.8 Appearance of uridine-labelled polyadenylated, L.M.W. and non-polyadenylated - L.M.W. RNA in cytoplasm following 36 hours of incubation with Con A. Coordinates as in Fig.3.5.


10^5
10^4
10^3
10^2
CPM
A. Con A, LMW-RNA
B. Control LMW-RNA
D. Con A, [poly (A)⁻]- LMW-RNA
36 h
C. Con A, poly (A)⁺
0
100
200
300
400
Minutes

3.7 PRE-INCUBATION WITH ISOTOPE BEFORE STIMULATION WITH CON A

Figure 3.9 shows the kinetics of appearance of non polyadenylated RNA in cytoplasm of cells incubated with [^{3}H]uridine prior to the addition of Con A. Curve A shows that cells pre-incubated with [^{3}H]uridine exhibit an increase in cytoplasmic acid-precipitable non-polyadenylated RNA after only 3 hours of contact with Con A. This is a significantly earlier increase than that measured without prior incubation with isotope and suggests that a fraction of a nuclear pool of transcripts, that turns over rapidly in resting cells, is conserved and transported to cytoplasm soon after stimulation with Con A.

3.8 APPEARANCE OF ADENINE-LABELLED RNA IN CYTOPLASM

Figure 3.10 is a comparison of the appearance of uridine and adenine labelled non-polyadenylated RNA and polyadenylated RNA in cytoplasm; isotope was added following 4 hours of incubation with Con A. Curves A,B and C are the same as described in section 3.6 (Figure 3.7). They are included here as I felt a direct comparison could only be made if the cells used for each labelling treatment were from the same batch from the same donor and treated identically. Curve F shows that utilising [^{3}H]adenine as label caused a significant increase in polyadenylated cytoplasmic RNA to be detected less than 5 hours after exposure to mitogen. Curve G shows lower levels of adenine-

labelled polyadenylated RNA were detectable in the cytoplasm of resting cells; as in the case of uridine label the lag between addition of isotope and cytoplasmic appearance of labelled molecules was longer than in stimulated cells. Figure 3.10 also shows that adenine label appeared in non-polyadenylated RNA with a shorter lag period than did uridine label. The relatively high degree of labelling of non-polyadenylated RNA with [^{3}H]adenine label could be due to CCA turnover in the 3'terminus of tRNA (Slater, Gillespie and Slater, 1973).

The data presented in figures 3.5,6,7,9 and 10 are summarised in table 3.2.

Fig. 3.9 Appearance of non-polyadenylated RNA in cytoplasm following 24 hours of incubation with 10uCi [^{3}H]uridine per millilitre then stimulation with Con A. Ordinate as in Fig.3.5. Abscissa represents time after addition of Con A.

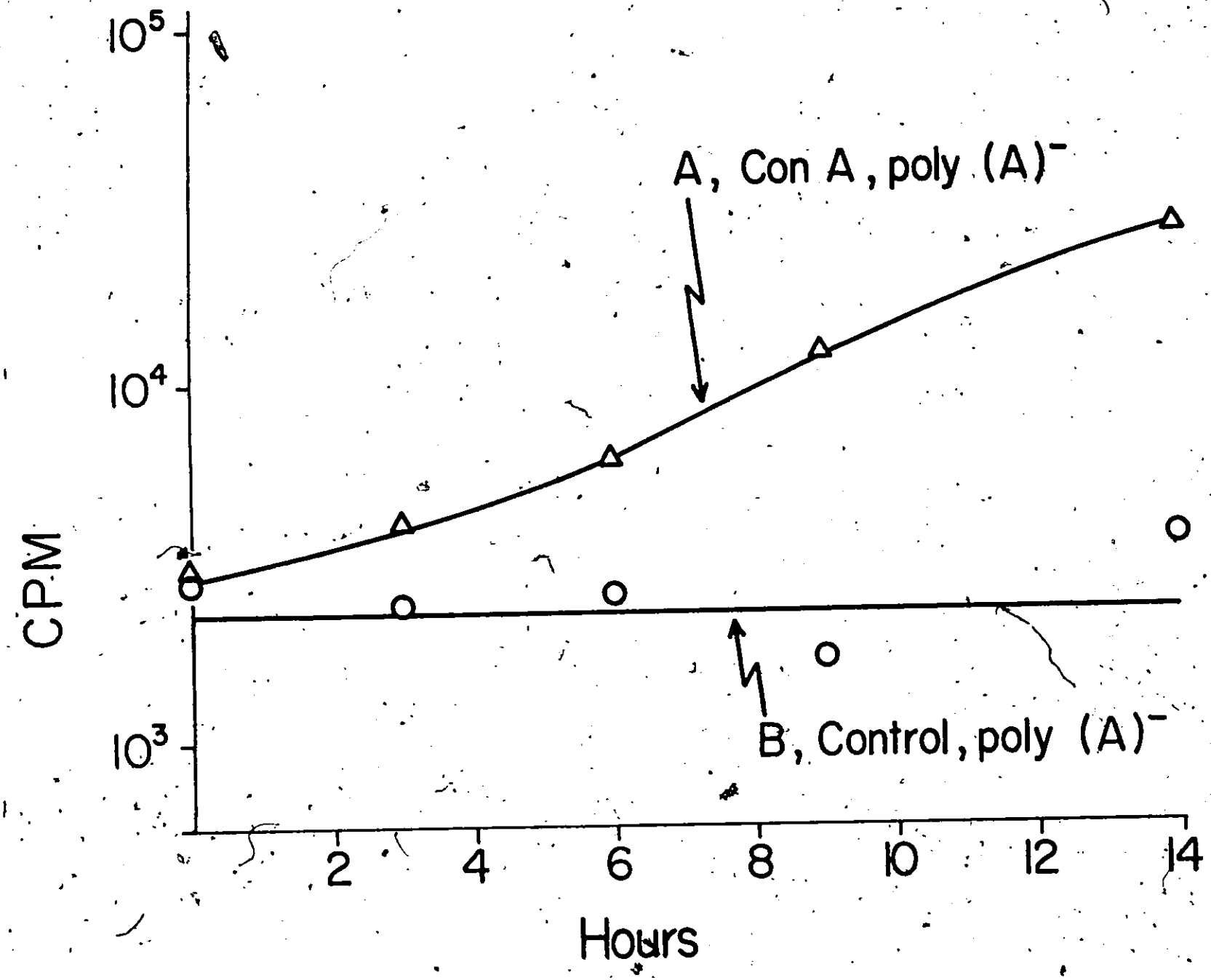

10^5
10^4
10^3
CPM
A, Con A, poly (A)⁻
B, Control, poly (A)⁻
2
4
6
8
10
12
14
Hours

Fig. 3.10 Appearance of uridine and adenine-labelled polyadenylated and non-polyadenylated RNA in cytoplasm following 4 hours of incubation with Con A. [^{3}H]uridine or [^{3}H]adenine counts per minute, minus background measured at 0 time, are expressed on the ordinate. Abscissa represents time after addition of 10uCi [^{3}H]uridine Or [^{3}H]adenine per millilitre.

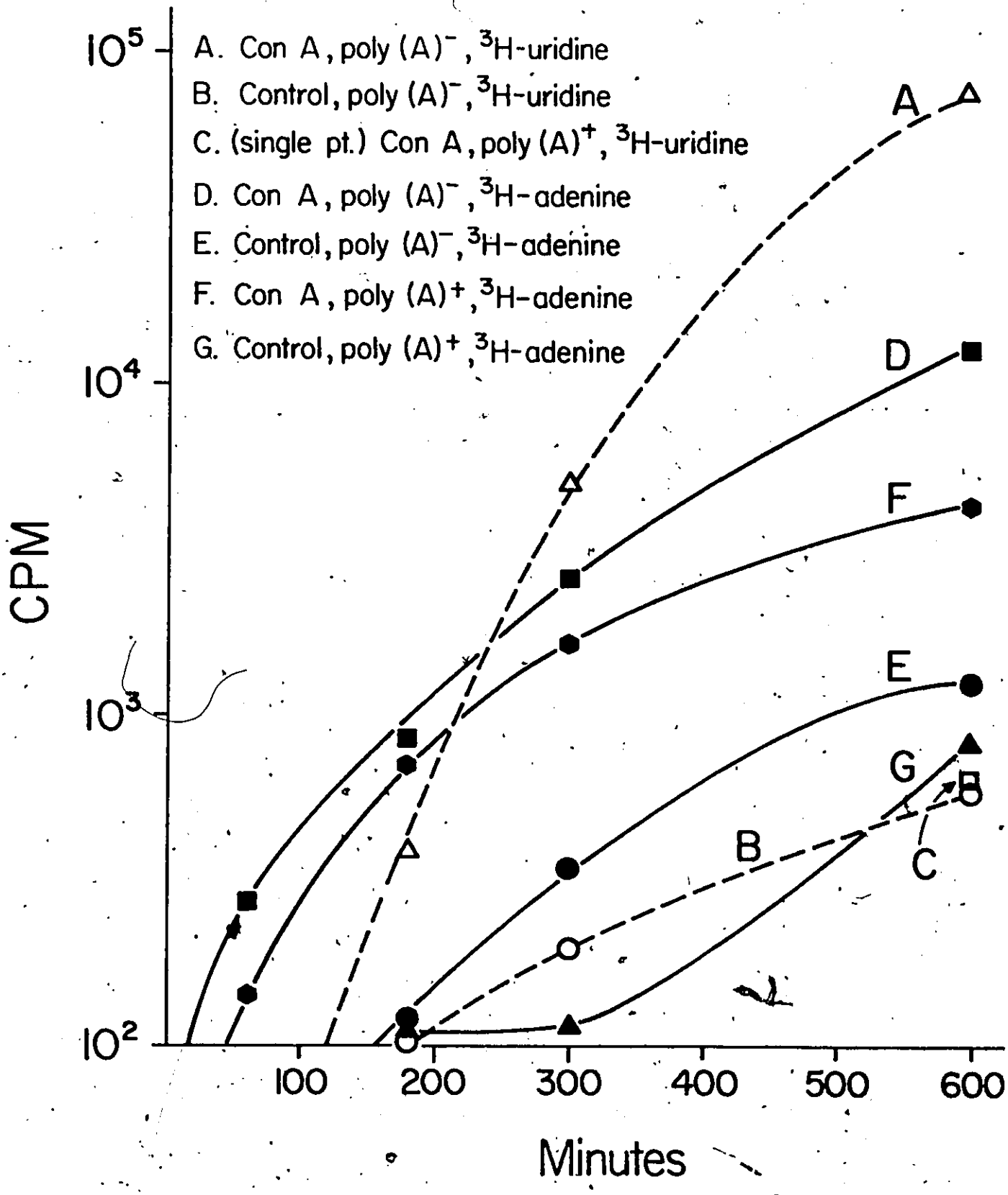

A. Con A, poly (A)⁻, ³H-uridine
B. Control, poly (A)⁻, ³H-uridine
C. (single pt.) Con A, poly (A)⁺, ³H-uridine
D. Con A, poly (A)⁻, ³H-adenine
E. Control, poly (A)⁻, ³H-adenine
F. Con A, poly (A)⁺, ³H-adenine
G. Control, poly (A)⁺, ³H-adenine
10⁵
10⁴
10³
10²
CPM
100
200
300
400
500
600
Minutes
A
B
C
D
E
F
G

Table 3.2

RNA	Label	Duration of incubation with mitogen before addition of label (hours).	Time required for 1st increase over resting cell levels of label in cytoplasmic RNA (minutes).	Total time required for 1st increase over resing cell levels of label in cytoplasmic RNA (hours).
poly(A)$^+$	^{3}H-U	4	400	
"	"	12	60	13
"	"	36	20	
"	^{3}H-A	4	50	4.8
poly(A)$^-$	^{3}H-U	4	110	5.8
"	"	12	60	
"	"	36	10	
"	^{3}H-A	4	50	4.8
"		Prelabel RNA before Con A addition		3

Chapter IV

DISCUSSION

In chapter 1 some of the paradoxes of the literature on RNA metabolism in stimulated lymphocytes were discussed. In this section I will attempt to resolve some of these paradoxes and present a new perspective of the early effects of mitogens on RNA metabolism. The data presented in this thesis suggest that the principal early effect of mitogen is to increase the rate of processing and transport of all classes of RNA and that there is little or no increase in the rate of transcription in the first ten hours following mitogenic stimulation of lymphocytes.

Some of the differences in work from different laboratories may be artifactual. I have mentioned some of the dangers in interpretation ascribable to changes in size of the nucleotide triphosphate pools. It has been claimed that pool sizes have, in fact, increased in stimulated cells which exhibit a marked increase in uridine kinase activity (Kay and Handmaker, 1976). Another problem in interpretation of data from different laboratories is the plethora of lymphocyte sources and methods of purification. A highly purified culture may respond quite differently to mitogen than a culture containing several types of cells.

Additionally, as described in chapter 2, certain purification steps are traumatic to the cells. It remains possible that the purity of my cultures (ie, loss of an accessory cell type) and/or the traumatic effects of centrifugation could be responsible for my failure to detect RNA synthesis in whole cell extracts before 10 hours; the incubation of cultures for at least 12 hours before manipulation should, however, have greatly reduced any effects of purification trauma. And even if there is a small amount of RNA synthesis during the early period, my data unambiguously show that increased processing and conservation of transcripts accounts for a large part of the increased cytoplasmic RNA pools. The actual time at which RNA synthesis may first be measured is less important than the observation that there is a larger increase in labelled cytoplasmic RNA than in whole cell RNA during the first 10 hours of mitogenic stimulation. Another reason for some of the ambiguities in the data dealing with high molecular weight RNA is doubtless the presence of high concentrations of nucleases in these cells. I suspect this is why the characteristic, multi-peaked polysome profiles observed in other growing cells have not been produced from lymphocytes (see appendix 1). Many authors seem reluctant to draw attention to these special difficulties which doubtless lead to misinterpretation of data. I am also suspicious of some of the cell fractionation methods described in the

literature. Other workers have used Triton-X-100 for homogenisation of lymphocytes at concentrations up to 0.5% (e.g. Johnson, Karn and Allfrey, 1974). I found that the detergent at this concentration caused leakage of nuclear material into the cytoplasmic fraction and that 0.005% was sufficient to damage the plasma membrane so as to permit extraction of the cytoplasmic fraction without nuclear contamination. (Clean nuclei could then be prepared by washing the pellet after this extraction).

These methodological differences may, in part, account for my results being in conflict with much of the literature reviewed in chapter 1. I have been able to detect no significant increase in [^{3}H]uridine labeling of total cell RNA untill 10h hours after exposure to mitogens (Fig.3.5). However, I can detect earlier increases in labelled RNA in cytoplasmic cell fractions; a significant increase in non-polyadenylated RNA occurs after 6 hours exposure to mitogen (Fig. 3.7) much of this being tRNA (Fig.3.8). The lag between addition of isotope and appearance of labelled RNA in cytoplasm was inversely related to the duration of incubation of the cells with mitogen. Fig.3.6 shows the kinetics of appearance of cytoplasmic label after 36 hours incubation with mitogen; the lag is only a few minutes; this should be compared with Fig.3.8. All these data, summarised in table 3.2, suggests that mitogen acts to accelerate the rate of processing and transport of RNA rather than to induce increased transcription.

I reasoned that in resting lymphocytes much of the rapidly synthesised RNA is constantly turning over with but little being transported to the cytoplasm. If an initial effect of mitogen is to effect the rapid processing, transport to the cytoplasm and thus conservation of such transcripts, then increased accumulation of labelled RNA should be detectable in the cytoplasm without necessitating any increase in transcription rates. I cannot exclude the possibility that there is a small increase in rate of transcription which is masked by incorporation of [^{3}H]uridine into the rapidly turning over pool of nuclear RNA in resting cells. I have shown that cells incubated with [^{3}H]uridine prior to the addition of Con A exhibit an increase in cytoplasmic acid precipitable RNA within 3 hours as shown in Fig.3.10. This is consistent with the hypothesis that a primary early effect of mitogen is to cause a fraction of a nuclear pool of transcripts turning over in resting cells to be transported to the cytoplasm soon after stimulation with Con A.

I found that at least 13 hours exposure to Con A was required before [^{3}H]uridine-labelled polyadenylated RNA appeared in the cytoplasm of stimulated cells and it did not appear in those of resting cells; compare Fig.3.7 with Fig.3.8. Utilising [^{3}H]adenine as label, I detected significant increases in cytoplasmic polyadenylated RNA less than 5 hours after exposure to mitogen (Fig.3.11). Lower

levels of adenine-labelled polyadenylated RNA were detectable in the cytoplasm of resting cells; as in the case of the uridine label, the lag between addition of isotope and cytoplasmic appearance of labelled molecules was considerably longer than in stimulated cells. Newly synthesised polyadenylated RNA tends to associate with polysomes close to, or attached to, the nuclear membrane so it is possible that some cytoplasmic polyadenylated RNA was removed along with the nuclear fraction from my preparations. This figure also shows that adenine label appears in non-polyadenylated RNA with a shorter lag period than did uridine label. The very high degree of label could be due to CCA turnover in the 3'terminus of tRNA. These data also suggest that the principal early effect of mitogen on messenger RNA metabolism is to induce processing, especially polyadenylation, and transport of transcripts which are also formed in the unstimulated controls.

4.1 FLUCTUATIONS IN NUCLEOTIDE POOLS

Radioactivity incorporated into RNA during any period of exposure to radioactive-nucleoside will depend on the average specific activity of the nucleotide-tri-phosphate (NTP) pool during that period as well as on the rate of RNA synthesis. It is not possible to accurately measure UTP pool sizes directly and, while the total cell ATP pool size can be determined by the luciferin-luciferase assay, it cannot

be assumed that this is related to the pool of ATP available for RNA synthesis. ATP is compartmentalised wihin the cell and many reactions break-down ATP at a faster rate than it is utilised in RNA synthesis.

The specific activity of NTP pools depends on the pool size, the rate of input of isotope to the pool and the rate of flow of isotope from the pool. Peters and Hausen (1971) have shown that mitogen causes an immediate increase in uridine uptake into bovine lymphocytes and that this increase is related to the external uridine concentration. They suggest that uridine is transported across the membrane by facilitated diffusion and that mitogens cause an increase in the number of functional carrier sites of the membrane transport system. It seems that the increase in uridine kinase activity measured soon after stimulation (Lucas, 1967; Hausen and Stein, 1968) occurs as a result of the increase in uridine incorporation rather than vice-versa. While there are discrepancies in the literature it seeems that at uridine concentrations normally used, mitogen stimulation rapidly leads to a twofold increase in thee rate of uridine incorporation. Presumably incorporation of other nucleosides is also affected by mitogen.

The other parameter establishing the specific activities of NTP pools, the rate of flow of isotope from the pool, is very complicated. It could be altered, for example, by a

change in the rate of RNA synthesis, a change in the average stability of newly synthesised RNA or a lower or higher efficiency of reutilisation of nucleotides from degraded RNA.

It is not possible to arrive at a firm conclusion but it seems quite likely that the increase in the rate of uridine incorporation during the stimulation of lymphocytes with mitogen may exaggerate measured increases in rates of RNA synthesis.

I now wish to consider how this affects interpretation of the data presented in this thesis. It must be conceded that part of the increases I measure could be due to increases in the specific activities of the NTP pools. However pool effects alone cannot possibly account for my results. Pool effects cannot be used to explain my finding that there are earlier increases in labelled cytoplasmic RNA than in labelled whole cell RNA or my pre-label experiment (Fig.3.9) which shows that if resting cell RNA is pre-labelled, labelled RNA accumulates in the cytoplasm sooner than if isotope is added at the same time as mitogen. In other experiments the differences I have measured are quite simply too big to be totally caused by a two fold increase in uridine uptake; consider, for example, figure 3.5 which shows that 36 hours in Con A reduces the lag between addition of isotope and appearance of labelled transcripts

in the cytoplasm from 150 to 20 minutes. This difference cannot be explained by a small increase in pool specific activity.

Using the amount of incorporation of radioactive nucleoside into RNA as a measure of RNA synthesis may lead to exaggeration of the increases in rates of RNA synthesis but if this is kept in mind while interpreting data, the assay is valid and is widely used.

4.2. SUPPORTING DATA FROM OTHER LABORATORIES

Simultaneously with my work, Schaefer and colleagues have also reported that while the rate of protein synthesis increases almost immediately after stimulation of lymphocytes from bovine lymph nodes, the rate of transcription does not change in the first 6 hours following addition of Con A (Hauser et al, 1976). Schaefer and colleagues also report an increased rate of RNA polyadenylation during the first few hours after addition of Con A to lymphocytes from bovine lymph nodes, before any increase in total RNA synthesis is detectable. Like Cooper, Schaefer et al report an increase in the fraction of polysomes formed at the expense of free ribosomes during the first 90min of protein synthesis. They showed that pre-existing RNA as well as RNA that was transcribed after Con A addition could be found in the monosomal, and subsequently in the polysomal fraction. They suggest that this shows that

at least part of the increase in polysomes is effected by a real increase in the number of translated mRNAs. Shaefer et al (1976) describe an increase in the rate of polyadenylation which leads to a higher polyadenylated RNA concentration within the nucleus and postulate that following stimulation, pre-existing RNA molecules are polyadenylated. This is very comparable to the situation in sea urchin eggs which, after fertilisation, will undergo many divisions in the presence of actinomycin D; pre-existing RNA molecules synthesised in the non-proliferative phase of oogenesis are polyadenylated after fertilisation. (Slater, Gillespie and Slater, 1973). Schaefer and colleagues also present data suggesting that poly(A) sequences leave the nucleus at an increased rate after Con A activation (Schaefer et al (1976). This suggests acceleration of the transport of polyadenylated RNA from the nucleus to the cytoplasm following addition of Con A. My results, and those of Schaefer, are mutually supportive. However our data do not exclude activation of mRNA already present in the cytoplasm. Wettenhal and London (1974) have published evidence for the presence of mRNA in the post-ribosomal cytoplasm of resting sheep lymphocytes. They suggest that this apparent pool of uncommited mRNA in resting lymphocytes may be utilised during the early stages of lymphocyte activation and that the mRNAs could be stored in forms similar to those postulated for other dormant

tissues (e.g.Bester, Kennedy and Heywood,1975; Gross et al, 1973).

Schaefer and colleagues have also investigated the in vitro synthesis and stability of RNA in isolated nuclei from bovine lymph nodes. As in their cellular assays, they detected no increase in the RNA synthesising capacity of isolated nuclei during the first 6 hours following stimulation. They found the same proportion, 14-20%, of all RNA synthesised in isolated nuclei from resting and from 30hour stimulated cells to contain poly(A) sequences. The absolute number of polyadenylated RNA molecules was higher in the stimulated cells. They found that considerably more non-polyadenylated RNA, mainly rRNA, was synthesised in nuclei from Con A stimulated cells than in nuclei from resting cells. In resting cell nuclei, 50% of polyadenylated RNA was degraded in a 30min chase. This RNA fraction was stable in nuclei from stimulated cells. It would seem that processing of polyadenylated RNA is rapid with no accumulation of smaller species or fragments. Shaefer et al. cite unpublished work by Berger which shows that the processing of polyadenylated RNA to yield mRNA in human lymphocytes is rapid and proceeds to completion without the accumulation of intermediates. Polyadenylated RNA was stable during a 30min chase in both types of nuclei. Shaefer and colleagues also found that nuclei from stimulated cells synthesised almost double the amount of

both polyadenylated RNA and non-polyadenylated RNA larger than 18S than did resting cell nuclei.

Further evidence for post-transcriptional rather than transcriptional control of early protein synthesis is its enhancement by mitogens even in the presence of high concentrations of actinomycin D (Kay, Leventhal and Cooper, 1969).

4.3 CONCLUSIONS

I feel that there is no conclusive evidence for any increase in the rate of transcription during the first six hours of mitogenic stimulation of lymphocytes. Investigators who have reported such an increase generally have worked with whole cell systems and measured total acid-precipitable material; in such systems, changes in the specific activity of nucleotide pools could well account for the increases measured. Investigators working with isolated nuclei and/or cytoplasm, who have extracted and characterised RNA, generally report no increase in uridine incorporation in the first 6-12 hours following stimulation. It has always been accepted that mobilisation of pre-existing ribosomes and accelerated processing of rRNA accounts for most of the early increase in protein synthesis. Recent evidence from several laboratories and this thesis strongly suggests that increased rates of maturation of pre-existing transcripts of all RNA classes, as well as an increase in the rate of

translocation of nuclear RNA to the cytoplasm, are an important early effect of mitogenic stimulation. It is perhaps at this level that important regulatory mechanisms exist which control the passage of the lymphocyte from the resting to the stimulated states.

My interpretation of the relative importance of processing versus extensive new transcription during the first hours of stimulation depends on the observed kinetics of incorporation of labeled precursors into RNA. No doubt the exact time at which increased transcription becomes evident in stimulated cells depends on a variety of experimental factors such as which label one uses and its specific radioactivity, which mitogen one uses, the type of cells used and how they were purified, etc. It is thus unwise too attach to much significance to the observed time of onset of increased RNA synthesis. What I feel to be significant is that within a single experimental system my data clearly show that increased rates of processing and transport of nuclear transcripts precede by many hours an observable increase in overall RNA synthesis.

I have developed a model, consistent with my data, of the changes in RNA metabolism that occur during the first 10 hours of mitogen stimulation (Fig.4.1). This model diagrams RNA metabolism in resting and stimulated lymphocytes and suggests that the early effects of mitogen on RNA metabolism are:

1. an increase in the rate of RNA processing; especially increased polyadenylation of pre-mRNA.
2. an increase in the number of RNA molecules transported from nucleus to cytoplasm and, possibly, an increase in the rate of transport.
3. a decrease in the amount of degradation of new RNA transcripts (Cooper, 1972).
4. An increase in the ratio of ribosomes:ribosomal subunits (Cooper and Braverman, 1977).
5. little, or no, increase in transcription.

4.4 FUTURE POTENTIALS OF LYMPHOCYTE MOLECULAR BIOLOGY

There are many experiments I could not do because of problems with the system. Some of these are discussed in the appendices to this thesis. I was never able to directly demonstrate that the rate or amount of RNA polyadenylation increased after mitogen stimulation. I spent several months trying to show this using a double label technique; I hoped to show that transcripts labelled with [^{3}H]uridine became labelled with [^{14}C]adenine added after stimulation but the cells never incorporated enough [^{14}C]adenine to obtain a significant amount of ^{14}C radioactivity in the extracted RNA. It may still be possible to demonstrate that resting cell transcripts are polyadenylated after stimulation. The

experiment will involve labelling resting cell transcripts with thiolated nucleosides. After washing the cells, and chasing the thiolated nucleoside with cold nucleoside, the cells will be stimulated and labelled with [^{3}H]adenine. The thiol-labelled RNA may be separated by affinity chromatography on Hg-Agarose; if this RNA had [^{3}H]labelled poly(A) it could be unambiguously concluded that polyadenylation of pre-existing transcripts does take place after stimulation. Experiments with isolated nuclei and cytoplasm could then determine how much of this polyadenylation occurs in the nucleus and how much occurs in the cytoplasm. I hope to do these experiments in collaboration with Dr. C. Schaefer.

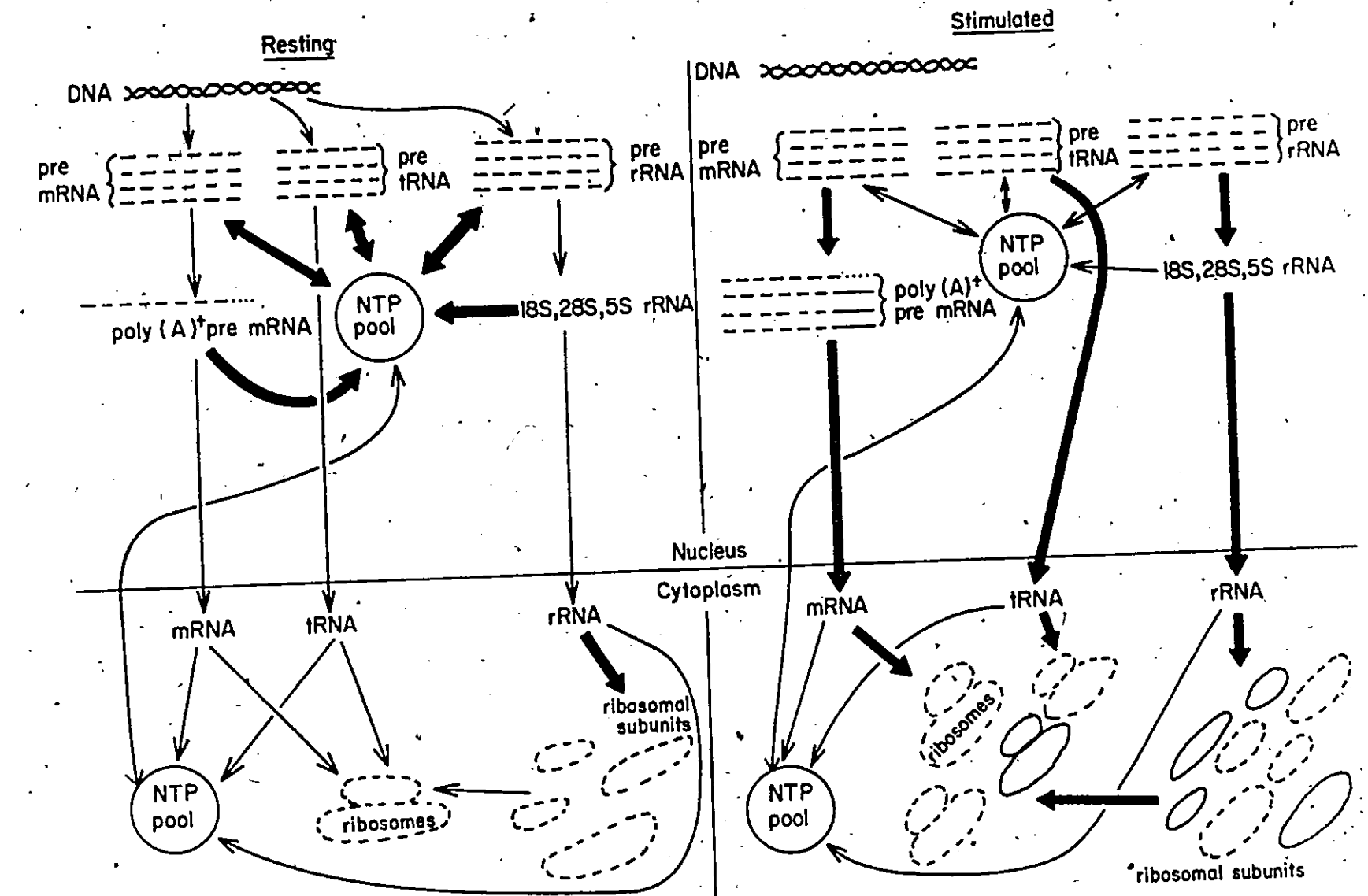
I have already discussed many of the difficulties encountered in studying molecular biology in the lymphocyte system. So far, most work with this system would be better labelled phenomenological than biochemical. Lymphomaniacs, including myself, can't help but feel a little discouraged when we look at the relative ease with which fundamental gene structure is being unravelled in other systems. However, knowledge of gene structure tells us remarkably little about how genes work and how the utilisation of a cells genetic information is controlled. The lymphocyte has great potential as a system for investigating these problems; it is a cell which has presumably evolved to facilitate the highest levels of control of gene expression.

No doubt the "turn on" of a lymphocyte involves control at all possible levels of gene expression.

Advances in other systems often involved the study of a single gene rather than the whole gene population. In lymphocytes it has, so far, not been possible to look at single genes. Messenger RNAs for proteins which do seem to increase significantly after stimulation, e.g. actin or tubulin, may be the "tools" for lymphocyte molecular biology in the future.

The lymphocyte system may not, now, be suitable for unravelling basic mysteries of the gene but it will almost certainly be the ideal system on which to apply that basic knowledge.

Fig. 4.1 Model of RNA metabolism in resting and 10 hours stimulated lymphocytes. ----------represents RNA sequences being produced in resting and in stimulated cells. ——————represents RNA sequences only produced in stimulated cells. The thickness of the arrow lines represents the relative amount of RNA following each pathway.


Resting
DNA
pre mRNA
pre tRNA
pre rRNA
poly (A)+pre mRNA
NTP pool
18S,28S,5S rRNA
Nucleus
Cytoplasm
mRNA
tRNA
rRNA
ribosomal subunits
ribosomes
NTP pool
Stimulated
DNA
pre mRNA
pre tRNA
pre rRNA
NTP pool
18S,28S,5S rRNA
poly (A)+ pre mRNA
mRNA
tRNA
rRNA
ribosomes
NTP pool
ribosomal subunits

Appendix A

EFFECTS OF ACTINOMYCIN D AND OUABAIN ON RNA SYNTHESIS OF HUMAN PERIPERHAL BLOOD LYMPHOCYTES

A.1 INTRODUCTION

I investigated the effects of two drugs, actinomycin D and ouabain on RNA synthesis in human peripheral blood lymphocytes.

A.1.1 Actinomycin D

In most cell types 0.05 ug/ml of actinomycin D specifically inhibits nucleolar transcription; the drug inhibits RNA polymerase by binding to chromatin and appears to bind preferentially in the nucleolar region. The exact molecular mechanism is, as yet, unknown. Almost all rRNA is nucleolar in origin while mRNA and tRNA are transcribed from the nucleoplasmic regions of chromatin. In most cell types nucleolar transcription may be inhibited by actinomycin D while mRNA transcription proceeds normally. Although it has been suggested that RNA processing is affected by actinomycin D, the drug has been used in many studies of mRNA metabolism. I wished to use the drug to facilitate my studies of the changes in mRNA metabolism concomitant with lymphocyte activation but found that the often used

concentration of 0.05ug/ml completely inhibited transcription of all types of RNA in lymphocytes. Experiments in which I attempted to find a concentration of actinomycin D that would specifically inhibit nucleolar transcription in lymphocytes are described here.

A.1.2 Ouabain

Ouabain is a cardiac glycoside which inhibits lymphocyte stimulation It has multiple effects on the cell; much research in our laboratory and others has been directed towards the inhibitory effect of ouabain on the membrane bound ATP dependent Na/K transport system.

Kay (1971) reported that some RNA synthesis was resistant to ouabain. He looked at the effect of ouabain on total RNA synthesis in resting and 26 hour PHA stimulated human peripheral blood lymphocytes and found a small percentage of RNA to be resistant to treatment with 10^{-5} M ouabain (a concentration which inhibits blast transformation). I wished to determine what type of RNA synthesis was ouabain resistant and therefore repeated Kays' experiment (except for stimulating the cells with Con A). I planned subsequently to prepare the resistant RNA in larger volume and subject it to sedimentation analysis. Instead, I found myself questioning Kay's data; as reported here, I find all transcription to be ouabain sensitive.

A.2 MATERIALS AND METHODS

A.2.1 Inhibition of RNA synthesis with actinomycin D

50ml cultures were incubated for 35 hours with Con A before actinomycin D was added in a range of concentrations (0.001-0.05ug/ml). After 30min further incubation 10uCi of [^{3}H]uridine per millilitre was added to the cultures for 2 hours before harvest. Control cultures were incubated without actinomycin D. Total acid precipitable radioactivity was determined in RNA extracted from cytoplasmic cell fractions. The different classes of RNA being produced were resolved by absorbing polyadenylated RNA to glassfibre filters saturated with poly(U) and by electrophoresis on 2.7% polyacrylamide gels (Perry and Kelley, 1968).

A.2.2 Inhibition of RNA synthesis with Ouabain

175ul cultures of resting and Con A stimulated lymphocytes were incubated on microplates (Falcon) for 26 hours. 10^{-5} or 10^{-4} M ouabain was added 15min before Con A. 10uCi of [^{3}H]uridine per millilitre was added 1hour before harvest.

A.3 RESULTS AND DISCUSSION

A.3.1 Effects of actinomycin D on RNA synthesis

I obtained highly variable results in 16 experiments utilising low concentrations of actinomycin D. In certain experiments 0.005ug actinomycin D per millilitre inhibited rRNA synthesis by over 80% while polyadenylated RNA synthesis was not affected (see fig.A.1). In other experiments, however, 0.002 ug actinomycin D per millilitre inhibited all RNA synthesis while in another experiment more than 50% of control levels of rRNA synthesis was measurable after incubation with 0.001ug actinomycin D per millilitre. The results from these experiments are summarised in table A.1.

L.Loeb (personal communication) has informed me that actinomycin D may be actively transported and accumulated by stimulated lymphocytes, and Darzynkiewicz and Ring (1969) have shown PHA stimulation to induce a marked increase in in actinomycin D binding to chromatin. Such events might account for my anomalous results; variations from one experiment to another may reflect differences in the capacity of the lymphocyte preparation to transport and concentrate actinomycin D rather than differences in sensitivity of nucleolar or overall gene transcription.

In any case, actinomycin D is not a suitable drug to use in studies of lymphocyte activation and any data obtained using it should be treated with caution.

Fig. A.1 Cytoplasmic RNA isolated from cells, either with or without a 30 min treatment of 0.005ug actinomycin D per millilitre was seperated by electrophoresis in 2.7% polyacrylamide gel (Perry and Kelley, 1978). Ordinate represents [^{3}H]uridine counts per minute in each solubilised gel fraction. Abcissa represents fraction number.

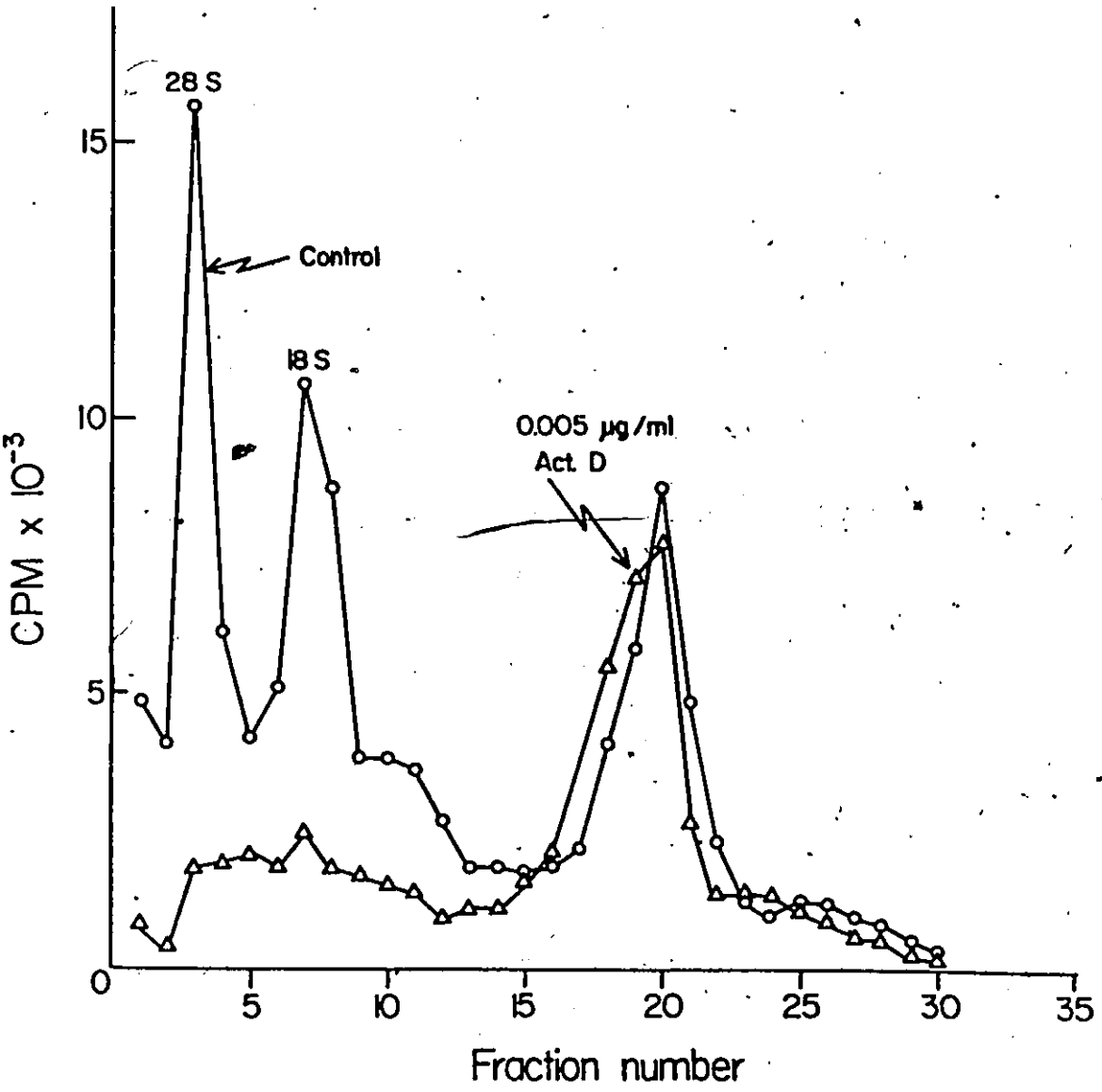

28 S
18 S
Control
0.005 µg/ml
Act. D
CPM x 10^{-3}
15
10
5
0
5
10
15
20
25
30
35
Fraction number

TABLE A.1

Inhibition of polyadenylated and non-polyadenylated RNA synthesis by different concentrations of actinomycin D

Blood Donor	[Act.D] ug/ml	%inhibition (A^-) RNA	(A^+) RNA
1	0.01	59	14
	0.005	36	0
2	o.05	85	59
	0.01	86	49
3	0.05	83	0
	0.01	45	0
	0.005	8	0
4	o.005	60	29
5	0.0025	19	27
	0.005	54	39
	0.01	68	42
6	0.005	34	55
	0.05	53	58
7	0.0025	17	7
	o.oo5	36	23
	0.001	16	25

A.3.2 Effect of Ouabain on RNA synthesis

Table A.2 shows the results of two experiments on the effect of 10^{-6} M ouabain on RNA synthesis. Ouabain, at this concentration, is seen to inhibit even the basal levels of RNA synthesis in resting cells. 10^{-5} M ouabain also, of course, caused such inhibition.

I therefore suggest that all RNA synthesis is ouabain sensitive and must question the validity of Kay's data on this subject.

Table A.2

Effect of 10^{-6} M ouabain on RNA synthesis.

	Acid precipitable cpm	
	Expt.1	Expt.2
Resting cells	4,300	5,000
Resting cells + ouabain	1,300	4,200
26h ConA cells	12,200	35,900
26h ConA cells + ouabain	1,400	2,100

Appendix B

PREPARATION OF POLYSOMES FROM MOUSE SPLENIC LYMPHOCYTES

B.1 INTRODUCTION

Polysome bound RNA is a good definition of mRNA involved in protein synthesis; an assay of polysomal RNA has thus more meaning than an assay of total cytoplasmic polyadenylated RNA. The desire to assay accurately polysomal RNA was the reason I spent several months trying to isolate mouse splenic lymphocyte polysomes. I also hoped to purify polysomal mRNA and use a reverse transcriptase enzyme to make complementary DNA (cDNA) copies so that, by doing cross hybridisation experiments, I could establish if qualitative differences in the messenger population are measurable following lymphocyte activation. Such a determination was made by Berke and Feldman (1971) but they used total cellular DNA and RNA that had been radioactively labelled for 5 hours so it is not surprising that they found no significant differences.

With one exception (Burrone and Algranati, 1977), all the lymphocyte polysome profiles I have been able to find in the literature have showed degradation of RNA. My aim was to find a combination of detergent and nuclease inhibitors,

that could be included in the isolation buffers, to facilitate isolation of polysomes.

B.2 MATERIALS AND METHODS

B.2.1 Preparation of cells

Two month old mice ("Paris 3 outbred") were obtained from the University of Ottawa animal house. Spleens were removed under sterile conditions and washed in 0.15M NaCl. Each spleen was immediately transfered to PBS + 10% fetal calf serum and cut and pressed gently but thoroughly with tweezers to yield a cell suspension. The suspension was layered on 100% fetal calf serum for 15 min to allow large debris to settle; then the cells in the 10% layer were relayered on 5ml of 100% fetal calf serum and centrifuged at 400g for 7mins. The pellet was resuspended in 0.17M NH_4Cl for 2min then repelleted and resuspended in culture medium.

The yield from this preparation was 9-16 x 10^7 cells per spleen. These cells were 95% lymphocytes, 95% viable as judged by Trypan Blue dye exclusion.

B.2.2 Culture Conditions

Cells were cultured in Corning or Falcon culture vessels at a density of $2x10^6$ cells/ml in RPMI 1640 culture medium (Flow Labs) supplemented with 5% fetal calf serum (Flow Labs), Penicillin (11U/ml)-Streptomycin (100ug/ml) (DIFCO). Cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C.

2ug of Con A per millilitre was added to stimulated cultures.

Sterile conditions and treatment of ribonuclease on glassware was as described in chapter 2.

B.2.3 Preparation of Rat Liver Supernatant

Approximately 1g of rat liver was added to two volumes 0.25M sucrose in TKM (20mM tris-HCL, 50mM KCl, 2mM $MgCl_2$; pH 7.6) and centrifuged at 10,000rpm in a Sorvall rotor for 15min. The supernatant was centrifuged for 3 hours at 37K in an SW41 rotor and a Beckman L5.50 centrifuge and again for 3 hours at 40K in a Ti60 rotor and a Beckman L5.50 centrifuge. The high speed supernatant was stored at -70°C.

B.2.4 Preparation of Polysomes

Cells were cultured for 50 hours with 2ug/ml Con A; 3uCi [^{3}H]uridine per millilitre was added two hours before harvest.

My strategy was to compare the effects of various concentrations and combinations of Triton-X-100, Poly vinyl sulphate (Sigma), Spermine (Calbiochem), diethyl-pirocarbonate (Sigma) and rat liver supernatant in the isolation medium for polysomes. I also tried inhibiting protein synthesis with cycloheximide (Sigma) so as to "freeze" the polysomes before harvesting the cells;

cycloheximide was added 10min prior to harvest as indicated in table B.1.

Cells were collected at harvest by centrifugation then washed in 3ml PBS (0.85M NaCl, 0.02M NaH_2PO_4; pH7) and incubated in 0.7ml low salt homogenisation buffer (10mM NaCl, 20mM TrisHCl, 1.4mM MgAcetate; pH 7.4) for 10min at 4 °C with concentrations of Triton, poly vinyl sulphate, spermine, dihetylpyrocarbonate, rat liver supernatant and cycloheximide varying in each experiment as described in table 1.

TABLE 1

Constitution of Homogenisation buffer used in isolation of polysomes

Expt. No.	Triton X100 %	PVS ug/ml	DEP 1%	Spermine ug/ml	RLS 20%	Cyclo-heximide ug/ml
1	0.02	50	-	100	+	-
2	0.01	50	-	100	+	-
3	0.005	50	-	100	+	-
4	0.02	-	-	-	+	-
5	0.01	-	-	-	+	-
6	0.005	-	-	-	+	-
8	0.01	35	-	55	-	-
9	0.005	35	-	55	-	-
10	0.02	50	-	100	-	-
11	0.50	50	-	100	+	-
12	0.10	50	-	100	+	-
13	0.05	50	-	100	+	-
14	0.005	50	+	100	-	-
15	0.005	50	+	100	-	50
16	0.005	50	+	100	-	100
17	0.005	50	+	100	-	150

After incubation in the homogenisation buffer the cell nuclei were pelleted twice and 0.7ml of the supernatant was applied o 12ml sucrose gradients (15-45%). Sedimentation was carried out in an SW41 rotor and a Beckman L5-50 centrifuge at 20.3K for 14 hours at 4°C.

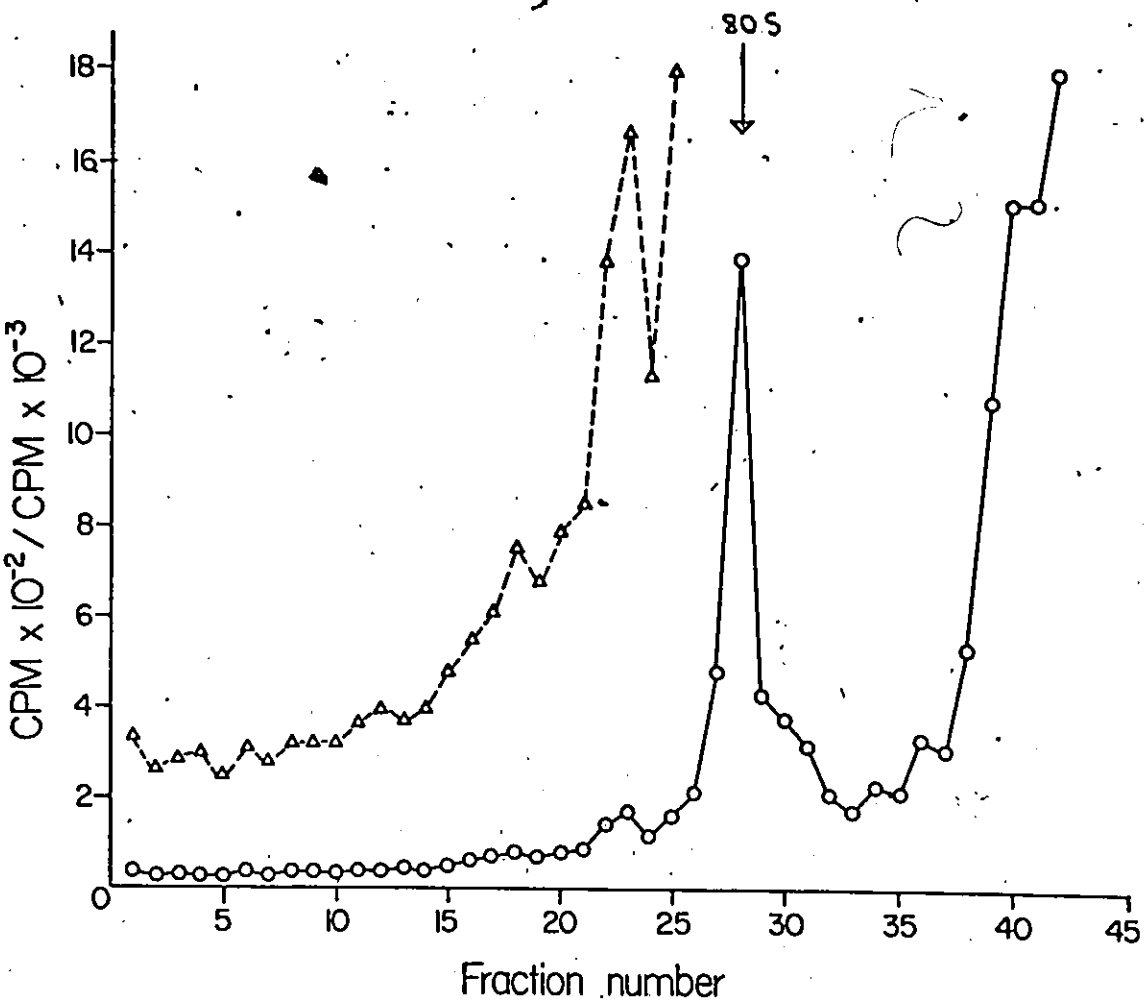
B.3 RESULTS AND DISCUSSION

In most experiments the polysome profiles I obtained were no better or worse than those published by other researchers; nearly all the material sedimented at 80S or as ribosomal subunits. In a few experiments I did obtain a small fraction of the total radioactive material sedimenting at higher density than 80S. Figure 1 shows the profile obtained in experiment 3. This is one of the "best" profiles I obtained. Exeriments number 4 and 15 resulted in profiles of similar quality.

A researcher working in our laboratory, Enzo Bard, carried out the following experiment. He isolated [^{14}C]uridine polysomes from HeLa cells which sedimented in the normal profile. He then incubated these HeLa cell polysomes with a preparation of [^{3}H]uridine-labelled lymphocyte polysomes. The HeLa cell polysomes suffered extensive degradation during this treatment and, like the lymphocyte polysomes, sedimented at lower density than 80S following the treatment.

We suspect that splenocytes contain exceptionally high quantities of ribonuclease and that this is is why the characteristic multipeaked polysome profiles observed in other cell types have not been obtained. Progress awaits establising an extraction medium with a combination of RNAase inhibitors sufficient to arrest this degradation.

Fig. B.1 Distribution of ribosomes in mouse splenic lymphocytes stimulated for 50 hours with Con A. Ordinate represents counts per minute x 10^{-3} or 10^{-3}. Abscissa represents gradient fraction number.


80 S
CPM x 10⁻² / CPM x 10⁻³
0
2
4
6
8
10
12
14
16
18
5
10
15
20
25
30
35
40
45
Fraction number

BIBLIOGRAPHY

Abelson, H.T., Johnson, L.F., Penman, S. and Green, H., Cell 1, 161 (1974).

Alford, R.H., J. Immunol. 104, 698 (1970).

Anderson, J., Sjoberg, O. and Muller, G. Transplant Rev. 11, 131 (1972).

Averdunk, R., Hoppe-Seyler's Z. Physiol. Chem. 353, 79 (1972).

Bandman, E. and Gurney, J., Exp. Cell Res. 90, 159 (1975).

Berger, S.L. and Cooper, H.L., Proc. Natl. Acad. Sci. USA 12, 3873 (1978).

Berget, S., Moore, C. and Sharp, P., Cell 12, 45 (1977).

Berke, S., Sarid, S. and Feldman, M., Biochim. Biophys. Acta 254, 440 (1971).

Bowman, L.H. and Emerson, C.B., Cell 10, 587 (1977).

Breathnach, P., Mandel and Chambon, R., Nature 270, 314 (1978).

Burrone, O and Algranati, I.P., Mol. Cell Biochem. 16, 105 (1977).

Cooper, H.L., Transplant Rev. 11, 3 (1972).

Cooper, H.L., in "Control of Proliferation in Animal Cells" (B. Clarkson andd R. Baserga, ads.), p. 769. Cold Spring Harbour Laboratory, (1974).

Cooper, H.L. and Braverman, R., J. Cell Physiol. 93, 213 (1977).

Cooper, H.L. and Kay, J.E., Biochim. Biophys. Acta 174, 503 (1969).

Cooper, H.L. and Rubin, A.D., Blood 25, 1014 (1965).

Crick, F., Science 204, 264 (1979).

Darnell, J., The 11th Miami Winter Symposium (abstract) 15 (1979).

Darzynkiewicz, B.L. and Ring, N.R., Exp. Cell Res. 55, 120 (1969).

Deutcher, M.P., Progr. Nucleic Acid Res. Mol. Biol. 13, 51 (1973).

Dunn . and Hassel, J., cell 12, 23 (1977).

Getz, M.J., Elder, P.K., Benz, E.W., Stephens, R.E. and Moses, H.L., Cell 7, 255 (1976).

Gilbert, W., Nature 271, 501 (1978).

Greenberg, H. and Penman, S., J. Mol. Biol. 21, 527 (1966).

Hadden, J.W., Hadden, E.M., Haddox, M.K. and Goldberg, N.D., Proc. Nat. Acad. Sci. 69, 3024 (1972).

Hauser, H., Knippers, R., Shaefer, K.P., Sans, W. and Unsold, H.J., Expt. Cell Res. 102, 79 (1976).

Hausen, P. and Stein, H., Eur. J. Biochem. 4, 401 (1968).

Jeffreys, A.J. and Flavell, R., Cell 12, 295 (1977).

Johnson, E.M., Karn, J. and Allfrey, V.G., J. Biol. Chem. 249, 4990 (1974).

Johnson, L.F., Abelson, H.T., Penman, S. and Green, H., Cell Physiol. 90, 465 (1976).

Johnson, L.F., Levis, R., Abelson, H.T., Green, H. and Penman, S., J. Cell Biol. 71, 933 (1976).

Johnson, L.F. and Meister, R., J.Cell Physiol.92, 57 (1977).

Johnson, L.F., Williams, J.G., Abelson, H.T., Green, H. and Penman, S., Cell 4, 69 (1975).

Kaplan, J.G., Mitchell, M. and Bard, E., Biochemistry and Molecular Biology of Lymphocyte Transformation (M.R. Quastel, ed.) Academic Press, N.Y. (1978).

Kay, J.E., European J. Biochem. 4, 225 (1968).

Kay, J.E. and Cooper, H.L., Biochim. Biophys. Acta 62 (1969).

Kay, J.E. and Hanelmaker, S.D., Expt. Cell Res. 63, 411 (1976).

Kay, J.E., Leventhal, B.G. and Cooper, H.L., Expt. Cell Res. 54, 94 (1969).

Land, H. and Shaefer, K.P., Biochem. Biophys. Res. Commun. 79, 947 (1977).

Lavi, S., Groner . and Weizman, S., Proc. Natl. Acad. Sci. 12., 5323 (1978).

Levis, R., McReynolds, L. and Penman, S., J. Cell Physiol. 90, 485 (1976).

Levy, R. and Rosenberg, S.A., Cell Immunol. 7, 92 (1973).

Liebhaber, S. A., Wolf, S. and Schlessinger, D., Cell 13, 121 (1978).

Loeb, L.A., Biology of Lymphoid Cells (Gottlieb, A.s. ed.) Academic Press, N.Y., 103 (1974).

Lucas, Z. J., Science 156, 1237 (1967).

Marbaix, G., Huez, G. and Soreq, H., Trends in Biochemical Science (1977).

Mauck, J.G. and Green, H., Proc. Nat. Acad. Sci. USA 70, 2819 (1973).

Mauck, J.G. and Green, H., Cell 3, 171 (1974).

Meedel, T.H. and Levine, E.M., J. Cell Physiol. 90, 211 (1976).

Mendelson, S., Skinner, A. and Kornfeld, S., J. Clin. Invest. 50, 816 (1971).

Mitchell, M., Bard, E., L'Anglais, R. and Kaplan, J.G., Can. J. Biochem. 56, 659 (1978).

Nobumichi, . and Tonegawa, ., Proc. Nat. Acad. Sci. 73, 2628 (1978).

Owen, J.J. and Ritler, M.A., J. Exp. Med. 129, 431 (1969).

Pago, B.G.T., Allfrey, V.G. and Mirsky, A.E., Proc. Nat. Acad. Sci. USA 55, 805 (1966).

Perry, R.P., Ann. Rev. Biochem. 45, 605 (1976).

Perry, R.P., Bard, E., Hames, B.D., Kelley, D.E. and Schibler, U., Prog. Nucleic Acid Res. and Molec. Biol. (E. Volkin and W. Cohn eds.) 19 275 (1976).

Perry, R.P. and Kelley, D., Cell 8, 433 (1976).

Peters, J.H. and Hausen, P., Eur. J. Biochem. 19, 502 (1971).

Quastel, M.R. and Kaplan, J.G., Exp. Cell Res. 63, 230 (1970).

Revel, M. and Groner, Y., Ann. Rev. Biochem. 47, 1079 (1978).

Roop, R., Tsai, A., Nordstrum, J.L., Tsai, S. and O'Malley, B., J. Cell Biol. 79, 359a (1978).

Roos, D. and Loos, D.a., Biochim. Biophys. Acta 222, 565 (1970).

Rosenfeld, M.G., Abruss, I.B., Mendelsohn, J., Roos, B.A., Boone, B.A. and Garreu, L.D., Proc. Nat. Acad Sci. USA 69, 2306 (1972).

Rudland, P.S., Proc. Nat. Acad. Sci. USA 71, 750 (1974).

Scholissec, C., Eur. J. Biochem. 28, 70 (1972).

Slater, J., Gillespie, D. and Slater, D.W., Proc. Natl. Acad Sci. USA 70, 406 (1973).

Smith, J.D., Prog. Nuc. Acid Res. and Mol. Biol. 16, 25 (1976).

Smith, J.W., Steiner, A.L. and Parker, C.W., Fed. Proc. 29, 369 (1970).

Stanners, C.P. and Becker, H., J. Cell Physiol. 77, 31 (1970).

Stein, J.B., Catterall, J.F., Lai, E.C.M., Woo, S.L.C., Means, A.R. and O'Malley, B.W., J. Cell Biol. 79, 144a (1978).

Wettenal, R.E.H. and London, D.R., Biochim. Biophys. Acta 349, 214 (1974).

Williamson, B., Nature 269, 9 (1977).