THE PROCESSING AND INTRACELLULAR TRANSPORT OF MESSENGER RNA IN A HIGHER PLANT

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I, David Baulcombe, declare that the work in this thesis was carried out by myself with the exception of the experiments on the comparison of turnover of nuclear and cytoplasmic RNA and the *in vitro* protein synthesis experiments. These were carried out in collaboration with Dr K. Chapman and Dr C. Leaver respectively.

Signed

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ABBREVIATIONS

All abbreviations are defined in the text at the first occurence. The following is a list of the most frequently used abbreviations.

RNA	=	ribonucleic acid
mRNA	=	messenger RNA
poly A(+) mRNA	=	mRNA containing poly A
poly A(-) mRNA	=	mRNA lacking poly A
hnRNA	=	heterogenous nuclear RNA
pre-mRNA	=	precursor of mRNA
0.7×10^6 rRNA	=	ribosomal RNA of 0.7×10^6 mol. wt.
1.3×10^6 rRNA	=	ribosomal RNA of 1.3×10^6 mol. wt.
2.4×10^6 pre-mRNA	=	precursor of rRNA of 2.4×10^6 mol. wt.
RNP	=	ribonucleoprotein
mRNP	=	RNP associated with mRNA
hnRNP	=	RNP associated with hnRNA
RNAase	=	ribonuclease
DNA	=	deoxyribonucleic acid
cDNA	=	a complementary DNA copy of RNA
rNTP	=	ribonucleoside triphosphate
P	=	density

SUMMARY

It is thought that structural features of eukaryotic mRNA, including 5' terminal caps, repeated sequence transcripts and 3' poly A tracts, may be involved in post-transcriptional regulation of gene expression. This thesis considers the possible role of the poly A on artichoke mRNA.

The poly A in the ribosome preparation of artichoke explants was associated with rapidly labelled heterodisperse RNA, although 50% of such RNA did not bind to poly U Sepharose. 88% of this heterodisperse RNA was associated with ribosomes in a sucrose gradient and there were no high specific activity components separate from the ribosome main band after fixation of a ribosome preparation from 1hr. pulse labelled explants. It was therefore concluded that the rapidly labelled heterodisperse RNA was mainly mRNA and that there was poly A (+)mRNA and poly A(-)mRNA which had no, or a very short poly A tract.

Messenger RNP, released from ribosomes by puromycin-KCl treatment and fixed, had a distinct buoyant density in CsCl from ribosomes and therefore a different protein : RNA ratio. Despite this, it was not possible to separate mRNP from ribosomes preparatively by equilibrium centrifugation in Cs_2SO_4 or Metrizamide. It was, however, possible to partially purify 29% of the mRNA from rRNA independently of poly A content by fractionation of EDTA released mRNP on sucrose gradients.

This 'mRNP-mRNA' was used to compare turnover rates of poly A(+) and poly A(-) mRNA. In dividing explants poly A(+)mRNA was more stable than poly A(-)mRNA, whereas in non-dividing explants there was no difference in the half life of these mRNA fractions. There was no detectable difference between poly A(+) and poly A(-) mRNA in terms of ribosome capacity, which suggested that translation efficiency in vivo was similar on poly A(+) and poly A(-) mRNA. There was no detectable intra nuclear turnover of poly A(+)hnRNA.

The results were discussed in relation to published work on poly

(+) and poly A(-)mRNA. It was concluded that poly A was not involved in selection of hnRNA sequences for cytoplasmic transport, but that it may affect the cytoplasmic metabolism of mRNA in a cell specific manner.

INTRODUCTION

Changes in the metabolic capacity of a cell are often coincident with changes in the enzymatic or structural protein complement of that cell. Related to this there may be alterations in the level of translatable messenger RNA (mRNA) for these proteins. In reticulocytes, for example, more than 90% of the protein synthesised is haemoglobin and the globin mRNA comprises a similar proportion of the cellular mRNA (Aviv et al 1976). Exposure of barley aleurone cells to gibberellic acid induces an increased rate of 'de novo' synthesis of α amylase (Filner and Varner 1967) and a higher level of translatable α amylase mRNA (Higgins et al 1976).

In non-green plant cells, most of the mRNA is assumed to be transcribed in the nucleus and transported to the cytoplasm before being translated. The rates of synthesis of a specific protein might therefore be regulated at four basic levels; transcription, nuclear transport, cytoplasmic transport and translation of mRNA or its precursor. In this introduction, I discuss evidence for control of mRNA production and translation at these levels and consider the possible mechanisms of control. The synthesis of proteins in cell organelles and the co-ordination of this synthesis with nuclear directed synthesis will not be considered.

Much of the recent progress in this research has been due to a few advances in experimental technology. The discovery that some mRNA is polyadenylated at the 3' end led to the development of techniques for isolating and purifying polyA containing RNA (poly A(+)RNA) and its putative poly A containing precursor, heterogenous nuclear RNA (hnRNA)(Aviv and Leder 1972, Firtel and Lodish 1973). In addition Roberts and Paterson (1973) improved a

cell free protein synthesising system from wheatgerm (the S.30 system) which translated exogenous mRNA with fidelity and produced only a minimum of endogenously coded polypeptides. Thus an additional criterion, the ability to stimulate the synthesis of a specific characterised protein in a cell free system, was added to the list of identifying properties of mRNA.

Previously, the functional definition of mRNA was a rapidly synthesised RNA which was released from polysomes by EDTA or puromycin (Penman et al 1968). The other main cell free protein synthesising system available before 1973 was prepared from reticulocytes. Although this system had a lower level of endogenous ribonuclease (RNAase) than the wheatgerm system and incorporated amino acids into polypeptides at a rate similar to the in vivo rate, most of this incorporation was into globin coded for by the endogenous mRNA. Pelham and Jackson (1976) have been able to overcome this problem by digesting the endogenous reticulocyte mRNA with a low level of calcium dependent nuclease. By removal of calcium with a chelating agent, EGTA, exogenous mRNA could be added to the lysate and remain undigested. The extremely efficient 'in vitro' protein synthesis was then due almost entirely to the exogenous mRNA. This improved reticulocyte system is superior to the wheatgerm system and may become the method of choice for translation of exogenous mRNA. Xenopus oocytes have also been used to translate exogenous mRNA which was introduced by micro-injection (eg Huez et al 1974).

High specific activity hybridisation probes have proved extremely useful in detecting low concentrations of mRNA sequences (Young et al 1974). The poly A of mRNA was annealed to oligo dT to

form a double stranded primer for reverse transcriptase in a reaction mixture containing radioactive deoxynucleotides and the DNA product (complementary DNA-cDNA) isolated. This technique may also be used to prepare copies of Poly A(+)RNA if the RNA is polyadenylated *in vitro* with 3' nucleotidylexotransferase (Mans and Huff, 1975; Hell et al, 1976).

The production of mRNA could be influenced at the transcriptional level by the availability of its gene for transcription by the RNA polymerase or by a change in the number of copies of that gene.

The approach of several laboratories testing the former of these alternatives has been to transcribe chromatin '*in vitro*' with *E.coli* RNA polymerase and then to assay the products for messenger sequences. A difference in the production of messenger sequences between different chromatin preparations has usually been taken to imply a difference in template (gene) availability '*in vivo*'. G.Stein et al (1975) using chromatin from different stages of the cell cycle in synchronised Hela cells and assaying the products by hybridisation to cDNA of histone mRNA found that histone genes were only available for *in vitro* transcription during 'S' phase. They extrapolated from this and concluded that histone genes were only transcribed *in vivo* during 'S' phase. The same technique was used by Gilmour and Paul (1973) to show that globin mRNA sequences were only found in '*in vitro*' transcripts of erythroid tissue chromatin. As chromatin may be contaminated with cellular RNA which, even at very low concentration would be detected by the sensitive cDNA probe, it is necessary to either eliminate this contamination or determine the 'background' for an experiment by assaying the chromatin before transcription. Crouse

et al (1976) were able to purify the newly synthesised RNA, after *in vitro* transcription using mercurated UTP, by passage through sulfydryl sepharose. Their experiments using E.coli RNA polymerase demonstrated that developing reticulocyte chromatin supported the synthesis *in vitro* of ten times the number of globin sequences produced *in vitro* by mature reticulocyte chromatin. Harris et al (1976) assayed the background ovalbumin sequence content in oviduct chromatin with a cDNA probe and found that E.coli polymerase only produced ovalbumin sequences *in vitro* from oestrogen treated tissue. A different approach by Gilmour et al (1974) was to estimate the number of globin sequences in nuclear RNA of Friend cells, again using a cDNA probe. After haemoglobin induction by dimethylsulphoxide, the number of globin sequences per nucleus increased threefold. This result was interpreted as showing an increased rate of transcription of globin sequences during induction, but could also be explained by increased stability of nuclear globin sequences. However, the *in vitro* transcripts of 'induced' chromatin contained a much more than threefold greater concentration of globin sequences compared with those of 'uninduced' chromatin as no globin sequences were detected in the 'uninduced' chromatin transcripts even at RNA/DNA ratio of 10^5:1 (Dot $\geq$ 0.1). Young et al (1974) have shown that at a Dot (DNA concentration (moles, ℓ^{-1}) x time (sec)) of 0.1, in RNA excess, essentially all of the homologous sequences would have hybridised. Since there evidently was transcription of globin sequences in uninduced cells, the *in vitro* transcription technique is clearly inadequate to show whether or not a gene is 'switched off' and results obtained in this manner are therefore not considered to demonstrate transcriptional control. Humphries et al (1976) described rigorously controlled

experiments in which nuclear RNA of various origins was hybridised to globin cDNA so that less than one sequence per cell would have been detected. Significant amounts of globin sequences were detected in nuclear RNA of all non erythroid tissues tested including liver, brain and lymphoid cells. This would suggest that genes may never be 'turned off'. The experiments which will measure changed rates of transcription in vitro have not yet been devised.

An increase in gene number has been demonstrated in amphibian oocytes where there is a massive increase in the number of ribosomal RNA genes compared to somatic cells (Brown and Dawid 1968). In a number of cases of endopolyploidy (Pearson et al 1974) or polyteney (Hennig 1972) nuclear DNA is replicated non uniformly. There is however, no evidence that the number of genes coding for a specific protein is ever changed in response to the requirement of the cell for that protein. Packman et al (1972), estimated with a cDNA probe, that both the liver and reticulocyte genomes of duck contained 2 to 3 copies of the globin gene and Harrison et al (1974i) showed that globin gene number in erythroid and non erythroid mouse cells remains constant and is less than five. Similarly, ovalbumin genes are present in the same low concentration in oestrogen induced and uninduced oviducts (O'Malley and Means 1974).

The second possible control level is the processing and transport of nuclear precursor mRNA (pre mRNA). It is concievable that in response to a stimulus, certain pre-mRNA sequences may be modified or may interact with 'activated' nuclear components such that they are no longer transported into the cytoplasm. Conversely, the stimulus may result in transport of new sequences into the cytoplasm. However, research into these possibilities has been fraught with the difficulty of identifying the nuclear precursor mRNA. These molecules will, of course, share some of the structural sequences of

mRNA, including the coding sequence. Imaizumi et al (1973) established a structural relationship between hnRNA and mRNA, by showing that duck erythroblast hnRNA contained sequences complementary to a DNA copy of globin mRNA. Stevens and Williamson (1972) injected poly A(+)hnRNA and mRNA from myeloma cells into Xenopus oocytes. Both samples induced synthesis of a product which was identified as immunoglobin by immuno-precipitation followed by electrophoresis, thus confirming the result of Imaizumi et al for a different mRNA. Levy and McCarthy (1976) showed that the structural relationship between poly A(+)hnRNA and poly A(+)mRNA applies to all sequences, by demonstrating that cDNA of poly A(+)mRNA hybridised to completion in a reaction driven by poly A(+)hnRNA. However, from kinetic data and sequence complexity estimations it is clear that some of the hnRNA turns over within the nucleus. Scherrer et al (1970) concluded from pulse chase experiments using actinomycin D to inhibit RNA synthesis during the chase period that only a few percent of hnRNA passed to the cytoplasm. Brandhorst and McConkey (1975) measured the kinetics of approach to steady state labelling of the poly A of hnRNA and mRNA. Unfortunately the results of this type of experiment are quite ambiguous and by changing the assumptions, the results of Brandhorst and McConkey were consistent with either intra nuclear turnover of most of the poly A(+)hnRNA or a cytoplasmic destination for most of the poly A(+)hnRNA. The latter is unlikely in the light of complexity estimations of hnRNA. Hough et al (1975) used single copy nuclear DNA as a probe for messenger RNA sequences and found that sea urchin hnRNA was ten times more complex than mRNA. Getz et al (1975) estimated that sequences next to poly A in hnRNA of Friend cells were five times more complex than those in mRNA. Thus there is substantial turnover of hnRNA sequences. Since only 60% of cDNA to poly A(+) hnRNA hybridised

to poly A(+) mRNA (Levy and McCarthy 1976), this intra nuclear turnover must amount to at least 30 to 40% of the poly A(+) hnRNA.

The status of the sequences which are turned over within the nucleus is of some interest. Are they potential messenger sequences, or are they sequences with a specifically nuclear rôle? The results of Green et al (1976) suggest that potential messenger sequences are turned over within the nucleus. Using highly radioactive adenovirus DNA as a probe for RNA from transformed hamster cells they detected 10% of the adenovirus genome in the cytoplasm and 38% in the nucleus. Since all of the adenovirus genome (ie 50% of the double stranded sequence) specifies mRNA, some potential mRNA must be turned over within the nucleus. Harrison et al (1974ii) found that in special clones of Friend cells, there was no increase in the number of nuclear globin sequences after DMSO induction of globin synthesis although the cytoplasmic sequences increased eightfold. The interpretation of this was that DMSO induced the transport of more nuclear globin sequences into the cytoplasm. Chan (1976) measured globin sequences within the nucleus and cytoplasm during maturation of cultured precursor chick erythroid cells. Before globin synthesis commenced, globin sequences were detected at high concentration within the nucleus. This level declined as cytoplasmic levels increased, just before globin synthesis was detected. Further work is needed to establish whether processing and transport of hnRNA are used extensively as regulatory steps.

Messenger like RNA occurs in the cytoplasm either associated with ribosomes as polyribosomes, or as free ribonucleoproteins (Spirin 1969). Spohr et al (1972) found that the kinetics of decay of free cytoplasmic sequences and of 9S polysomal globin mRNA were

consistent with a precursor-product relationship. They also noted that the electrophoretic profile of the messenger like RNA from the free RNP was more complex than that of polysomal mRNA. Their experiments used avian erythroblasts in which most polysomal mRNA was 9S globin mRNA, whereas the informosomal RNA contained an additional prominent 12S component. These results suggested the possibility of control of mRNA production at the level of cytoplasmic mRNA transport. Zahringer et al (1976), assaying ferritin mRNA by ferritin production in a cell free protein synthesising showed that, concomitant with iron stimulation of ferritin synthesis, there was a decrease in the concentration of ferritin 'mRNA' activity in 'subribosomal RNP' and an increase in polysomes. As a further example of cytoplasmic transport control, Buckingham et al (1976) found that in differentiating skeletal muscle and muscle cell cultures, myosin 'mRNA' was [illegible] in 70-90s cytoplasmic RNP and unattached to ribosomes long before myosin synthesis commenced (Buckingham et al 1976). Rudland et al (1975) showed that in cultured fibroblasts, the transition from the quiescent to the proliferating state was characterised by mobilisation of free cytoplasmic RNP containing poly A(+) RNA, into polysomes.

A further possible level of regulation is at the level of translation. Translation control is especially attractive as a rapid response regulation mechanism. Lodish (1974) has formulated an interesting model in which the relative synthesis of different proteins on a constant mRNA population would change depending only on the concentration of the (met-$tRNA_I^{met}$ - 40S subunit) complex (initiation complex). He proposed that on a mRNA the number of

ribosomes in a polysome would increase with the rate of initiation per mRNA. Up to a maximum rate of initiation, the rate of protein synthesis per mRNA would also increase, but if the rate of initiation per mRNA became such that the mRNA was fully loaded, elongation would become the limiting process. At very high rates of initiation per mRNA, ribosomes would be packed so closely that they would 'interfere' with each other and the rate of protein synthesis per mRNA would decline. Thus for a mRNA there would be an optimal rate of initiation. The rate of initiation per mRNA would depend on the concentration of initiation complex and the rate constant of initiation (K_I) for that mRNA. If the population of mRNA contained molecules with different K_I values, increasing the initiation complex concentration would make the rate of initiation per mRNA optimal on some mRNAs and supra optimal on others. Experimental values of the translation of α and β globin mRNA in a reticulocyte cell free system were in good agreement with the theoretical predictions, suggesting that this theory may be relevant to the situation in vivo. Messenger RNA specific factors (eg Bester et al 1975) would act, according to this model by changing the rate constant of initiation for a specific mRNA.

The rate of production of a protein may also depend on the intra cellular concentration of translatable mRNA. At a steady state, the rate of synthesis of a component is equal to the rate of degradation (dM/dt, M = component concentration). $dM/dt = KM$ where K is the rate constant of degradation. Thus the concentration of a mRNA, which is synthesised (degraded) at a constant rate will change if the rate constant of degradation is changed. The rate constant of degradation is related to the half life thus:

$K=\ln 2/T_{\frac{1}{2}}$. In a number of cell types the mRNA population contains components with different half lives, implying the existence of mRNA molecules with different rate constants of degradation. Singer and Penman (1973) compared the decay of labelled Hela cell mRNA in a chase experiment, with the decay of rRNA which was assumed to be infinitely stable. The decay plot was interpreted as showing components with half lives of 7 and 24hr. More recently, Puckett et al (1975) have produced data from Hela cells also, which is consistent with the existence of a third unstable class of mRNA with a half life of 1hr. Unpublished work on soyabean mRNA has shown 3 stability components in pulse chase experiments (J.L.Key personal communication) and in yeast there was a suggestion of 2 stability classes (Fraser 1975). A number of workers have, however, described only one stability class of mRNA. Abelson et al (1974) concluded that in resting and growing mouse fibroblasts, the mRNA decayed as a single component with a half life of 9hr. Greenberg (1972) measured the kinetics of approach to steady state labelling of mRNA in a transformed human cell line and detected one component decaying with a half life of 10 hr. However, with only a limited number of points in the kinetic analysis, such experiments are quite insensitive, and a 'single component' may be the sum total of several components (Brandhorst and McConkey 1974).

Apparently, therefore, several diverse cell lines contain mRNA classes with different rate constants of degradation. Experiments are needed to determine whether this is a fixed property of mRNA or one which may be modulated such that the decay of mRNA serves as a regulatory process. Certainly, the results of Lodish and Small (1976) suggest that this may be so. RNA from reticulocytes (which are not synthesising RNA) of different ages, was translated in vitro

in a wheatgerm S$_{30}$ and the products were analysed electrophoretically. The amount of a 64000 dalton molecular weight protein which was synthesised in vitro decreased more rapidly than globin with the increasing age of the reticulocytes from which the RNA was derived. This suggested that the ageing of the reticulocyte was characterised by an increased turnover of certain mRNAs compared to globin mRNA. In differentiating muscle cell cultures, the level of untranslated myosin messenger like RNA increased markedly just before myosin synthesis commenced. Buckingham et al (1976) suggested that this may in part have been due to reduced turnover of the myosin sequences.

There is therefore, the potential for regulation of gene expression at several levels subsequent to transcription. Such regulation is undoubtedly the result of an interaction between RNA and other cellular components. Several predictions may be made concerning 'regulatory' RNA sequences and proteins. The regulatory RNA sequences may well be associated with several messenger RNAs, so that production of functionally related proteins may be modulated with the same 'switch'. Because of this it is likely that the regulatory sequences are located outside the coding sequence. Structural features of mRNA or hnRNA which may be important in regulation include poly A tracts, internal oligo U or oligo A sequences, repeated sequence transcripts (Brawerman 1974), and methylation and 5' 'caps' (Rottman et al 1974). Regulatory proteins may interact with the regulatory RNA and be cell type specific or subject to post-translational modification. Either the modification or the protein may turnover quite rapidly so that the regulation is flexible.

In 1970, Lim and Canellakis showed that mouse globin mRNA

contained a poly A sequence. Kates (1970) showed also that vaccinia virions were associated with poly A synthesising activity and that vaccinia mRNA was polyadenylated. These observations were extended by Edmonds et al (1971) who demonstrated that polyadenylation of both mRNA and hnRNA of uninfected Hela cells. Poly A has been found on several purified animal and viral mRNAs (Brawerman 1974) and also on plant mRNA (eg Higgins et al 1973). The poly A tract has been shown to be covalently attached to the rest of the molecule (Edmonds et al 1971) and digestion with 3' exonuclease (Molloy et al 1972), periodate oxidation followed by ^{3}H borohydride reduction (Burr and Lingrel 1971), and alkaline hydrolysis followed by AMP analysis (Kates 1970) have established that it is at the 3' end of the molecule. The length of the poly A has been extensively studied. Digestion of mRNA with RNAases A and T_1 in high salt does not affect the poly A core of the molecule. Sedimentation in sucrose gradients of a poly A core prepared in this manner indicated a length of about 4S (Lee et al 1971; Darnell et al 1971), but gel electrophoresis of newly synthesised poly A isolated from the nuclear or cytoplasmic RNA of Hela cells showed the poly A to be longer than 5S RNA, ie about 200 nucleotides (Sheiness and Darnell 1973). Mouse globin poly A showed electrophoretic properties which, when compared to poly A standards, were consistent with a length of only 50 bases (Morrison et al 1973). Pulse labelled plant poly A migrated heterogenously with a mean size of 5.8S RNA in polyacrylamide gels (Key and Silflow 1975). This corresponded to a number average length of 100 nucleotides. Smaller tracts of poly A than in Hela cells were also found in Dictyostelium (Firtel, Jacobson and Lodish, 1972) and Yeast (Groner et al 1974).

In their original communication, Lim and Cannelakis (1970) showed that globin mRNA isolated from smaller polysomes contained a

shorter poly A tract, which suggested that poly A could be shortened. Sheiness and Darnell (1973) and Brawerman (1973) confirmed this, but found the shortening process to be independent of protein synthesis, as it was detected in free cytoplasmic poly A(+)RNA and in polysomal poly A(+)RNA and also in mRNA from emetine inhibited cells.

In a study of the origin of the poly A tract, Jelinek et al (1973) found that even the longest nuclear poly A was labelled after pulse periods shorter than the transcription time and that poly A synthesis was initially insensitive to actinomycin D. This suggested nuclear, post-transcriptional synthesis of poly A. No free adenylic acid polymers could be detected, so a polymerisation reaction rather than a stepwise addition of oligo A seemed more likely. The necessary enzyme for this, a primer dependent nucleotidyl (AMP) transferase has been found widely in eukaryotic cells. Although polydA:dT sequences are present within the duck genome, Bishop et al(1974) concluded that such sequences were insufficient to account for poly A by direct hybridisation. However, in Hela cells, Nakazato et al (1974) reported an Actinomycin D sensitive, cordycepin insensitive addition to Hela cell HnRNA. Lodish et al (1974) concluded that the genome organisation of Dictyostelium was consistent with there being a transcribed poly A sequence 20 nucleotides long in each mRNA. A procedure involving differential binding to oligo dT cellulose and poly U filters enabled Dubroff and Nemer (1975) to fractionate early blastula hnRNA from sea urchin into subclasses of poly A containing molecules. Alkaline hydrolysis of the poly A core from one of these fractions gave an AMP/adenosine ratio 35 times greater than predicted for a 3' terminal polymer the length of this core (25 bases). The poly A from this subclass of hnRNA was deduced to be internal and therefore transcribed. Thus, there is evidence for transcriptional and post-transcriptional origin of poly A synthesis. There is also

evidence for cytoplasmic poly A synthesis. Slater et al (1973) estimated the amount of poly A in the nucleus and cytoplasm of developing sea urchin emrbyos by ^{3}H poly U hybridisation. In early embryogenisis there was a net synthesis of poly A on cytoplasmic RNA during a period of nuclear inactivity. More recently Diez and Brawerman (1974) noted that in actinomycin D inhibited Chinese Hamster cells, the initial rate of radioactivity accumulation in cytoplasmic poly A was greater than could be accounted for by apparent nuclear turnover of poly A. Nucleotide/nucleoside ratio analysis showed cytoplasmic poly A synthesis in mouse sarcoma cells to involve the 3' terminal addition of 8 bases. Vesicular stomatisis virus mRNA has a 3' poly A tract and is formed by copying the virion RNA which lacks any poly U sequences. A cytoplasmic extract of V.S.V. infected cells contains a poly A synthesising enzyme (Galet and Prevec, 1973). There is therefore the potential for: a) transcription of oligo A; b) post-transcriptional nuclear synthesis of poly A; c) cytoplasmic synthesis of poly A and d) cytoplasmic degradation of poly A.

On the basis of pulse-chase experiments in which polyadenylation during the chase was inhibited by cordycepin, Jelinek et al (1973) concluded that most of poly A(+)hnRNA was destined to become mRNA. It was proposed that polyadenylation of hnRNA regulated the nucleocytoplasmic transport of hnRNA (Darnell et al, 1973). Very similar results to those of Jelinek et al (1973) were interpreted by Latorre and Perry (1974) as showing that approximately half of the nuclear poly A turned over within the nucleus in a cordycepin 'chase'. The experiments of Getz et al (1975) and Levy and McCarthey (1976) (see page 6) showed that the complexity of poly A(+)hnRNA and abundance of poly A(+)mRNA sequences in poly A(+)hnRNA were such that there must be substantial intra-nuclear turnover of poly A(+)

hnRNA. Hela cells (Milcarek et al, 1974) and sea urchin embryos (Nemer et al, 1974) contain substantial amounts of poly A(-)mRNA. Histone mRNA lacks poly A (Adesnik et al, 1972). It is therefore unlikely that polyadenylation controls the nucleocytoplasmic transport of mRNA. Since even cytoplasmically produced viral mRNA is polyadenylated (Galet and Prevec, 1973), a cytoplasmic role for the poly A is more likely.

Several workers have investigated the effect of poly A on the translation of mRNA. Bard et al (1974) analysed the rate of initiation of protein synthesis on new (^{3}H labelled) with a long poly A tract and old (^{32}P labelled) mRNA with a short poly A tract after *in vivo* polysome dissociation by heat shock. The ^{3}H : ^{32}P ratio in the polysomal mRNA remained constant showing that the rate of reinitiation of protein synthesis was independent of the length of poly A. Williamson et al (1974) showed that in a Krebs Ascites cell free protein synthesising system there was no difference in the translation of native mRNA and mRNA which had been deadenylated using polynucleotide phosphorylase. This may however have been due to the low efficiency of the Krebs Ascites cell free system, as Doel and Carey (1976) have shown poly A(-) ovalbumin mRNA to translate less efficiently in a reticulocyte lysate protein synthesising system than poly A(+) ovalbumin mRNA. In sea urchin embryos there may be a difference in the translational efficiency of poly A(+) and non histone poly A(-) mRNA *in vivo* as Nemer et al (1975) have shown that the non histone poly A(-) mRNA was less loaded with ribosomes than the poly A(+)mRNA. Bester et al (1975) have devised a novel scheme of translational regulation in the developing myoblast which involves poly A and a small ribonuclease sensitive molecule isolated from the subribosomal fraction of cultured developing myoblasts. This RNA inhibited translation

in vitro of poly A(+)RNA from the subribosomal fraction, but not of poly A(+)mRNA from the polysomes. This inhibition was dependent on the mRNA being polyadenylated (Heywood and Kennedy, 1976). This translational control RNA(tcRNA)was rich in UMP and it was suggested (Bester and Heywood, 1975) that tc RNA interacted with poly A(+)RNA. The UMP rich region may have bound to the poly A and the rest of the tcRNA may have associated with another region of the mRNA molecule, probably at the 5' end such that initiation was not possible.

Several workers have looked for an effect of poly A on mRNA stability. In sea urchin embryos, the ratio of poly A(+) : non histone poly A(-)mRNA did not change during a pulse-chase experiment (Nemer et al, 1975) and in Hela cells, this ratio remained constant over a long period of labelling. These results suggested that poly A had no effect on mRNA stability. However, when Xenopus oocytes were injected with either poly A(+) globin mRNA or poly A(-) globin mRNA, then globin was produced for a longer period in the oocytes containing the poly A(+)mRNA (Huez et al 1974). By using cDNA to assay globin mRNA in oocytes, it was found that poly A(-) globin mRNA was degraded more rapidly than poly A(+) globin mRNA (Marbaix et al, 1975). It was shown further, that readenylation of the poly A(-)mRNA restored its stability and that the stability was related to the length of the restored poly A tract above a threshold length of 16 bases (Nudel et al, 1976). The results of Hieter et al (1976) who found that 3' poly A affects the RNAase sensitivity of RNA are quite consistent with an effect of poly A on mRNA stability. They found that endoribonuclease was reversibly inhibited by 3' poly A to an extent dependent on the length of the poly A. Jeffrey and Brawerman (1975) have demonstrated a weak interaction between

poly A and the 5' terminus of mRNA which may confer resistance to exonucleases onto the mRNA.

A second type of postranscriptional modification of mRNA involves methylation of base and ribose moieties (Perry and Kelley 1974). This modification has now been observed in several types of animal mRNA (Adams and Cory 1975; Furuichi et al 1975) and animal and plant viral mRNA (Roman et al 1976). Methylation is associated with an unusual 5' structure, which due to resistance to alkali and 5' exonuclease has been designated a 'cap'. Rottman et al (1974) described this cap in many mRNAs as a structure in which the terminal 5' 7 methylguanosine is joined via the 5' hydroxyl in a triphosphate linkage to the 5' hydroxyl of an O-methylated riboside, to give the structure $m^7G5'pppNmpNp$. Messenger RNA of Newcastle disease virus (Colonno and Stone 1976) or Dictyostelium (Dottin et al 1976) may not be O-methylated on the penultimate 5' nucleoside. Knowing the number average size of mRNA and the molar amount of 5' caps in a mRNA sample, it was estimated that in mouse myeloma (Adams and Cory 1975) and in Hela (Salditt-Georgieff et al 1976) cells, all of the mRNA was capped. However, poliovirus mRNA (Nomoto et al 1976) and satellite tobacco necrosis virus (STNV) mRNA (Roman et al 1976) are uncapped.

HnRNA is also capped, although less extensively than mRNA (Salditt-Georgieff et al 1976) and the 5' capped termini are similar in the nucleus and cytoplasm (Perry et al 1976). McGuire et al (1976) showed in adenovirus infected cells, that caps were present only on the fraction of viral hnRNA which showed a precursor-product relationship with viral mRNA. Caps on the cytoplasmic viral RNA were found only on the polysome associated sequences. This result suggests quite strongly that messenger sequences are capped in the nucleus and that the cap function is related to the

translation of mRNA. A number of recent papers have suggested that caps are involved in the formation of initiation complexes. Muthukrishnan et al (1975) compared the in vitro protein synthesis activity of capped reovirus mRNA and 'de capped' reovirus mRNA in a wheatgerm S-30 system. The latter was only 15% as efficient in stimulating protein synthesis as the capped mRNA. When S-adenosyl methionine was included as a methyl donor in the mixture only the 5' ppG termini of the decapped molecules became recapped. The main product of the decapping reaction which was 5' $pppG^{m}$ was largely unaffected and the extent of recapping paralleled exactly the restoration of protein synthesis activity in the wheatgerm system. A detailed analysis by Both et al (1976) studied the formation of 40S and 80S initiation complexes with synthetic polyribonucleotides. 40S complex formation was dependent on a 5' cap next to a UMP rich sequence. 80S complex formation required an AMP-UMP rich sequence next to the 5' cap. This is especially interesting in the light of the finding that the 5' terminus of Brome Mosaic Virus mRNA, the only eukaryotic mRNA studied in this respect, contains an AMP-UMP rich region between the cap and the initiation codon (Dagsuptra et al 1975). It is tempting to compare this with the situation in bacteria where there may be some homology between the ribosome binding site on mRNA and the 3' terminus of 16S rRNA (Shine and Dalgarno 1975). Filipowicz et al (1976) demonstrated a cap binding activity in the salt wash of Artemia embryo ribosomes. The activity bound m^{7}pppGmc in preference to analogues lacking methylation of G and could be competed with methyl capped mRNA but not uncapped (STNV) or methyl poor mRNA. It is possible that the initial reaction in initiation complex formation involves alignment of complementary (AMP-UMP rich?) sequences on mRNA and 0.7 x 10^{6} rRNA to form a

40S initiation complex. An interaction between the cap and the cap binding protein might then mediate the formation of an 80S initiation complex. A change in the activity of the cap binding protein might affect the rate constant of initiation of protein synthesis and possibly result in a changed spectrum of protein synthesis (see Lodish 1974, page 8). The report of Roman et al (1976) is quite consistent with the proposed basic mechanism. They found that 7-methyl guanosine inhibited in vitro protein synthesis of capped reovirus mRNA, but not capless STNV mRNA, thus showing that the inhibition was related to the m^7G-cap structure. This analogue had no effect on formation of 40S-met $tRNA_I^{met}$ complex formation but inhibited formation of the message dependent 80S-met $tRNA_I^{met}$ complex. Translation of V.S.V. mRNA in vitro did not depend entirely on 5' capping and presumably initiation of protein synthesis on capless mRNA is via a different mechanism. (Rose and Lodish, 1976)

Base methylation of adenosine has been demonstrated in mRNA and hnRNA (Adams and Cory 1975; Furuichi et al 1975). By analysing the 6 methyl adenosine content of partially cleaved mRNA molecules which had been fractionated into poly A(+) (3' of native mRNA) and poly A(-) (5' of native mRNA) fragments, the 6mA was located at the 5' end. Di and trimethyl nucleosides have been detected in large hnRNA (Salditt-Georgieff et al 1976).

If any transcribed sequences are of regulatory significance and if these sequences fulfil the predictions for regulatory sequences (page 11), they may be detected by DNA-RNA hybridisation as repeated sequence transcripts (r.s.t.). If DNA is denatured and renatured in the presence of trace amounts of radioactive homologous mRNA, the rate of mRNA hybridisation is related to the repetition of the sequence coding for the mRNA. Greenberg and

Perry (1971) assayed the amount of hybridisation by digestion with ribonuclease and concluded that 80% of L cell mRNA was transcribed on 'non-repeated' DNA, and Goldberg et al (1973) similarly detected that sea urchin mRNA was nearly all 'non-repeated' DNA transcript. Campo and Bishop (1974) separated alkali cleaved rat liver mRNA into poly A(+) (ie 3' native mRNA) and poly A(-) (ie 5' native mRNA) fragments and found that the small r.s.t. component was not enriched in either of these fractions. They concluded therefore that the r.s.t. in mRNA was a separate class of molecules and not a regulatory region of some molecules. Dina et al (1974) however, concluded that r.s.t. were located at the 5' terminus of all Xenopus mRNA. After only a short hybridisation period, the DNA excess reaction mixture was fractionated into molecules with a hybridised region and unhybridised molecules by passage through hydroxylapatite. All of the mRNA apparently contained r.s.t.. When 5' labelled and randomly labelled mRNA were hybridised to DNA and the hybridisation assayed by ribonuclease digestion, the 5' sequences nearly all hybridised rapidly ('Cot'=100), but the randomly labelled molecules had not completely annealed to DNA at a 'Cot' of 10^4 (Cot = concentration x time). This result suggested that the r.s.t. component of Xenopus mRNA was at the 5' end of the molecule. Davidson and Britten (1974) have shown that the length of Hela cell mRNA is no greater than expected for the size range of Hela proteins and so the extent of any r.s.t. regulatory regions of the mRNA must be quite limited. Of course there is no reason to expect regulatory sequences to be long, and it may be necessary to use RNA sequencing to study short regulatory sequences which would not be detected by DNA-RNA hybridisation. Proudfoot and Brownlee (1976) have sequenced the 3' termini of 4 different

mRNAs and have shown considerable homology between these, including a AAUAA sequence common to all.

Repetitive sequence transcripts are a more prominent component of hnRNA. HnRNA of L cells contains 32% r.s.t. (Greenberg and Perry, 1971) and in Dictyostelium there is at least 25% r.s.t. in hnRNA (Firtel and Lodish, 1973). Smith et al (1974) measured the amount of sea urchin hnRNA complementary to excess DNA by binding to hydroxylapatite. 25% of the hnRNA had hybridised at low Cot, but hnRNA had degraded during the hybridisation. Allowing for this it was possible that each hnRNA molecule contained an r.s.t. sequence.

HnRNA from HeLa cells (Jelinek and Darnell, 1972) and Ehrlich carcinomas (Ryskov et al, 1973) contains an RNAase resistant component which may be double stranded and which is complementary to repetitive DNA. In HeLa cells the 'double stranded' regions are located at the 5' end of hnRNA (Molloy et al, 1974) and are absent from mRNA. The r.s.t. of Dictyostelium hnRNA are also at the 5' end (Firtel and Lodish, 1973). A large proportion of the genome of animals (Lewin, 1974) and also a plant (Walbot and Dure, 1976) is made up of 'unique' sequences approximately 1000 base pairs long (the length of mRNA) interspersed with 'repeated' sequences. In sea urchin DNA, Davidson et al (1975) have demonstrated that this arrangement applies to structural genes. Thus, transcription of hnRNA is probably initiated in repeated sequences and runs into the gene.

In all organisms studied so far, the hnRNA is larger than the mRNA although the extent of this size difference is far from clear, due to the marked tendency of hnRNA to form aggregates. HnRNA of Dictyostelium (Firtel and Lodish, 1973) and artichoke RNA (Chapman and Ingle, 1976) is only fractionally larger than mRNA

and the size distributions of hnRNA and mRNA overlap. In many animal cell types the hnRNA appears very large with an apparent molecular weight of up to 20 x 10^6 daltons (Imaizumi et al 1973) even in denaturing,DMSO gradients. Schminke et al (1976) showed that the number average size of pulse labelled Ehrlich Ascites cell hnRNA in formamide gels was 0.9 x 10^6, which was in good agreement with the number average molecular weight estimated by pppNp determination. This suggests that the pulse labelled hnRNA was the primary transcript and that analysis of hnRNA is valid. It is possible that in denaturing conditions a hidden break like that between 5.8S and 28S RNA (Pene et al 1968) would open when the hnRNA was denatured and the size of hnRNA would be underestimated. These large hnRNA molecules contain sequences complementary to the smaller cytoplasmic mRNA. Imaizumi et al (1973) detected globin sequences in >28S hnRNA of duck erythroblasts and McNaughton and Bishop (1974) located nuclear globin sequences in a 14S peak on formamide gradients. HnRNA containing adenovirus 2 sequences is certainly larger than the cytoplasmic mRNA (Bachenheimer and Darnell 1975), but this is an example of a polycistronic transcript. Using E co RI fragments of the adenovirus genome it was shown that longer hnRNA contained all adenovirus sequences and that short hnRNA contained only those near to the 3' end of the genome. It is not known whether non viral genomes are transcribed into polycistronic molecules. In order to reconcile the size difference between hnRNA and mRNA, several authors (eg Lewin 1975) have proposed that mRNA is derived by cleavage of large hnRNA which may involve loss of r.s.t.. It has long been known that rRNA in animals (Perry 1969) and plants (Leaver and Key 1970) is cleaved out of a large precursor molecule in a process involving intra nucleolar turnover

of 'spacer' transcripts. However, McKnight and Schimke (1974) using a cDNA copy of ovalbumin mRNA could not detect any ovalbumin sequences larger than the ovalbumin mRNA in total oviduct RNA, even under hybridisation conditions which would detect 1 molecule per cell. This implied that the primary transcript of the ovalbumin gene was similar in size to ovalbumin mRNA.

Spirin (1969) introduced the concept that messenger like RNA of eukaryotic cells is associated with protein. If there are any protein-RNA interactions of regulatory significance one might expect them to be contained in these ribonucleoproteins. Free cytoplasmic messenger like RNA exists in rat liver (Henshaw and Loebenstein, 1970), in plant cells (Ajtkhozin et al, 1973) and in avian erythroblasts (Gander et al, 1973) as RNP called informosomes. In Avian erythroblasts the informosomes contain globin specific sequences. Informosomes are generally characterised by a low buoyant density (~1.4g cm^{-3} compared with 1.5-1.6g cm^{-3} for ribosomes) after aldehyde fixation and centrifugation in CsCl. The RNA and proteins of informosomes are electrophoretically distinct from nuclear ribonucleoproteins and from ribosomes.

When polysomes were dissociated with puromycin (Bryan and Hyashi, 1973) EDTA (Olsnes, 1970) or heat stock (Schoechman and Perry, 1972), mRNA was released as a RNP (messenger RNP-mRNA). The mRNP have similar buoyant density properties to informosomes but contain different proteins to informosomes (Gander et al, 1973). The role of the proteins in mRNP is not clear. Chen et al (1976) found that lens crystallin and globin mRNP were translated *in vitro* in a wheatgerm S-30 system with the same efficiency as mRNA. However, a different result may have been obtained if a more efficient cell free protein synthesis system had been

used (c.f. Doel and Carey 1976). The protein of mRNP invariably includes a protein of molecular weight 73 to 78 x 10^3 daltons, which Blobel (1973) and Brawerman and Kwan (1972) have shown to be associated with the poly A core of mRNA. Gander et al (1973) showed that a protein of molecular weight 73000 associated with duck erythroblast mRNP was phosphorylated in some of the serine residues. Phosphosylation of a protein is a post-translational modification which often affects the biological activity of the proteins (Trewavas 1976). Depending on the salt concentration during isolation, various other proteins are associated with mRNA. Using affinity chromatography on oligo dT cellulose to isolate mRNP, Lindberg and Sundquist (1974), demonstrated a high molecular weight mRNP protein which was specifically associated with adenovirus infection of KB cells.

HnRNA is also associated with protein to form complexes with a similar buoyant density to informosomes (Samarina et al 1973). By adding trace amounts of deproteinised hnRNA to the extraction buffer, Pederson (1974i) showed that the hnRNP are not artifacts. In vivo the hnRNP were closely associated with chromatin (Kimmel et al 1976), but by examining the distribution of radioactive hnRNP proteins after stimulation of RNA synthesis, Pederson (1974ii) showed that they were functionally distinct from chromatin non-histone proteins. Samarina et al (1973) concluded that the major protein component of hnRNP had a molecular weight of 40000 daltons. Other workers have found a more diverse protein complement in hnRNP (Neissing and Sekeris 1971) even using the methods of Samarina et al. In Dictyostelium, Firtel and Pederson (1975) demonstrated a protein of molecular weight 72000 dalton associated with poly A and Pederson (1974i) showed the protein complement of hnRNP to vary with the tissue and organism of origin. Niessing and Sekeris (1973) found

endonucleolytic and ribohomopolymer synthetase activity on hnRNP, which perhaps suggests an involvement of hnRNP in the processing of hnRNA. Price et al (1974) found a 74S hnRNP which was extracted from chromatin in high salt. This hnRNP contained hnRNA which was enriched for poly A(+) molecules compared with total hnRNA and which turned over rapidly. These are both properties which are predicted for a nuclear pre-mRNA fraction.

There is considerable potential for regulation of eukaryotic gene expression at several post-transcriptional levels. HnRNA and mRNA contain RNA sequences which may well be involved in this regulation and they interact with proteins in RNP. In this project the possible biological activity of one of the features of artichoke mRNA, the poly A tract, has been studied. The work has involved characterisation of mRNA and a kinetic study of the turnover of mRNA and hnRNA. The experiments involving hnRNA were carried out in collaboration with Dr K. Chapman. Cultured artichoke explants have been used in this department for several investigations, so the basic growth characteristics were well defined. Hepburn (1974) had already shown that some cytoplasmic heterodisperse RNA was polyadenylated and that some did not bind to oligo dT cellulose.

METHODS

1) Growth of Tissue

Artichoke (Helianthus tuberosus var. Bunyards Round) plants derived from a single clone, were grown in the departmental garden. Tubers were harvested each winter and stored in moist sand at 0-4°C. Under these conditions the tubers remained usable until the following winter. Tubers were selected for uniformity of shape and size, freedom from lesions and were then scrubbed clean. After surface sterilization for 20 to 30 minutes in 20% sodium hypochlorite (2 to 3% available chlorine) the tubers were rinsed thoroughly in sterile distilled water. This and subsequent preparation was performed in a sterile room which was maintained at a slight positive pressure and sterilised between usage by U.V. radiation. Culture medium in the roller bottles, and tissue paper had been previously sterilised by autoclaving. Metallic instruments and foil tops for the roller bottle had been heated at 150°C for at least 4 hours. The ends of the artichoke tubers were removed and cylinders of tissue were cut parallel to the longitudinal axis of the tuber with a canula (diameter 2mm). The cylinders were placed on a ridged block and cut into 2.4mm lengths using a series of mounted razor blades. The explants were then transferred to roller bottles, which were then sealed with sterile foil caps. All manipulations were carried out under a green safe sight, as exposure of the explants to red light results in inhibition of the cell division capacity of the cultures (Fraser 1968). Cultures were grown in either 7.5 or 15.0ml of medium in 250 or 500ml roller bottles with 10 explants per ml. of medium. Table 1 shows the final composition of the medium. Solutions A and B were stored cold at 100 times final concentration. 2,4-D was stored in ethanol at 1000 times final concentration and was omitted from the medium only when stated.

TABLE I - THE COMPOSITION OF ARTICHOKE EXPLANT CULTURE MEDIUM

Solution Code	Component	Final Concentration
A	$MgSO_4,7H_{20}$	0.146mM
	KNO_3	0.800mM
	KCl	0.880mM
B	$FeCl_3$	0.006mM
	$Ca(NO_3)_2$	1.440mM
2,4-D	2,4-dichlorophenoxy acetic acid	10^{-6}M
Sucrose	Sucrose	4%

Radioactive label (Amersham) and other substances were introduced into the culture bottles with a sterile syringe through the foil cap. The hole was sealed with autoclave tape. ^{3}H uridine was introduced carrier free. ^{32}P phosphate was added with carrier phosphate (pH 6.0, 20^{o}C) at the concentrations shown.

The number of cells per explant was determined by digesting 5 explants for 2 to 3 days in 3ml of chromium trioxide at 0^{o}C. The tissue was then macerated by repeated passage through a pasteur pipette and the uniform suspension was introduced into the cavity of a Fuchs-Rosenthall haemocytometer slide. 5 grids from each of two replicate samples were counted, and the cell number per explant calculated, knowing that the volume of the haemocytometer slide was 3.2µl.

2) Polysome Preparation

Explants were harvested and rinsed with ice cold distilled water. Homogenisation was in five volumes of a high Tris, high pH buffer

modified from Davies et al (1974), (200mM Tris-HCl (pH 8.5, 0°C, 50mM KCl, 10mM M_9Cl_2, 0.3M sucrose, 10mM dithiothreitol (Koch Light), 1mg ml^{-1} polyvinylpyrollidone (Sigma). All operations were carried out in a cold room at 0 to 4°C using pre-cooled apparatus. Initial homogenisation was in a pestle and mortar, followed by two half turns in a Dounce conical glass in glass homogeniser. A more rigorous homogenisation caused breakage of nuclei and contamination of the post mitochondrial supernatent with nuclear RNA. The homogenate was then made to 2% final concentration Triton X100 by addition of a 20% stock solution, stirred and filtered through 1 layer of miracloth (Calbiochem). The filtrate was centrifuged for 7 min. at 13000rpm in an MSE 8 x 50 rotor at 0°C, in order to pellet cell debris, nuclei and mitochondria. The supernatant was removed using a wide bore 25ml pipette and layered over 1 ml of 200mM Tris-HCl (pH 8.5 0°C) 50mM KCl, 10mM Mg Cl_2, 1M sucrose, via a 10ml syringe. The needle of the syringe passed through the central hole of the centrifuge cap which had already been tightened onto the tube. 7.7ml of supernatent were thus allowed to flow into the tube under gravity and the tube was sealed. The advantage of this method of filling centrifuge tubes was that several tubes could be filled in minimal time without disturbing the sucrose cushion. Ribosomes and other RNP were recovered from the 13000rpm supernatant by centrifugation at 65000rpm (300,000g av.) for the indicated time period in an MSE 10 x 10 Ti rotor. This ribosome pellet was suspended in either 20mM Hepes (pH 8.0, 0°C) or 10mM Tris-HCl (pH 8.5, 0°C) 50mM KCl, 10mM Mg Cl_2 (= TKM buffer). The suspension was clarified by centrifugation at 10000rpm for 5 min. in an MSE 10 x 50 rotor. The E_{260nm} of the clarified supernatant was assayed by diluting an aliquot to 1.0ml and measuring the U.V. absorbance in a Pye Unicam SP8000 spectrophotometer. Up to 10 OD_{260nm} in 0.2ml were loaded onto a 10 to 60% sucrose gradient in TKM and centrifuged

at 40000rpm for 70min in an MSE 6 x 14 rotor at 0°C. Alternatively a sample containing up to 20 OD_{260nm} in 1.0ml was centrifuged through 10 to 60% gradient in an MSE 3 x 25 rotor at 28000rpm for 2 hr 20 min at 0°C. Sucrose gradients were preformed by layering sucrose solutions of decreasing density and allowing to stand for several hours before use. Gradients were pumped out by upward displacement with dense sucrose (70%) or "Fluorinert FC-43" (I.S.C.O.) through an LKB 'Uvicord' with a 254nm filter. The E_{254nm} was recorded on a Vitatron potentiometric recorder. Gradients were pumped out at either 100ml hr^{-1} (14ml gradient) or 160ml hr^{-1} (25ml gradient).

3) Ribosome Dissociation

a) EDTA

The ribosome pellet was suspended in the centrifuge tube with a close fitting PTFE pestle in 100mM Tris-HCl, 50mM KCl, 15mM EDTA (disodium ethylenediamine tetracetic acid) pH 8.5 0°C (TKE buffer) containing 0.2% diethyl pyrocarbonate (DEP, Sigma) which was added immediately before use of the solutions. After standing the suspension on ice for five minutes, the sample was layered over a 10 to 35% sucrose gradient (in TKE) and centrifuged at 25000rpm in an MSE 3 x 25 rotor for 16hr at 0°C. In some experiments, a 10 to 50% gradient was centrifuged for 16hr at 40000rpm at 0°C in an MSE 6 x 14 rotor.

b) Puromycin - KCl

The ribosome pellet was resuspended in 10mM Tris-HCl (pH 8.5 at 0°C) 500mM KCl, 10mM $MgCl_2$, allowed to stand on ice for 10 minutes and clarified by centrifugation. One tenth volume of 10mM puromycin-diHCl (Sigma) neutralised to pH 7.5 with KOH was added and the suspension was incubated for 10 min at 37°C. For analysis by sucrose gradient centrifugation, the sample was then layered over a 10 to 35% sucrose gradient in 10mM Tris-HCl (pH 8.5 0°C), 500mM KCl, 10mM $MgCl_2$ buffer

and centrifuged at 25000rpm for 16hr at 0°C in an MSE 3 x 25ml rotor.

c) MDMP Dissociation

0.75ml of 10^{-2}M DMDMP (2-(4- methyl- 2,6 dinitroanilino-N-methyl propropamide) was added to a 250ml roller bottle culture containing 7.5ml of medium, 10 min before harvesting. The explants were collected, homogenised as described, and the RNP pelleted by centrifugation of the post-mitochondrial supernatant through 1M sucrose for 6hr at 65000rpm (MSE 10 x 10Ti rotor). The pellet was resuspended in 20mM Hepes for centrifugation through sucrose or in Metrizamide gradients.

4) Buoyant density centrifugation of artichoke RNP

a) CsCl

RNP samples were fixed in aldehyde (Spirin, 1965). Tris,which must be avoided in the presence of aldehydes (Hamilton, 1971) was replaced with triethanolamine-HCl (Sigma). Otherwise ribosome pellets were resuspended and dissociated as described. Tris and sucrose were removed from sucrose gradient fractions by extensive dialysis of the sample against an equivalent buffer lacking sucrose and containing triethanolamine-HCl in place of Tris-HCl. Alternatively, the RNP was pelleted from the diluted sample at 65000rpm for 6hr (0°C MSE 10 x 10Ti rotor) and resuspended in a triethanolamine-HCl containing buffer. Samples were fixed at 0°C for at least 16hr in 6% formaldehyde, which had been neutralised with bicarbonate.

The CsCl gradient was either formed during centrifugation or was pre-formed by layering. In the former case, Brij 58 and CsCl were added to form a solution of density 1.320g cm^{-3} (0.2% Brij 58). This solution was layered over an equal volume of CsCl solution (density 1.68g cm^{-3}) containing 0.2% Brij 58 and 4% formaldehyde and centrifuged as described in the figure legends. The density of CsCl solutions was determined by the relationship:

$P = 10.8601\ n_D^{25} - 13.4974$ (n_D^{25} = refractive index at 25°C)

The refractive index of the solution was corrected for salt and formamide in the buffer. Preformed gradients were made by layering CsCl solutions of density 1.4, 1.5, 1.6, 1.7g cm^{-3}, in buffer and containing 0.2% Brij 58 and 4% formaldehyde, which were left to stand for several hours. The fixed RNP was layered in 200µl over the top layer and the gradients were centrifuged at 25000rpm for 40hr at 0°C in an MSE 6 x 5.5 rotor. Both methods of gradient formation produced similar profiles. After centrifugation, gradients were fractionated by upward displacement with Fluorinert FC-43. 5.5ml gradients were pumped out at 40ml hr^{-1}.

b) Cs_2SO_4

Dissociated and undissociated samples were centrifuged unfixed in Cs_2SO_4 gradients. The ribosome pellets were resuspended and dissociated as described. Gradients of Cs_2SO_4 (1.35 to 1.65g cm^{-3}) were preformed by layering and the sample in 200µl put on the top layer in 200µl. The density of Cs_2SO_4 was determined by refractometry at 25°C using a standard curve prepared from the data of Ludlum and Warner (1965). Refractive index readings were corrected for the salt content of the buffer. The centrifugation was at 35000rpm for 40hr (0°C) in an MSE 6 x 5.5 rotor.

c) Metrizamide

Solutions of Metrizamide (2-(3 acetamido-5-N-methyl acetamide -2,4,6-tri iodobenzamido)-2-deoxy-D-glucose (Nyegaard) form a density gradient during centrifugation in a fixed angle rotor. (Rickwood and Birnie, 1975). The ribosome pellets were resuspended and dissociated as described and Metrizamide added to form a solution of density 1.22g cm^{-3}. The density of Metrizamide solutions was determined by refractometry at 20°C using the relationships : $P = 3.453\ n_D^{20} - 3.601$, and making a correction for the contribution of the buffer.

Metrizamide solutions are light sensitive, so care was taken to avoid unnecessary exposure to strong light. The ribosome preparations in Metrizamide (5ml) were overlayed with DEP treated liquid paraffin and centrifuged in an MSE 10 x 10 Al rotor at 35000rpm for 68 hr. Preformed gradients were prepared by layering and the ribosome pellet preparation in 200µl was layered over the top layer. Gradients were centrifuged at 40000rpm for 40hr at $0^{o}C$ in an MSE 6 x 5.5 rotor.

5. RNA Preparation

Gradient fractions were diluted and the RNP collected by centrifugation at 65000rpm for 6hr ($0^{o}C$). All RNA containing pellets were treated in the same way. The pellets were resuspended in 10mM Tris-HCl (pH 8.0), 50mM KCl, containing 6% sodium paraamino salicylate (P.A.S.) and 1% sodium tri-iso-propylnapthalene-sulphonate (TNS. Eastman-Kodak), then shaken with an equal volume of phenol mixture. The phenol mixture contained 15% redistilled m-cresol, 0.1% 8-hydroxyquinoline and phenol saturated with 10mM Tris-HCl(pH 8.0.) The mixture was centrifuged at 2500g (MSE Mistral 4L) for 10 min and the top aqueous phase reextracted with phenol. After a second centrifugation, the aqueous phase was removed and the RNA precipitated by the addition of 2 volumes of ethanol and storage overnight at $0^{o}C$. The RNA was collected by centrifugation (2500g 10 min) washed twice with 80% aqueous ethanol 0.2% SLS (sodium lauryl sulphate BDH 'specially pure'), redissolved in 0.15M sodium acetate (pH 6.0) 0.5% SLS and reprecipitated by the addition of 2 volumes of ethanol and storage at $-20^{o}C$ for more than 2hr. This procedure extracted all poly A containing RNA (Hepburn 1974) without the use of high pH buffers (Brawerman et al 1972) due to the inclusion of detergents. Total nucleic acid was prepared from intact explants, by homogenisation in a pestle and mortar in 5 volumes of PAS/TNS buffer, with subsequent phenol deproteinisation

Cold buffers and precooled apparatus were used. After a second phenol extraction, the aqueous phase was removed and re-extracted with phenol. The nucleic acid was precipitated and washed as described above. In some experiments, when the RNA from gradient fractions was to be used only for polyacrylamide gel electrophoresis, SLS (0.5% final concentration) was added to the fraction and the RNA directly precipitated by the addition of two volumes of ethanol.

6) Poly acrylamide gel fractionation of nucleic acid

The method of Loening (1967) was used. Acrylamide and bis acrylamide (both Eastman-Kodak) were purified by recrystallisation. 70g of acrylamide were dissolved in 1l of chloroform at 50°C and the solution was filtered. After cooling to -20°C, the crystals were transferred to a cold filter funnel and washed with cold chloroform and heptane, then dried. Bisacrylamide was recrystallised from acetone (10g l^{-1} 45°C) by cooling to -20°C. The crystals were collected and washed with cold acetone. Solid acrylamide and bis acrylamide were handled in draught free rooms and great care was taken to avoid skin contact or inhalation.

For dilute gels (2.3 or 2.4%) a stock solution of 15% acrylamide and 0.75% bisacrylamide was made and stored at 0°C in darkness. For 7.5% gels a stock solution of 30% acrylamide and 0.375% bis acrylamide was used. Stocks were diluted to the required concentration with water and 1/5 final volume of 5x electrophoresis buffer (5 x 'E' buffer was 150mM sodium dihydrogen orthophosphate, 80mM Trizma base, 5mM EDTA (final pH 7.6 to 7.8). This mixture was degassed for 20 to 30 sec. with gentle swirling. Freshly prepared 10% ammonium persulphate and TEMED (N, N, N^1, N^1 tetramethylethylene diamine) were added (0.25ml and 25µl respectively for 5ml of stock acrylamide) and 2.7ml of the solution was pipetted into a 9cm long plexiglass tube (0.6cm

internal diameter) which was stoppered at one end with a plastic support ring and a short length of narrow glass rod. The top of the gel was carefully overlayed with water to ensure a flat surface, and to prevent air contact with the acrylamide. Ammonium persulphate and TEMED concentrations were reduced by half for 7.5% acrylamide gels. The gels were polymerised for 2hr at 25°C, after which the gels and then pre-electrophoresed in 'E' buffer for 30 min containing 0.2% SLS (50V 6mA $tube^{-1}$) to run SLS into the gel and to remove the catalysts. The RNA was dissolved in 'E' buffer containing 0.2% SLS and 10% sucrose. Up to 75µg of nucleic acid in less than 100µl were loaded onto the gels under a low voltage. Electrophoresis at 50V, 6mA $tube^{-1}$ at room temperature was for the period indicated in figure legends. sRNA was electrophoresed off the end of the 2.3% gels after 2hr 15min. After electrophoresis the gels were removed from the tubes and soaked in distilled water for 30min to 2hr. When estimation of the low molecular weight RNA was required, the washing step was omitted since sRNA rapidly diffuses out of 2.3% and 2.4% gels. The gels were scanned at 7.5 cm length in a quartz cell in a Joyce Loebl U.V. scanner. The light source was a medium pressure mercury lamp with wave-length selection through a freshly prepared liquid filter of p-dimethylaminobenzoic acid (PDAB) in methanol (100µg ml^{-1}). The absorbance at 265 nm was recorded by a 'Servoscribe' potentiometric recorder which was geared to give scans 1 x, 2 x and 4 x the gel length. The recording was linear up to 5V f.s.d.

The recording system had been calibrated by electrophoresis of known amounts of RNA on gels which were scanned at 4 x gel length. The peak areas were measured by cutting out and weighing the tracings of the peaks. The amount of RNA loaded was calculated from the absorbance of the sample (25 OD_{260} units mg^{-1} RNA). If standard tracing paper was used, at 5V f.s.d. and 4:1 scan, 1mg of paper was equivalent to 0.357µg RNA.

7) Isolation of poly A(+)RNA

a) Oligo dT cellulose

Oligo dT cellulose (Collaborative Research T2) was washed with ten volumes of elution buffer (10mM Tris-HCl, 10mM EDTA (pH 7.5) 0.05% SLS) and ten volumes of load buffer (10mM Tris-HCl, 500mM NaCl, 10mM EDTA, (pH 7.5) 0.05% SLS) and packed under gravity in 1ml or 2ml polypropylene syringes with a pad of Whatman GFC in the bottom. RNA was dissolved in load buffer and passed twice through the column (1mg of total RNA per ml of oligo dT cellulose). More than 90% of the unbound RNA was present in the first 3ml after the sample volume. This RNA was recovered by ethanol precipitation at -20°C. The column was washed with a further 5 to 10ml of 'load' buffer and the bound RNA was eluted off in 3ml of elution buffer. More than 90% of eluted RNA was contained in this volume. One tenth volume of 5M NaCl was added to this elution fraction, and the sample was again passed through the column which had been re-equilibrated with load buffer. The bound RNA was re eluted and collected by ethanol precipitation with carrier RNA. When analysed on polyacrylamide gels this bound poly A(+)RNA was often very aggregated. This could be eliminated by loading the RNA onto the gels in 90% formamide. Low concentrations of RNA ($<1.0\mu g\ ml^{-1}$) could be collected as ethanol precipitates by centrifugation at 40000rpm for 4hr at 0°C in an MSE 6 x 5.5 rotor after standing at -20°C for longer than 1 hr. Poly A(+)RNA collected without carrier, did not appear aggregated. After use, oligo dT cellulose was washed and stored at 0°C in 0.1N NaOH. Each preparation was used several times.

b) Poly U Sepharose

Poly U Sepharose 4B (Pharmacia) was washed with 10 volumes of elution buffer (90% formamide, 10mM Tris-HCl, 10mM EDTA (pH 7.5), 0.05% SLS) and ten volumes of load buffer (125mM NaCl, 10mM Tris-HCl, 10mM EDTA pH 7.5) and packed in 1ml or 2ml polypropylene syringes with a GFC pad in the bottom. The RNA was applied to the column

(1mg total RNA to 1ml of poly U Sepharose) and unbound RNA was collected in the first 4ml of buffer. The column was then washed with a further 10ml of load buffer. Bound RNA was eluted off the column with 3ml of elution buffer. The salt concentration of this fraction was raised to 150mM NaCl and 2 volumes of ethanol were added. The RNA was collected by centrifugation at 40000rpm for 4hr ($0^{o}C$) in an MSE 6 x 5.5 rotor. With one batch of poly U Sepharose, a considerable portion of the labelled RNA was irreversibly bound to the column. In order to check whether this was due to specific binding of poly A(+)RNA, mRNA isolated from mRNP particles was fractionated on oligo dT cellulose into poly A(+) and poly A(-) RNA which were then passed individually through the poly U Sepharose. 88% of the poly A(-) mRNP mRNA passed through the column and was recovered. 12.1% of the poly A(+)RNA passed through the column, but only 14.1% was eluted off. The remainder could not be recovered even with warm formamide. This indicated that poly A(+)RNA was specifically and irreversibly bound to the poly U Sepharose. On the basis of this recovery, a correction was made to the poly A(+) mRNA values in the experiment involving analysis of the kinetics of the approach to steady state labelling of mRNA in non-dividing tissue. This experiment was only performed once. The manufacturers of poly U Sepharose suggested a pre-equilibration of the gel with 1.0MNaCl pH 7.5, followed by washing with 0.1M NaCl pH 7.5 before washing with elution buffer. This batch of poly U Sepharose then gave good recovery of bound RNA (>90%). The reason for this is not clear, especially as the previous batch of poly U Sepharose reversibly bound poly A(+)RNA without this pretreatment.

The poly U Sepharose was used 2 or 3 times and was stored between use in 50% ethanol at $-20^{o}C$.

8) Isolation of the Poly A Tract

SLS was removed from RNA preparations by repeated washing in 95% aqueous ethanol. In some cases this was also followed by reprecipitation from 0.15M sodium acetate. The RNA was then dissolved in 10mM Tris-HCl, 10mM EDTA pH 7.5 150mM NaCl (TEN buffer) to concentrations up to 0.1 mg ml^{-1}. Ribonucleases A, T_1 and T_2 were stored frozen in TEN at concentrations of 2mg ml^{-1}, 10000 U ml^{-1} and 500 U ml^{-1} respectively, and were used at final concentrations of 10µg ml^{-1}, 100 U ml^{-1} and 10 U ml^{-1} respectively.

RNAse A (Sigma EC2.7.7.16) is an endonuclease attacking specifically the linkage between the 3' phosphate of pyrimidine nucleotides and the 5' C of the adjacent nucleotide leaving pyrimidine 3' phosphate and oligo nucleotides with a pyrimidine 3' phosphate end group. RNAase T_1 (Sigma EC. 2.7.7.26) attacks the 3' phosphate of guanosine nucleotides in a similar way and RNAase T_2 (Sigma EC 2.7.7.17) shows a preference for the 3' phosphate of adenosine nucleotides. Thus, digestion with RNAases A and T_1 produces UMP, GMP, CMP, dinucleotides with a 5' AMP and poly A sequences. Addition of RNAse T_2 to the reaction results in digestion of the poly A and 5' AMP dinucleotides. The amount of poly A in a sample was estimated as:

$$\frac{(\text{cpm A},T_1 \text{ resistant} - \text{cpm A},T_1T_2 \text{ resistant})}{(\text{Total cpm} - \text{cpm A}, T_1\ T_2 \text{ resistant})}$$

The RNA was digested at 37°C for 2hr to ensure complete digestion of non poly A components (Hepburn 1974). The RNAase A, T_1,T_2 residue was routinely less than 0.1% of total radioactivity. The reaction was stopped by the addition of 10% trichloroacetic acid (TCA). When the residue was to be analysed by electrophoresis, by addition of 0.1 volume 10% SLS, 0.2% DEP, 100µg carrier/marker total artichoke nucleic acid and 2 volumes of ethanol. In the absence of DEP, the marker RNA was degraded due to residual nuclease activity.

9) Base Composition Analysis

RNA samples were collected by TCA precipitation onto GFC (section 11), dried and hydrolysed in 1 to 2ml of 10% piperidine 10mM EDTA for 48hr at 60°C in sealed vials. The liquid was centrifuged to remove GFC fibres and then dried down. The residue was redissolved in 2 to 3 drops of 7.5% glacial acetic acid, 0.75% pyridine (pH 3.5 - 4.0) and loaded onto Whatman No. 1 chromatography paper as 2cm streaks which were 10cm from the end of a 40cm x 14cm sheet (3 samples per sheet). A marker dye, 0.5% orange G was also spotted on the base line. The paper was moistened with the acetic acid/pyridine buffer and electrophoresed at 1200V until the marker dye ran off the end (about 2 hr).

During electrophoresis the paper was cooled by immersion in white spirit. After electrophoresis the paper was removed and dried in air.

10) 'In vitro' Protein Synthesis

A wheatgerm 'S.30' was prepared by Dr C.J.Leaver by the method of Marcu and Dudock (1974). RNA for use in 'in vitro' protein synthesis was prepared from ribosome pellets or from sucrose gradient fractions as described and was fractionated into poly A(+) and poly A(-) components on poly U Sepharose or oligo dT cellulose. As the wheatgerm S.30 system is inhibited by detergents, SLS was omitted from the poly U Sepharose or oligo dT cellulose buffers, and total fractions were repecipitated from 0.15M NaCl. The RNA was dissolved in sterile distilled water to a concentration of more than 1mg ml^{-1} and an aliquot was incubated at 25°C for 60min with the wheatgerm S.30 system containing salts, amino acids including ^{35}S methionine and an energy source (Table II). Incorporation of ^{35}S methionine was estimated by drying aliquots of the reaction mixture onto filter paper discs (Whatman 3MM). The discs were then soaked in ice cold 10% TCA

TABLE II - The Composition of the wheatgerm S.30 'in vitro' protein synthesis incubation;

Component	Final Concentration	Storage Concentration
Hepes	40mM	X20
KCl	30mM	
$Mg(Ac)_2$	0.25mM	
Spermidine	0.25mM	
ATP	1mM	X20
GTP	0.05mM	
Creatine phosphate	8mM	
Creatine phosphokinase	0.1mg ml^{-1}	5mg ml^{-1} in 50% glycerol
19 amino acids each (except methionine)	0.025mM	X20
dithiothreitol	2mM	200mM
^{35}S methionine	5-10μCi/50μl	~5mCi ml^{-1}
Wheatgerm S-30	20μl/50μl	
mRNA or	<5μg/50μl	
total RNA	<20μg/50μl	

for 30min., transferred to 5% TCA at 90°C for 15 min. and passed through four changes of 5% TCA allowing at least 5min. in each change. TCA was washed out of the filters by soaking in ethanol:ether (1:1 v/v) for 15min at 37°C and then in ether for 10min at 37°C.

Products of the 'in vitro' protein synthesis were separated on slab gels (Laemmli 1970). The gels were 2mm thick, 15cm across, 20cm long, and were made from 0.375 M Tris-HCl (pH 8.8), 15% acrylamide and 0.1% bisacrylamide. These reagents were degassed and 10% SLS, TEMED

and fresh ammonium persulphate (0.1ml, 4µl and 50µl respectively) to 10ml of mixture). The mixture was poured into the gel holder which was made of glass plates held apart by strips of plastic placed round the edges and sealed with a minimal amount of silicon grease. The apparatus was held upright during polymerisation and the gel was overlayed with distilled water. After 45min, the surface of the gel was washed with water and a 5% stacking gel was prepared by degassing the acrylamide/bis acrylamide with Tris-HCl pH 6.8 (60mM final concentration) and adding 10% SLS, TEMED and 10% freshly prepared ammonium persulphate (100µl, 10µl and 50µl respectively) to 10ml of mixture). This mixture was poured over the separating gel and a shaped plastic comb was inserted into the mould to outline the shape of the loading slots. After polymerisation, the comb was removed. Electrophoresis buffer was 50mM Tris-HCl, 0.384M glycine, 0.1% SLS. The *in vitro* S.30 reaction mixture was prepared for electrophoresis by addition of 60mM Tris-HCl pH 6.8, 2% SLS, 5% β mercaptoethanol and 10% sucrose. After electrophoresis the gel was stained, dried onto 3MM filter paper and autoradiographed with Kodak Blue Band Xray film for 3 to 6 days.

11) Radioactive Determination

a) Sample Preparation

Gels containing labelled RNA were frozen on solid CO_2 in aluminium troughs to a length of 7.5cm and cut into 1.0mm slices using a Mickle gel slicer. Slices of dilute (2.3% or 2.4%) gels were dried onto 16mm cine film. Slices of 7.5% gels were dried onto filter paper discs.

Radioactive samples in solution (gradient fractions or enzyme digests) were precipitated by the addition of an equal volume of 10% TCA containing 20mM phosphate. After 1 hr at $0^{o}C$ the precipitate was collected by suction filtration onto GFC discs (2.1cm Whatman) and washed with 6 x 5.0ml of 5% TCA, 10mM phosphate. The filters were sucked dry and dried under an infra-red lamp.

b) Sample Assay

Gel slices on cine film were counted in a lead castle, by a thin window Geiger tube which was coupled to a 'J and P Engineering' scalar unit. The film was automatically moved on after counting each slice. The radioactivity was recorded by a Freiden print out calculator and plotted directly on a 'Servoscribe' potentiometric recorder. Counting time and chart speed were generally set such that the record was the same length as the E_{265nm} scan of the gel.

The background of this apparatus was 9cpm and the efficiency 34% of that obtained by counting ^{32}P labelled samples on GFC discs in liquid scintillant in the Intertechnique SL31.

Samples dried onto GFC and filter paper were counted in 10ml of liquid scintillant (2-(4'-t-butylphenyl)-5-(4" biphenyl)-1,3,4,-oxadiazole (= butyl PDB) in toluene (4g l^{-1}) in an Intertechnique SL31 liquid scintillation spectrometer using the preset ^{32}P single isotope window. The experiments described in this thesis used only highly labelled ^{3}H samples which were counted as precipitates on GFC at low efficiency using the preset ^{3}H channel of the Intertechnique. ^{35}S labelled samples were counted as precipitates on 3MM filter paper (see section 10) using the preset ^{14}C channel.

RESULTS

1) Growth of Tissue

In this study it was intended to investigate messenger RNA metabolism in asynchronously dividing cells. It was therefore necessary to characterise the growth of the artichoke explant cultures. This had been done by previous investigators of the nucleic acid metabolism of artichoke explants (Gore, 1972; Hepburn, 1974) but it was apparent from their data that the growth characteristics of the cultures varied from year to year. The primary requirements for my experiments were that the growth should have extended beyond the initial, partially synchronous cell division and that the cultures should still be accumulating RNA and undergoing cell division. The explants were cultured for increasing periods of time and the phenol prepared nucleic acid from a known number of explants was quantitated from the electrophoretic profile. The values obtained are not accurate in absolute terms, as nucleic acid was lost in the phenol phase during extraction. However, since all preparations were carried out under standard conditions, these values are considered comparable. Cell division in cultures derived from tubers harvested in the winter of 1975-6 (1976 cultures) was monitored by a change in the cell number. The growth of cultures from tubers harvested in 1974-5 (1975 cultures) was monitored by a less precise method, the DNA being estimated from the electrophoretic profile of total nucleic acid from a known number of explants. Figures 1 and 2 pertain to the 1975 and 1976 cultures respectively, and show that there was an initial lag before cell division (1976) or DNA synthesis (1975) started. The 1976 cultures showed the more vigorous and prolonged growth. The initial lag before cell division was 15hr. compared to 24hr. in the 1975 cultures, and RNA per explant increased throughout the first 85hr. of culture of the 1976 cultures up to 16μg explant^{-1} (fig. 2) compared with a

FIGURE 1 : The culture of 1975 Artichoke Explants

Samples taken from cultures at the indicated times were extracted with phenol and the nucleic acid analysed on 2.4% polyacrylamide gels. The amount of nucleic acid was estimated from the area of the peaks on the electrophoretic profile and expressed per explant. Each point is the mean of two replicates.

X —— X RNA explant^{-1}

O —— O DNA explant^{-1}

FIGURE 2 : The culture of 1976 Artichoke Explants

Samples were taken from cultures at the indicated times and RNA per explant estimated as described for Fig. 1. Cell number was determined by macerating explants in a known volume of chromium trioxide and counting an aliquot of the suspension in a haemocytometer slide.

Each point is the mean of two replicates.

X —— X RNA explant^{-1} (+ 24D culture)

O —— O cell number explant^{-1} (+ 24D culture)

⊕ —— ⊕ cell number explant^{-1} (- 24D culture)

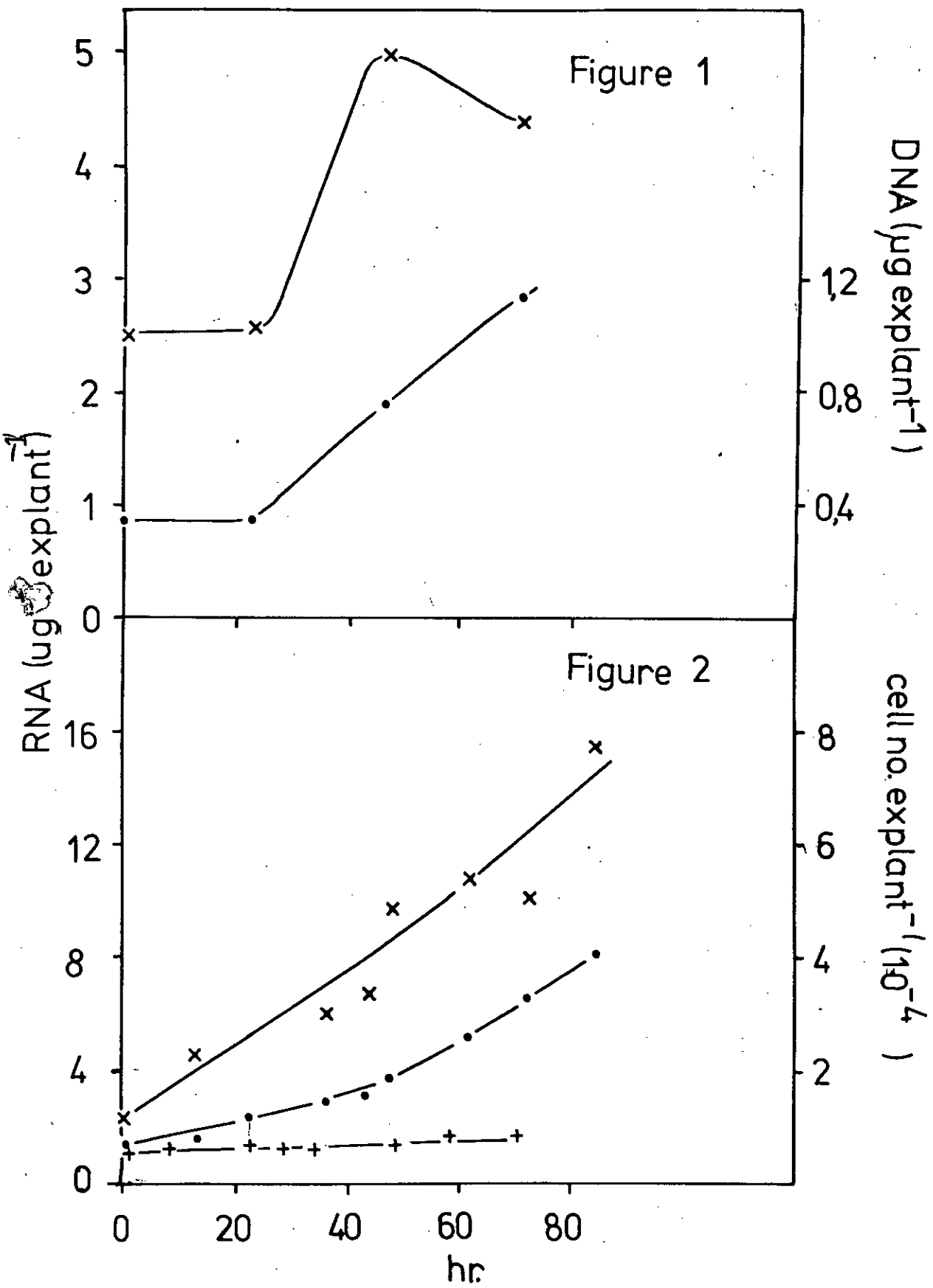


Figure 1
RNA (μg explant⁻¹)
DNA (μg explant⁻¹)
5
4
3
2
1
0
1,2
0,8
0,4
Figure 2
cell no. explant⁻¹ (10⁻⁴)
16
12
8
4
0
8
6
4
2
0
20
40
60
80
hr.

flattening accumulation of RNA in the 1975 cultures at 5µg per explant (fig. 1). Expressed on a per cell basis, however, the RNA content of 1976 cultures declined after an initial rapid increase (fig. 3). This is probably a reflection of heterogeneity of cells within the explant, in that only the peripheral cells are thought to divide (Mitchell and Yeoman, 1970). Thus as the explant grows, the proportion of dividing, RNA rich cells would decline, resulting in a reduced average RNA content per cell. In the cultures of 1975 material, cell division, assessed by DNA content of the explants, was less vigorous than cell division in the cultures of the 1975 material. The DNA content of the 1975 material, which is a maximal estimate of cell number increase if the cells were becoming endopolyploid, increased only threefold in 72hr., compared with a four-fold increase in cell number over an equivalent period with the 1976 material. The decrease in RNA per explant observed after 50hr. culture of 1975 explants (fig. 1) may be related to this slower rate of cell division. A similar decline was observed by Hepburn (1974) in cultures from earlier years. Thus 1975 cultures were suitable for study of RNA metabolism in asynchronously dividing plant cells after 48hr., before the cultures had started to degenerate, and after completion of the first partially synchronous cell division (Yeoman, 1974). The 1976 cultures could be used between 48 to 80hr. of growth. Division growth in 1976 cultures was entirely dependent on inclusion of 10^{-6}M 2'4' dichlorophenoxyacetic acid in the growth medium (fig. 2).

2) Labelling of Tissue

The conditions for radioactive labelling of tissue were adjusted according to the type of experiment. Experiments involving characterisation of rapidly labelled RNA were designed to yield high specific activity RNA after a short pulse label (1hr). Experiments involving kinetic analysis of RNA turnover were designed such that the

FIGURE 3 : The RNA Content per cell of Cultured Artichoke Explants

The data of figure 2 are replotted as RNA per cell (+ 2,4,D)

FIGURE 4 : The effect of ^{32}P Phosphate Specific Activity on the Specific Activity of Newly Synthesised RNA

RNA was prepared from 48hr cultures of artichoke explants which had been pulse labelled for 1hr in ^{32}P phosphate of indicated specific activity. The RNA specific activity was determined by TCA precipitating a sample of known 260nm absorbance.

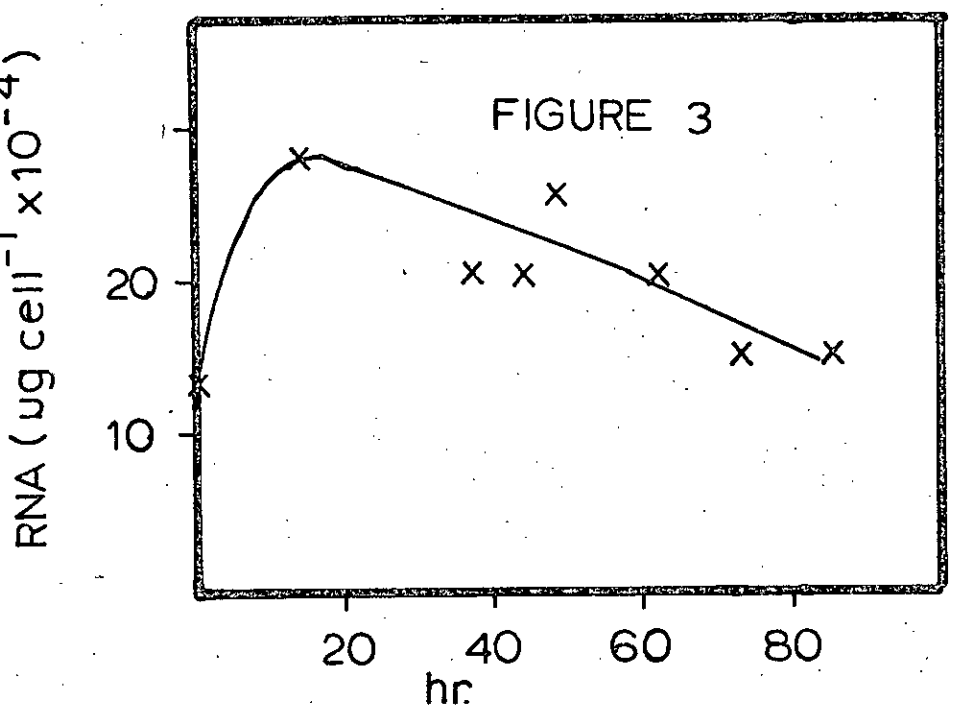


FIGURE 3
RNA (ug cell^{-1} x10^{-4})
20
10
20
40
60
80
hr.

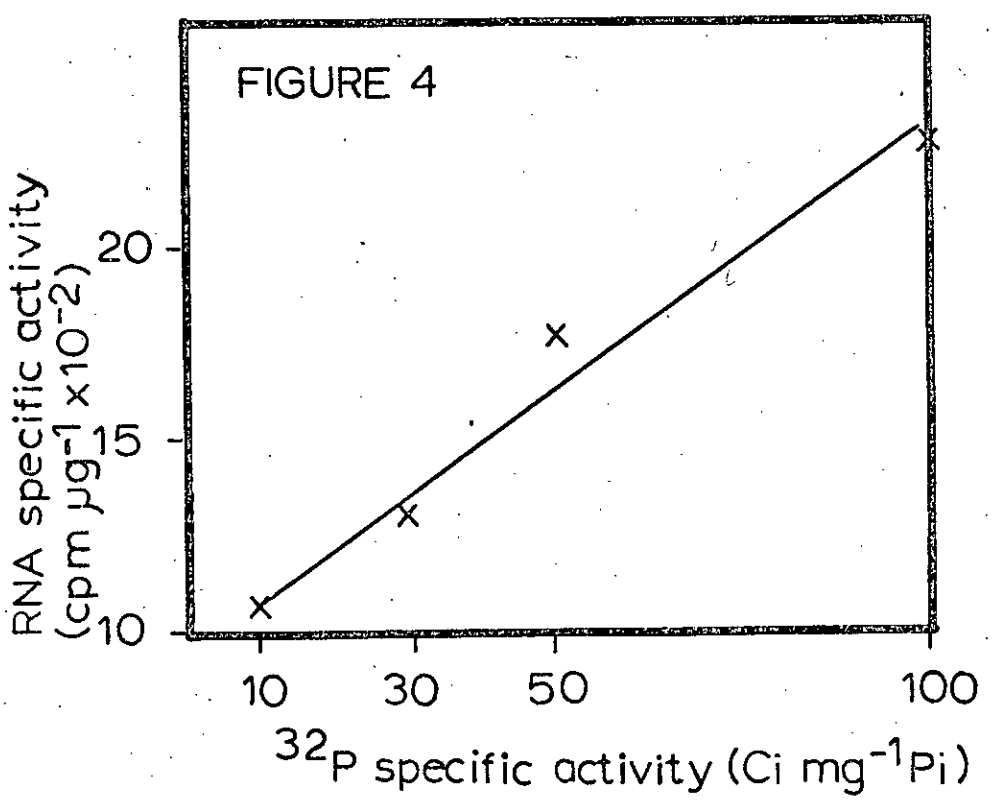


FIGURE 4
RNA specific activity
(cpm µg^{-1} x10^{-2})
20
15
10
10
30
50
100
^{32}P specific activity (Ci mg^{-1}Pi)

nucleotides were of constant specific activity throughout the duration of the experiment. Figure 4 shows the specific activity of RNA prepared from explants incubated for 1hr. in ^{32}P phosphate of decreasing specific activity. Decreasing the specific activity of the ^{32}P phosphate resulted in lower specific activity of 1hr labelled RNA. This contrasts with the result of Hepburn (1974) who found that 10Ci mg^{-1} ^{32}P phosphate produced higher specific activity RNA than 100 Ci mg^{-1} phosphate. This again is probably a reflection of the annual variation of the artichokes themselves. In fact, a ten-fold decrease in specific activity from 100 ci mg^{-1} phosphate resulted in only a two-fold reduction in specific activity of the RNA and 10 Ci mg^{-1} phosphate was used routinely. The much greater uptake ^{32}P phosphate in the presence of carrier phosphate may have resulted from the reduced losses of isotope due to absorption onto glassware which occurs when high specific activity ^{32}P phosphate (low molar concentration) is used.

In order to determine whether or not the phosphate concentration was adequate to support a constant rate of labelling of RNA, the specific activity of rRNA was measured from tissue incubated for increasing periods of time in varying specific activity ^{32}P phosphate. The specific activity of a stable RNA being labelled at a constant rate should increase exponentially at a decreasing rate. When the phosphate was present at 10Ci mg^{-1} phosphate (50μCi ml^{-1}) the rRNA specific activity increased for only 6hr (fig. 5a). If the phosphate concentration was increased ten-fold (and as a result the ^{32}P phosphate specific activity decreased), the incorporation into rRNA did show an exponential increase with time at a decreasing rate after a short lag (fig. 5b). In several experiments the rate of change of rRNA specific activity increased with time (fig. 5c). No explanation is offered for this and the results of these experiments were not considered in analysis of mRNA turnover kinetics.

FIGURE 5 : The Effect of ^{32}P Phosphate Specific Activity On Long Term Incorporation of Label into rRNA

48hr old cultured explants were incubated for the indicated time periods in 50μCi ml^{-1} ^{32}P Phosphate and the RNA prepared. The specific of rRNA was determined from the 2.3% polyacrylamide gel fractionation.

a) 10 Ci mg^{-1} phosphate, b) 1 Ci mg^{-1} phosphate, c) 1 Ci mg^{-1} phosphate

FIGURE 6 : Centrifugation of rRNA containing RNP from the post-mitochondrial supernatant of artichoke explants

60hr old cultures were incubated in 50μCi ^{32}P phosphate for 1hr. and harvested. After homogenisation and filtration through miracloth, the homogenate was centrifuged at 13000rpm (MSE 8 x 50 rotor). The supernatant was layered over 1ml 1M sucrose and centrifuged for the indicated times at 65000rpm (MSE 10 x 10 rotor). The recovery of each RNA component was calculated from the amount of each ^{32}P labelled RNA species in the pellet and supernatant after phenol extraction.

Each value is the mean of two replicates.

X —— X 1.3×10^6 rRNA

O —— O 0.7×10^6 rRNA

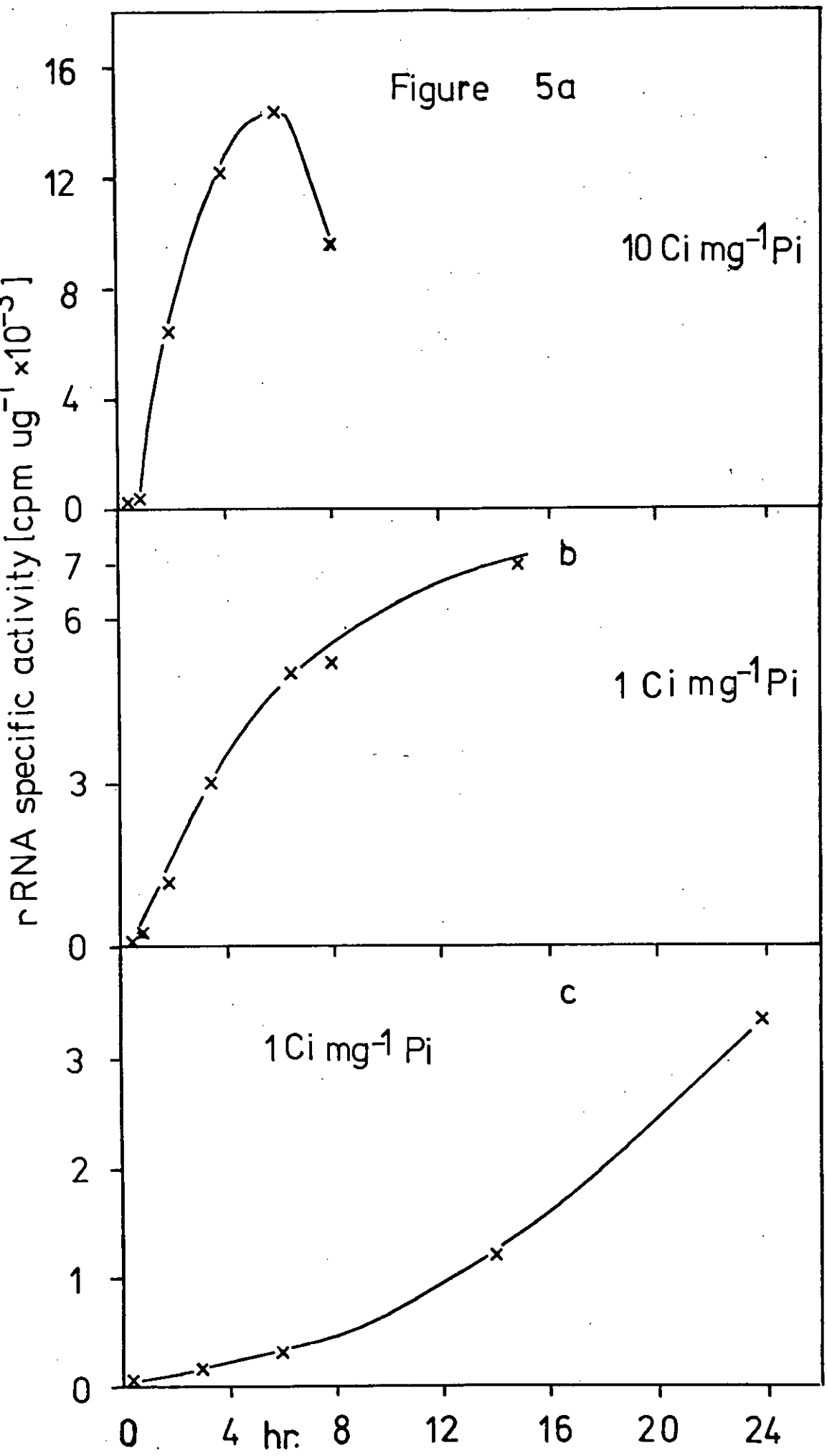


Figure 5a
16
12
8
4
0
10 Ci mg⁻¹Pi
rRNA specific activity [cpm ug⁻¹ x10⁻³]
b
7
6
3
0
1 Ci mg⁻¹Pi
c
3
2
1
0
1 Ci mg⁻¹ Pi
0
4 hr
8
12
16
20
24

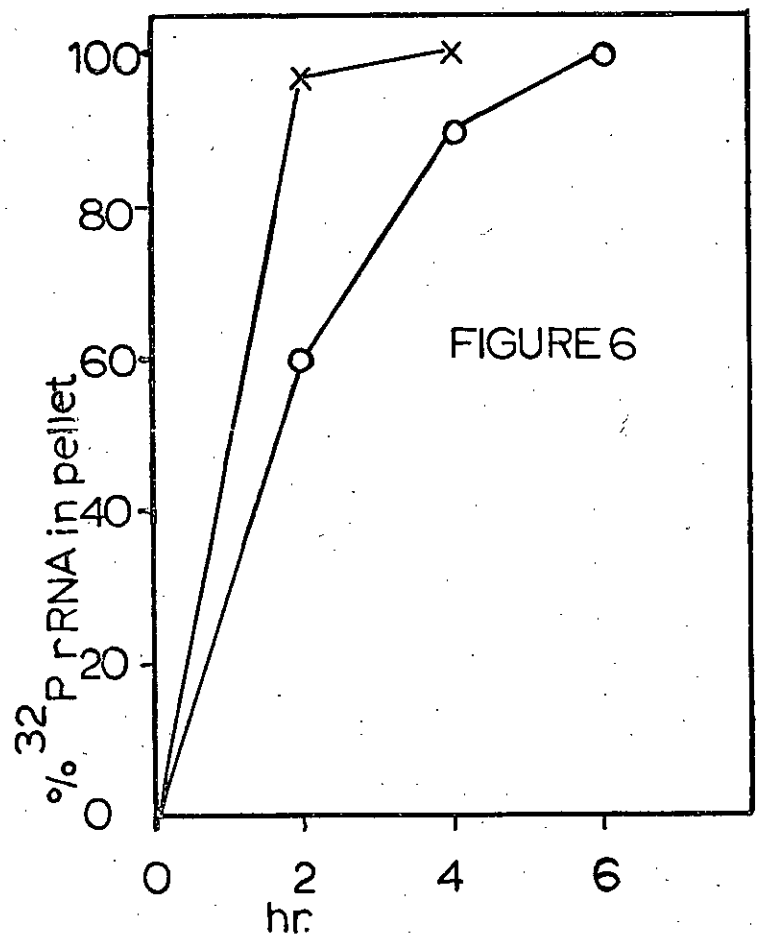


FIGURE 6

3) Preparation of Polysomes and Cytoplasmic RNA

The 13000rpm post mitochondrial supernatant contained substances which co-purified with RNA through ethanol precipitations and which inhibited the binding of poly A(+)RNA to poly U Sepharose. It was therefore considered necessary to prepare RNA from purified cytoplasmic RNP. The magnesium precipitation technique of Palmiter (1974) was effective in precipitating large polysomes but only at very low efficiency. A more conventional method involves centrifugation of the post mitochondrial supernatant through a sucrose cushion. Leaver and Dyer (1974) showed the importance of an extensive centrifugation of the post mitochondrial supernatant for the quantitative recovery of ribosomes. During long periods of centrifugation, however, the possibility of RNA digestion by endogenous nucleases is increased. By decreasing the depth of the sucrose cushion over that used by Leaver and Dyer (1974), and using very high centrifugation speed (and therefore 'g' force), it was possible to reduce the time of centrifugation considerably. The reduction in depth of the sucrose cushion did not appear to affect the purity of the ribosome pellet, as the $A_{260/280}$ was routinely 1.8 to 1.9 compared with ∿1.8 reported by Leaver and Dyer (1974). Figure 6 shows the recovery of newly synthesised rRNA (as ribosomes and free ribosomal subunits) through a 1ml cushion of 1M sucrose after centrifugation at 65000rpm (300000g_{av}) in an MSE 10 x 10Ti rotor for increasing periods. The degree of ribosome subunit pelleting was determined by comparing radioactivity in the rRNA components in the 65000rpm supernatant and pellet. Total recoveries varied between 95 and 106% of RNA in the original post mitochondrial supernatant. It was not possible to follow the recovery of heterodisperse RNA due to the presence of a highly radioactive background on gels containing '13000rpm' or '65000rpm' supernatant samples. Since essentially all of the 1.3 x 10^6rRNA was pelleted

after 2hr (fig. 6), all of the polysomes would also have pelleted after this time. Only 60% of the 0.7 x 10^6rRNA had pelleted after 2hr centrifugation, implying that a large portion of the newly synthesised 0.7 x 10^6rRNA in these cultures was present in free ribosomal subunits. The U.V. absorbing profile of such a 2hr pellet after resuspension and centrifugation through a sucrose gradient (fig 7) showed prominent 60S and 40S ribosomal subunit peaks, the specific activities of which were higher than the 80S monomer.

Although satisfactory for purifying cytoplasmic RNP for RNA preparation and analysis, a disadvantage of this high speed centrifugation method was the resistance of the ribosome pellet to resuspension in TKM buffer. A ribosome pellet (65000rpm, 2hr) was resuspended in buffer and divided into two parts. One half was centrifuged at 10000g for 5min and the supernatant removed and dissolved in warm 0.1% SLS. The other half was dissolved directly in SLS. Only 64-68% of the SLS soluble 260nm absorbing material remained in suspension after the low speed clearing centrifugation. However, following the method of Palmiter (1974), 83 to 89% of the 260nm absorbing material was fully resuspended in 20mM Hepes buffer (pH 8.0 0^{o}C). Thus Hepes buffer was used routinely for the resuspension of pelleted ribosomes. The absorbance profile of sucrose gradients (fig. 7) was identical for Hepes and TKM resuspended samples.

A feature of the sedimentation profile of ribosomes from artichoke explants was the high proportion of 80S monomer and 60S and 40S subunits (fig. 7). If these were an artifact and were derived by nuclease action on polysomes during extraction, it should have been possible to obtain a higher proportion of polysomes by inhibiting ribonuclease during the extraction. Diethylpyrocarbonate is an effective RNAase inhibitor (Weeks and Marcus, 1969). However, inclusion of DEP (0.2%) in the homogenisation or resuspension buffers

had a deleterious effect on the polysome profile, causing dissociation of some monomers and polysomes into ribosome subunits. This observation has also been reported by Anderson and Key (1971) working with soybean ribosomes. Morton et al (1975) showed that inclusion of purified yeast sRNA in the homogenisation buffer used to extract rat liver polysomes, greatly improved the yield of large polysomes. Inclusion in the homogenisation buffer of yeast sRNA (Sigma) which had been purified by phenol extraction had no effect on the sedimentation of artichoke polysomes.

4) Fractionation of Poly A(+)RNA

The most widely used methods for separation of poly A(+) and poly A(-) RNA involve use of poly U Sepharose (Firtel and Lodish, 1973) or oligo dT cellulose (Aviv and Leder, 1972). I checked both methods for the efficiency of fractionation of RNA prepared from ribosome pellets. Pulse labelled (1hr) RNA was resuspended in the appropriate loading buffer and passed through a column of either oligo dT cellulose or poly U Sepharose. The TCA precipitable radioactivity of column fractions showed that some RNA was bound and could be eluted in low salt (oligo dT cellulose) or formamide (poly U Sepharose) buffer. Recycling unbound RNA through oligo dT cellulose failed to increase the amount of RNA bound. When the RNA bound to dT cellulose was eluted and recycled in high salt, only 30% annealed in the second cycle. The poly A content of bound, unbound and total RNA was determined by digesting the RNA in high salt with ribonucleases A and T_1. The poly U Sepharose-bound RNA contained 12.2% poly A and the unbound RNA 0.14% compared with 10.5% and 0.79% poly A respectively from the oligo dT cellulose fractions. These results, summarised in Table III, show that poly U Sepharose was more efficient in binding artichoke poly A(+)RNA. Poly U Sepharose bound 81% of the total poly A compared with 47% bound

TABLE III - The distribution of poly A in RNA fractionated on oligo dT cellulose or poly U Sepharose

	RNA (cpm)	Poly A (cpm)	% Poly A
Total ribosome pellet RNA	118440 (100%)	2787 (100%)	2.4
Poly U Sepharose i) bound RNA	18403 (15%)	2245 (81%)	12.2
ii) unbound RNA	108460 (91%)	152 (6%)	0.14
Oligo dT Cellulose i) bound RNA	12553 (10%)	1316 (47%)	10.5
ii) unbound RNA	92383 (78%)	727 (26%)	0.79

Artichoke explants were cultured for 48hr and pulse labelled with 100μCi ml^{-1} ^{32}P phosphate for 1hr. The ribosome pellet was isolated and RNA prepared and fractionated on poly U Sepharose or oligo dT cellulose. The RNA of the column fractions and of total RNA was digested with RNAases A and T_1 as described in the methods section to enable determination of the poly A content of the RNA.

FIGURE 7 : Rate Zonal Centrifugation of Artichoke Ribosomes

A ribosome pellet (2hr 65000rpm) was prepared from the post mitochondrial supernatant of explants which were cultured for 60hr and labelled for 1hr (50μCi ml^{-1}) immediately before harvesting. The pellet was resuspended in TKM and layered over a 10-60% sucrose gradient. After centrifugation at 28000rpm for 140 min. (MSE 3 x 25 rotor) the gradient was pumped out, the 254nm absorbance (continuous line) recorded and ^{32}P radioactivity of fractions (dotted line) determined. Sedimentation values are arbitrary, '60S' and '40S' ribosome subunits were identified by the presence of either 1.3 or 0.7 x 10^6 rRNA.

~~~ : E_{254} •—• : ^{32}Pcpm

FIGURE 8 : The Size of the Poly A Tract on Artichoke Cytoplasmic RNA

48hr cultures of artichoke explants were pulse labelled for 1hr or 6hr with 100μCi ml^{-1} ^{32}P phosphate and RNA was prepared from the ribosome pellets. The RNA was digested with RNAases A and T_1 in TEN buffer either with or without pre-fractionation on poly U Sepharose. The enzyme resistant residue was collected and electrophoresed for 3hr on 7.5% polyacrylamide gels (50V) with total nucleic acid as marker. The histogram is ^{32}P radioactivity and the continuous line the E_{265}nm profile.

a) Poly A from the total ribosome pellet RNA of 6hr pulse labelled explants

b) Poly A from the poly U Sepharose bound RNA from 6hr pulse labelled explants

c) Poly A from the total ribosome pellet RNA of 1hr labelled explants
~~~

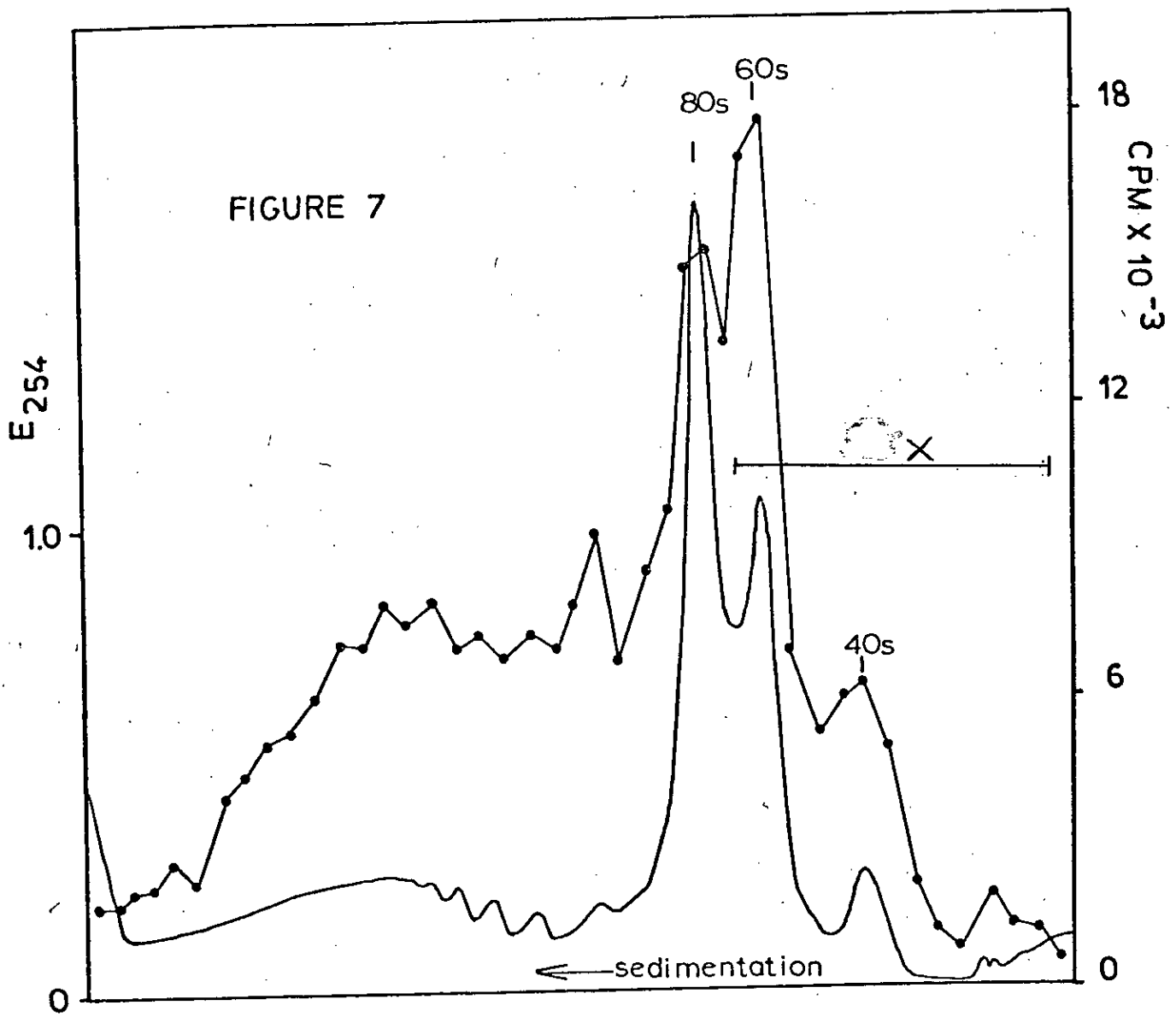


FIGURE 7
80s
60s
40s
X
E_{254}
1.0
0
CPM X 10^{-3}
18
12
6
0
sedimentation

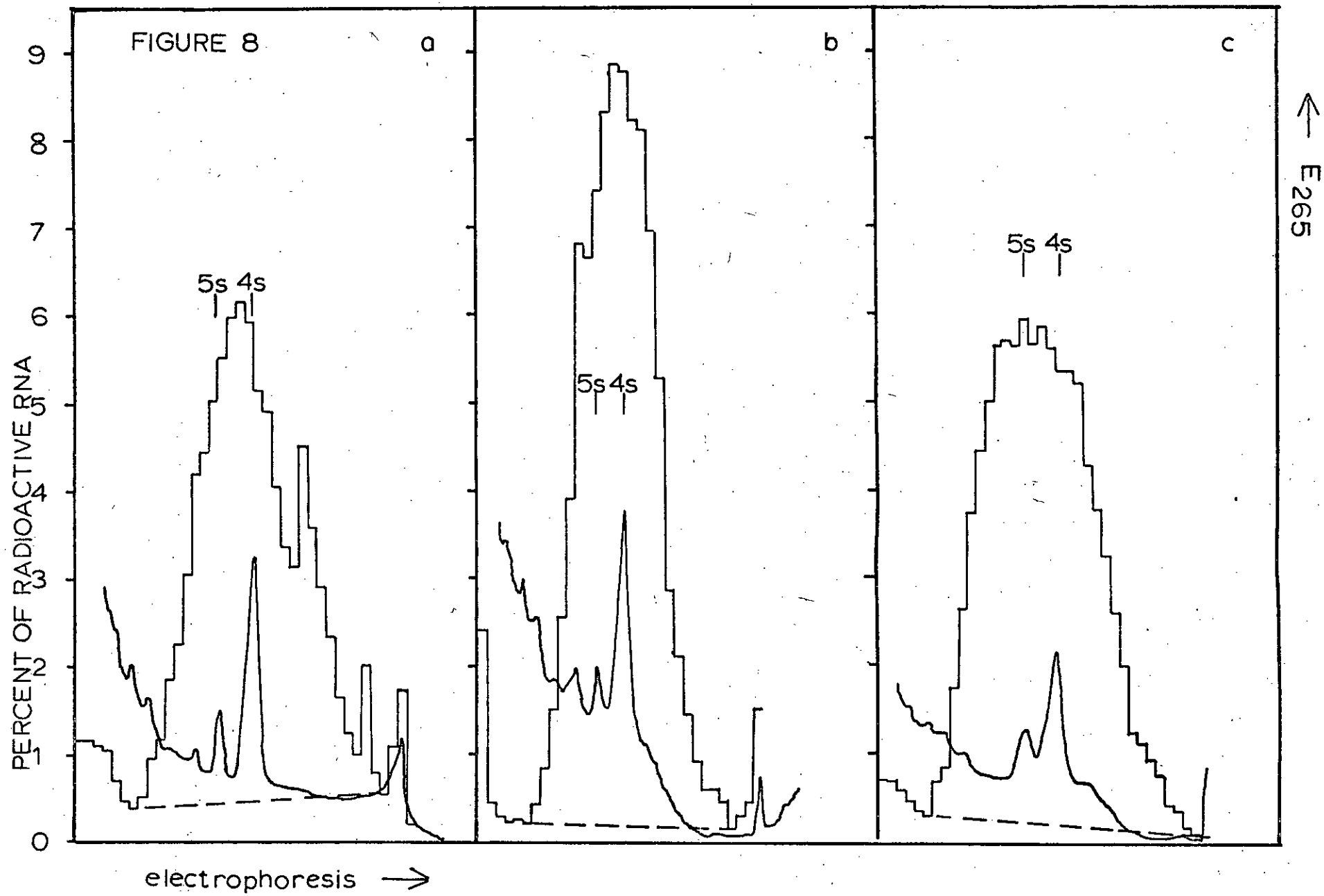


FIGURE 8
a
b
c
5s 4s
5s 4s
5s 4s
PERCENT OF RADIOACTIVE RNA
0
1
2
3
4
5
6
7
8
9
E 265
electrophoresis

after 2 cycles through oligo dT cellulose. A significant portion of poly A (26%) was recovered in the oligo dT cellulose wash fraction, and the total recovery of poly A was only 73%, compared with 87% from poly U Sepharose.

5) The Size of Poly A

Short poly A tracts would not bind to poly U Sepharose as readily as long tracts and so the size of the poly A tracts in total and bound poly A(+)RNA were compared, in order to detect possible selective loss of the shorter tracts in the poly U Sepharose bound RNA. Figures 8a and 8b illustrate the electrophoretic profile of the ribonuclease resistant core from total RNA prepared from the ribosome pellet and from the poly U Sepharose bound RNA. Sheiness and Darnell (1973) have reported that the poly A tract is shortened with age, so in order to illustrate more clearly any 'flow through' of small poly A sequences, the tissue was labelled for a relatively long period, 6hr. 43% of the poly A from the poly U Sepharose-bound RNA and 44% from the total RNA had a mobility less than 4S RNA (80 bases), suggesting that the small poly A sequences had bound quantitatively to the poly U Sepharose. It should be noted, however, that if 50% of the molecules had poly A tracts of 5 to 10 bases long they would comprise only 5 to 10% of the total radioactivity which would hardly be detected by this analysis. Comparison of the electrophoretic profile of '6hr labelled' (fig. 8a) and '1hr labelled' (fig. 8c) poly A shows that poly A on artichoke RNA shortened with time. The number average molecular weight of the newly synthesised poly A tract corresponded to 52 nucleotides, compared with 45 nucleotides for the 6hr pulse-labelled poly A. In a replicate experiment the number average molecular weight of total ribosome pellet poly A decreased from 61 bases long in 1hr tissue to 51 bases in 6hr tissue. The shoulder, which in figure 8a electrophoresed

more rapidly than 4S RNA, was not found in any other preparations. The method of computation of number average molecular weight is shown in figure 9. The relative number of molecules in each slice was calculated by dividing the radioactivity by the molecular weight of RNA in that slice. Molecular weight determinations assumed an inverse linear relationship between log (molecular weight) and electrophoretic mobility using 4S and 5S RNA as molecular weight markets (Loening, 1967). The cumulative percentage of poly A molecules was plotted versus molecular weight (fig. 9) and the number average molecular weight is that value which is the 50% cumulative total of all molecules. Comparison of the 6hr pulse labelled (fig. 9a) and 1hr pulse labelled (fig. 9b) poly A by this method of analysis clearly illustrates the size difference.

These estimations of the number average molecular weight of the poly A tract assumed that in non-denaturing conditions, poly A electrophoresed at the same rate as a riboheteropolymer of the same size. Morrisson et al (1973) found that in 12% gels, poly A standards electrophoresed at half the rate predicted from the migration of 4 and 5S RNAs.

Knowing the number average molecular weight of poly A(+)RNA and the percentage of poly A, it is possible to obtain an independent estimation of the number average size of the poly A. The electrophoretic profile of poly U Sepharose bound RNA is shown in figure 11c. This corresponds to a number average molecular weight of 310000 by comparison with artichoke rRNA markers. Digestion of 6hr labelled ribosome pellet RNA with RNAases A and T_1 in high salt, left 6.3% of the TCA precipitable radioactivity. 6.3% of 310000 is 19350 which corresponds to a number average poly A sequence of 59 nucleotides length. This compares with 45 to 51 nucleotides based on the direct electrophoretic analysis, suggesting that in 7.5% polyacrilamide gels poly A migrated more rapidly than a riboheteropolymer of equivalent

FIGURE 9 : The Number Average Molecular Weight of Artichoke Poly A

Total RNA was prepared from the ribosome pellet of cultured artichoke explants and digested with RNAases A and T_1 in TEN buffer. The residue was electrophoresed in 7.5% polyacrylamide gels (data in Figure 8). The molecular weight of RNA in each slide was determined assuming an inverse relationship between log (molecular weight) and electrophoretic mobility using 4S (25000) and 5S (38000) RNAs as markers. The relative number of molecules in each slice was calculated by dividing radioactivity in each slice by the molecular weight of that slice and the data is plotted as cumulative percent of poly A molecules (ordinate) versus the increasing molecular weight of the poly A (abscissa).

a) Poly A prepared from explants labelled for 6hr (data in Fig. 8a)

b) Poly A prepared from explants labelled for 1hr (data in Fig. 8c)

FIGURE 10 : The Base Composition of the Poly A Tract

48hr cultures of artichoke explants were labelled for 1hr with ^{32}P phosphate ($100\mu Ci\ ml^{-1}$) and total ribosome pellet RNA was digested with RNAases A and T_1 in NET buffer. The TCA insoluble material was collected on GFC discs and hydrolysed in piperidine-EDTA for 48hr. The hydrolysate was electrophoresed at 1200V for approximately 2hr in pyridine-acetic acid. The electropherogram was cut into 1cm strips and counted.

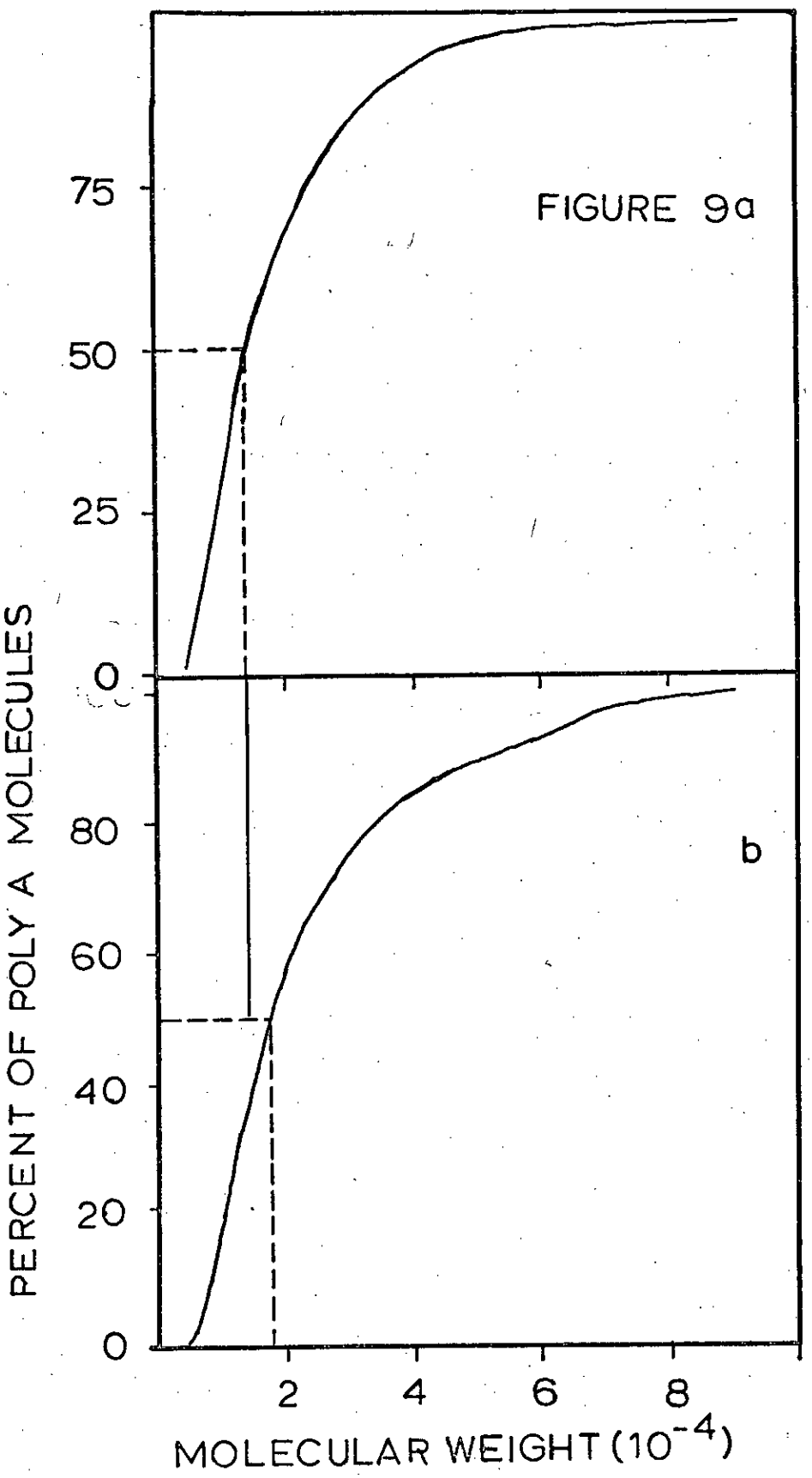
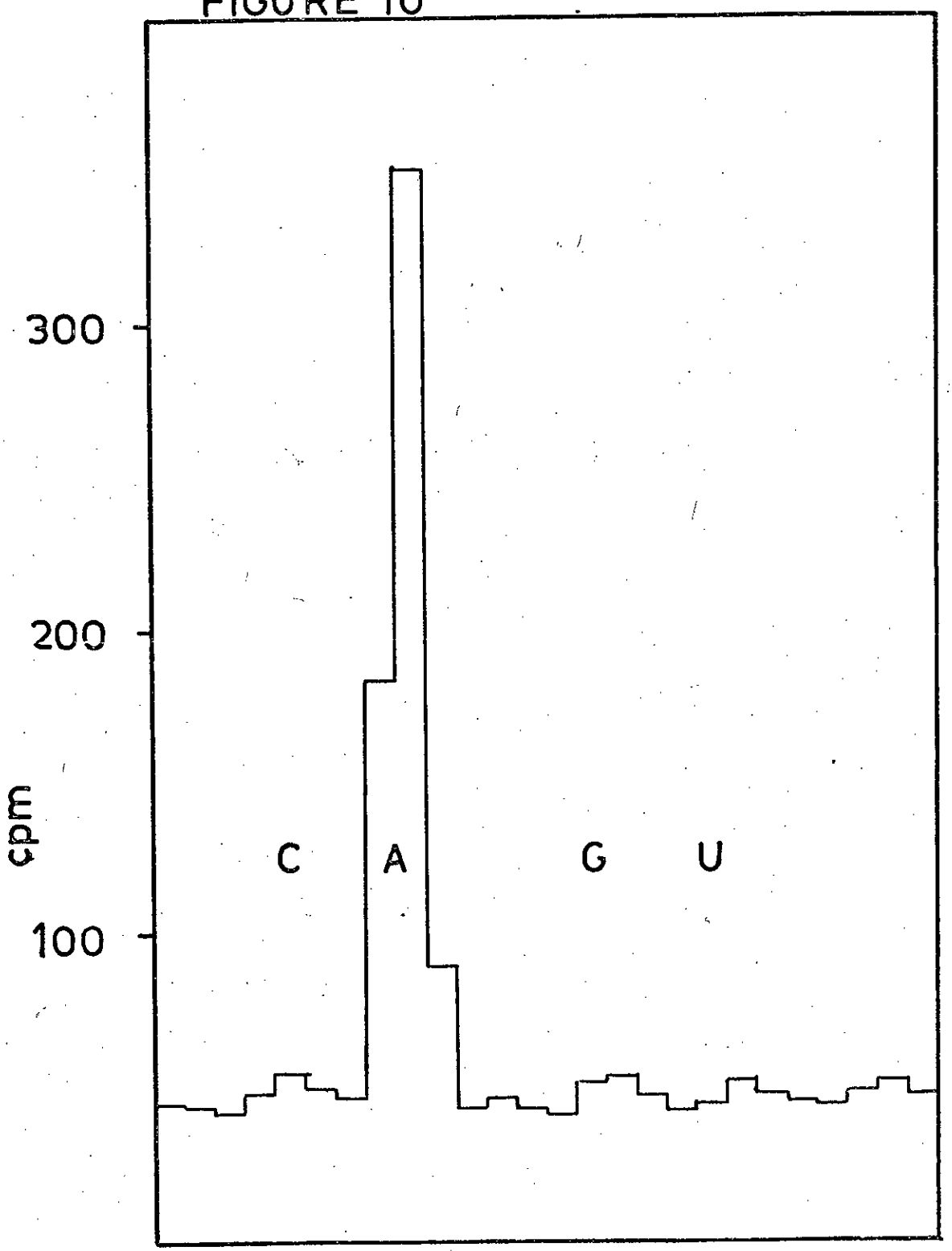


FIGURE 9a
75
50
25
0
80
60
40
20
0
b
PERCENT OF POLY A MOLECULES
2
4
6
8
MOLECULAR WEIGHT (10^{-4})

FIGURE 10



length, and that the estimation of poly A length from these gels is only 76% ($^{45}/59$) to 86% ($^{51}/59$) of the true value.

In order to confirm the identity of the RNAase resistant core as poly A, the residue of the enzyme reaction was collected by TCA precipitation, hydrolysed with alkali and electrophoresed in pyridine/acetic acid (fig.10). More than 90% of the radioactivity was identified as AMP.

6) The Synthesis of Poly A

The radioactive poly A content of poly A(+)RNA prepared from a ribosome pellet after a 1hr pulse label, varied in several preparations between 9% and 16%. This was much higher than predicted from the size distribution of 1hr labelled poly A (figs. 8c, 9b) even when the error of the gel method was considered. A possible explanation of this is that the AMP of this RNA had a higher specific activity than the other nucleotides. This is quite consistent with a post-transcriptional addition of poly A (Molloy and Puckett, 1976). If the half-life of a non adenylated precursor to poly A(+)mRNA is significant relative to the pulse period, then newly synthesised, radioactive poly A would be added to non-labelled molecules and the proportion of poly A would be higher than if the proportion of poly A were estimated after a long pulse period when nearly all of the mRNA molecules would be labelled. After a 6hr pulse label, the percentage of poly A declined to 6.3%. This post-transcriptional addition of poly A may also explain the AMP rich base composition of total and poly A(+) ribosome pellet RNA prepared from 1hr pulse labelled tissue (figs. 11a and 11c).

(7) Poly A(+) and Poly A(-) mRNA

The electrophoretic profiles of total and poly U Sepharose bound and unbound RNA from the ribosome pellet of tissue labelled for 1hr

are shown in figure 11. In the total RNA, 1.3 and 0.7 x 10^6 mol wt rRNA were seen on both the absorbance and radioactivity profiles, whereas the heterodisperse component was seen only as radioactivity. Heterodisperse RNA was the only component bound to poly U (fig. 11c). Ribosomal RNA and approximately 50% of the heterodisperse RNA did not bind to the Poly U Sepharose (fig. 11b). In order to establish that heterodisperse RNA was mRNA, it was necessary to consider whether the ribosome pellet contained possible RNP sources of non messenger, heterodisperse RNA. Chapman and Ingle (1976) have shown that hnRNP may be released from artichoke nuclei. These sedimented heterogenously and contained hnRNA, 80% of which lacked poly A. Free cytoplasmic 'informosome' like particles, containing heterodisperse RNA, have been demonstrated in plant cells (Ajtkhozhin et al, 1973). If either of these RNP types were present in the ribosome pellet from explants labelled for 1hr., they should have been detected in CsCl gradients as high specific activity components with a buoyant density distinct from ribosomes (Chapman and Ingle, 1976; Ajtkhozhin et al, 1973) which generally band at 1.5 to 1.6g cm^{-3} (Spirin et al, 1965). It is clear from the data in figure 12 that the ribosome pellet comprised one quite homogenous component. Essentially all of the radioactivity banded at 1.53g cm^{-3} with the UV absorbing peak. Analysis of the unfixed ribosome pellet by centrifugation in Metrizamide also suggested one homogenous component. Since the results in the literature suggest that hnRNP would sediment more slowly than 80S (Ajtkhozhin et al, 1973; Pederson, 1974), the 60S/40S ribosome subunit region of a sucrose gradient (fig. 7 fraction X) should be enriched for any of these components relative to the total ribosome pellet. It would therefore be easier to detect small amounts of informosomes and hnRNP by pre-fractionating the ribosome pellet before fixation and CsCl centrifugation. The analysis of this ribosome subfraction is shown in figure 13. The

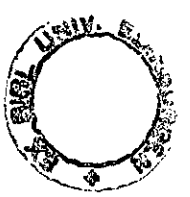


FIGURE 11 : Polyacrylamide gel electrophoresis of Ribosome Pellet RNA subfractionated on Poly U Sepharose

48hr artichoke explant cultures were labelled for 1hr with ^{32}P phosphate ($100\mu Ci\ ml^{-1}$) and the RNA extracted from the ribosome pellet. The RNA was fractionated on poly U Sepharose and the bound and unbound components collected by ethanol precipitation and electrophoresed on 2.3% polyacrylamide gels for 2.5hr at 50V. Gels were scanned at 265nm, sliced and counted in the strip counter.

a) total ribosome pellet RNA

b) poly U Sepharose unbound ribosome pellet RNA

c) poly U Sepharose bound ribosome pellet RNA

The histogram is the ^{32}P radioactivity and the smooth line the E_{265nm}. The percentage base composition of each RNA in terms of AMP (A), CMP (C), GMP (G), UMP (U) is indicated by the figures above each figure and are the mean of 2 determinations.

The $E_{265}nm$ trace on figure 11c is carrier RNA. The figure (5V or 2V) on the top right hand corner of each profile indicates the f.s.d. setting on the 'Servoscribe' potentiometric recorder.

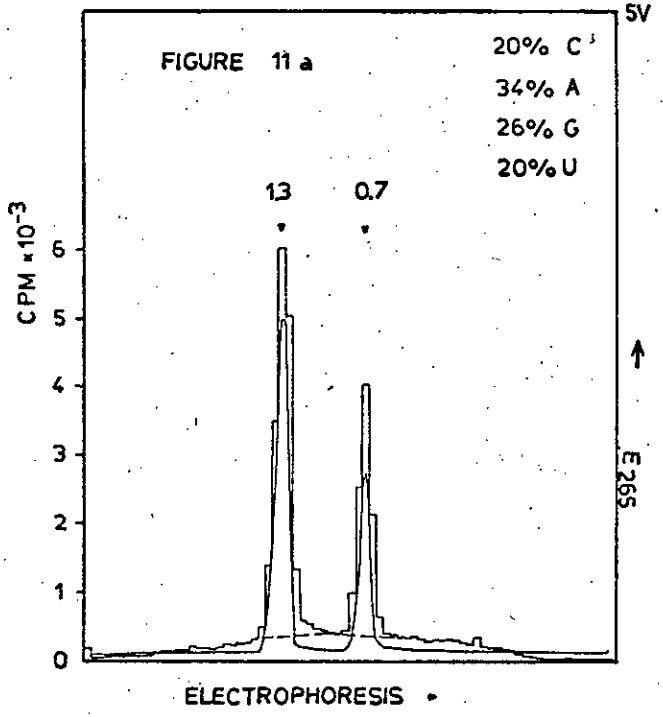
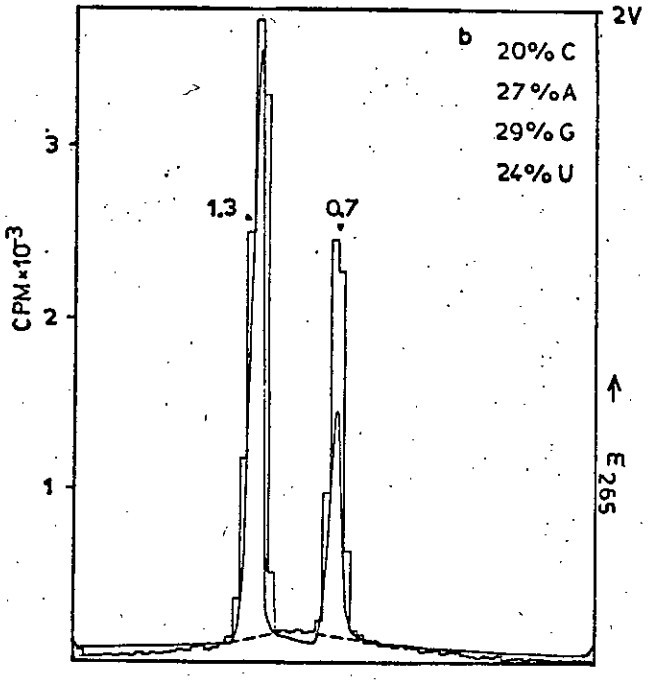


FIGURE 11 a
20% C
34% A
26% G
20% U
1.3
0.7
5V
CPM ×10⁻³
E 265
ELECTROPHORESIS


b
20% C
27% A
29% G
24% U
1.3
0.7
2V
CPM×10⁻³
E 265

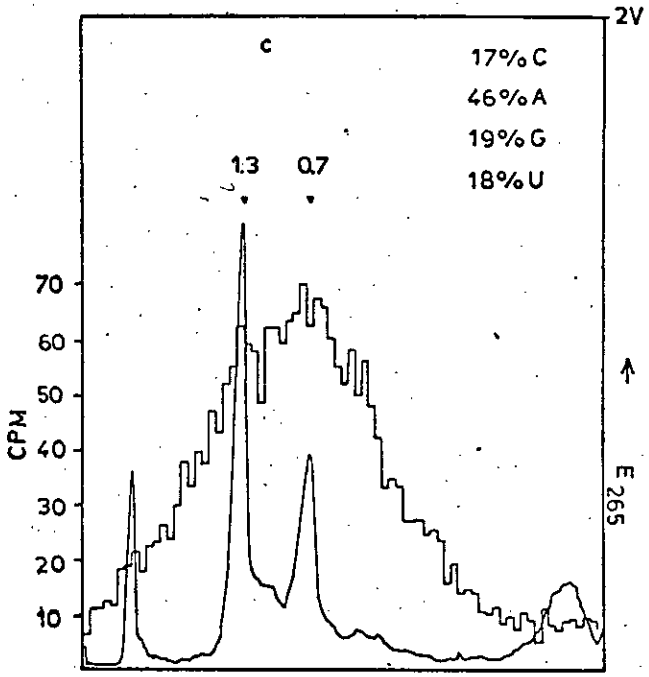

c
17% C
46% A
19% G
18% U
1.3
0.7
2V
CPM
E 265

FIGURE 12 : Buoyant density gradient centrifugation of total cytoplasmic RNP in CsCl

Cultures of explants were grown for 48hr, pulse labelled for 1hr in ^{32}P phosphate ($50\mu Ci\ ml^{-1}$). The ribosome pellet (2hr, 65000rpm) was prepared, resuspended in Triethanolamine-HCl (pH8.0), 50mM KCl, 10mM $MgCl_2$ (Tet K M) and fixed with formaldehyde (6%) for 16hr at 0^oC. The sample was then layered over a preformed 1.35 to 1.65 $g\ an^{-3}$ gradient of CsCl in Tet K M buffer containing 4% formaldehyde which was then centrifuged at 35000rpm for 40hr at 2^oC (MSE 6 x 5.5 rotor)

—— : E_{254nm}

•——• : ^{32}P cpm

X——X : density gradient ($g\ cm^{-3}$)

FIGURE 13 : Buoyant density gradient centrifugation in CsCl of a <80S fraction of artichoke cytoplasmic RNP

40hr cultures of artichoke were labelled for 1hr in ^{32}P phosphate ($50\mu Ci\ ml^{-1}$) and the ribosome pellet (2hr 65000rpm) layered over 10-60% sucrose gradients. After centrifugation for 70min (40000rpm 6 x 14 rotor) the gradients were fractionated and the region corresponding to fraction X of figure 7 dialysed against Tet K M. The dialysate was fixed in 6% formaldehyde and the sample layered over a cushion of CsCl in Tet K M + 4% formaldehyde and centrifuged for 3 days (25000rpm MSE 6 x 14 rotor).

—— : E_{254nm}

•——• : ^{32}Pcpm

X——X : density gradient

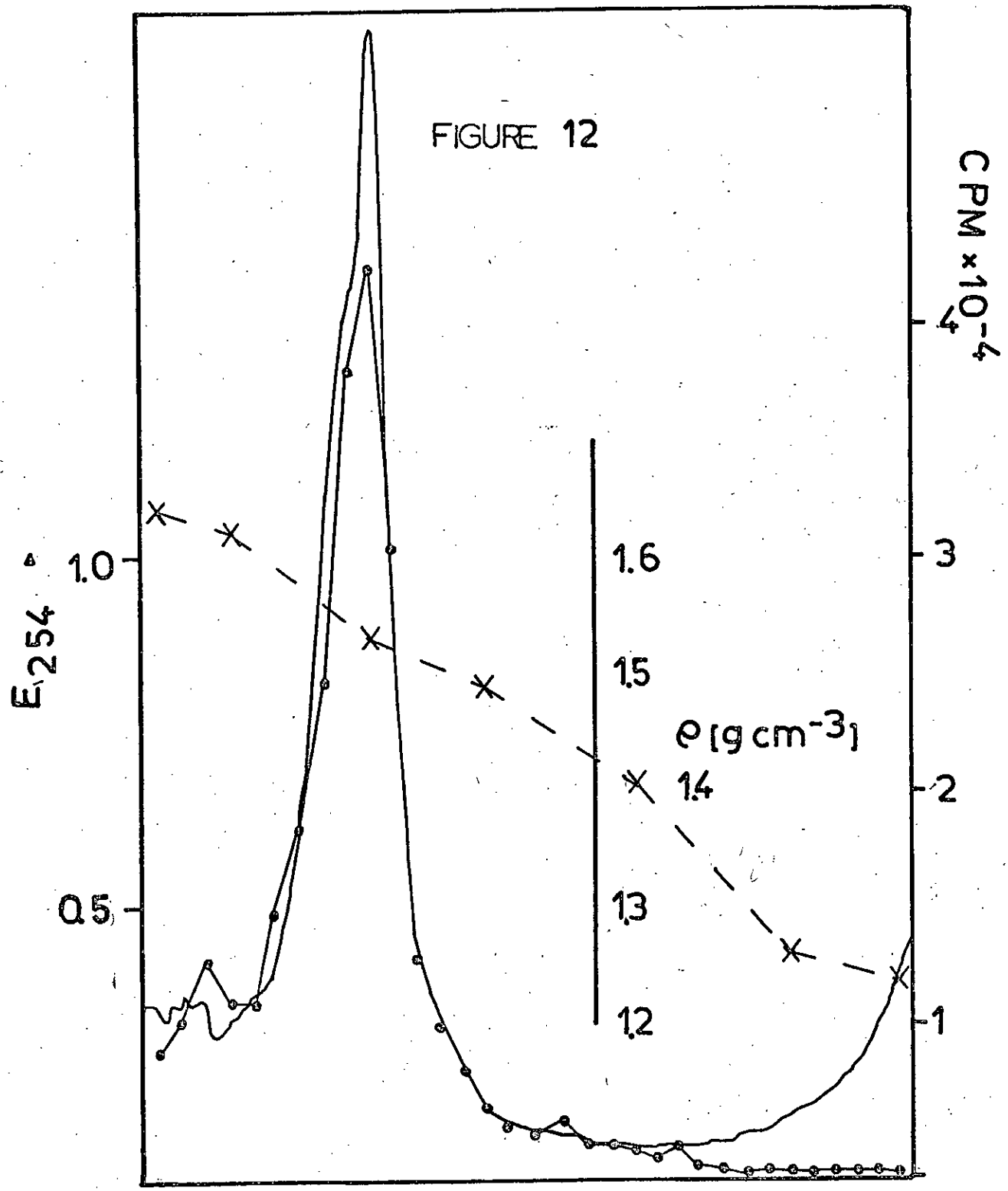
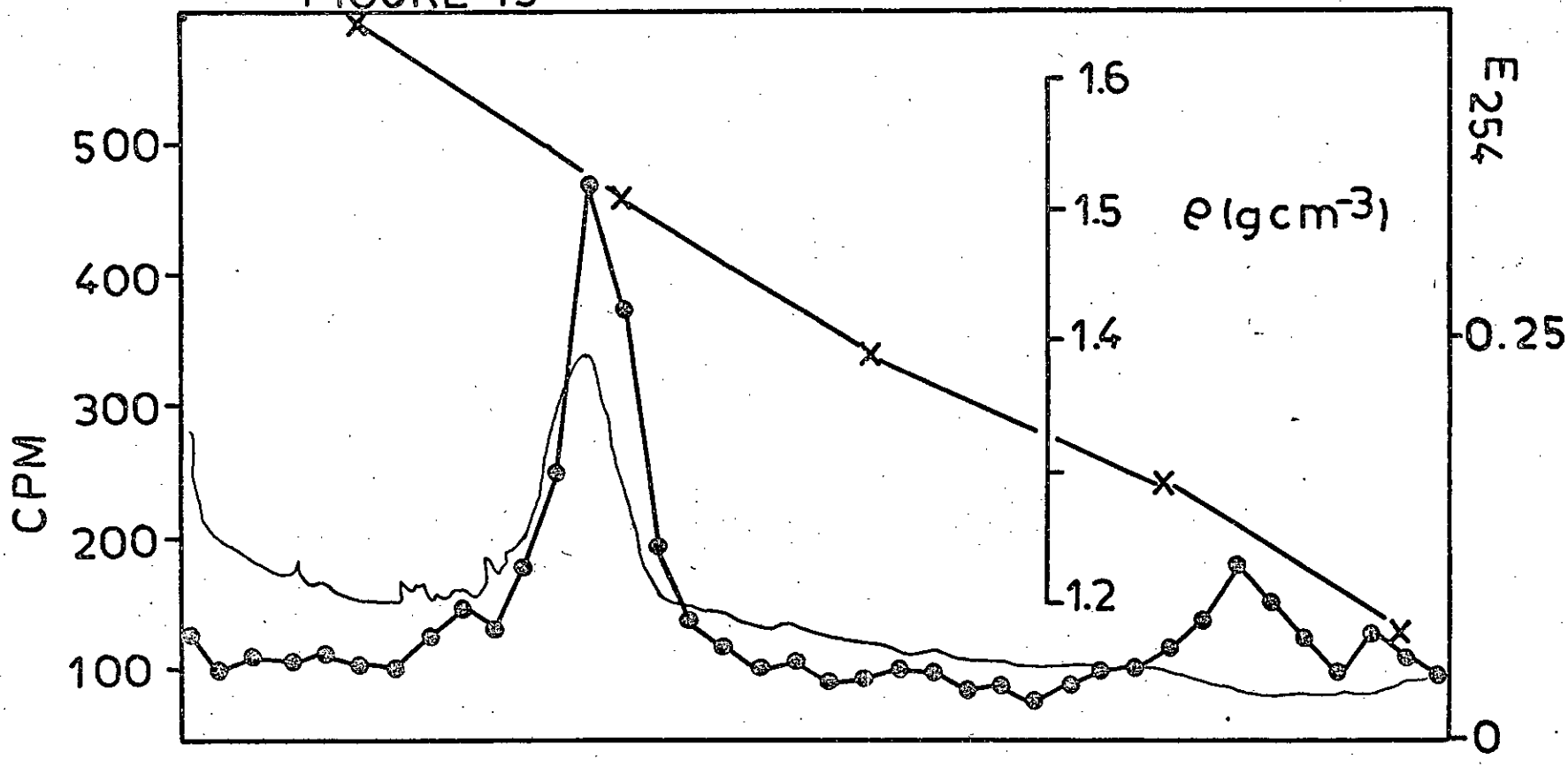


FIGURE 12
E 254 Δ
1.0
0.5
1.6
1.5
ρ [g cm⁻³]
1.4
1.3
1.2
CPM ×10⁻⁴
4
3
2
1

FIGURE 13



radioactivity profile paralleled the UV absorbing profile and there was one major low specific activity component ($P = 1.51g\ cm^{-3}$). The high specific activity region at $P = 1.27g\ cm^{-3}$ was lighter than the reported density for informosomes or hnRNP ($\geq 1.4g\ cm^{-3}$) and may, in fact, be phosphorylated protein. Protein bands at $1.26g\ cm^{-3}$ in CsCl (Hamilton, 1971). When a ribosome pellet prepared from explants labelled for 1hr was centrifuged through a sucrose gradient (fig. 7), 88% of the heterodisperse RNA was found to co-sediment with 80S or heavier RNP. On the basis of this, and the buoyant density evidence, it is seen that nearly all of the rapidly labelled (1hr) heterodisperse RNA of the ribosome pellet satisfied an important criterion of mRNA, namely, that it was structurally associated with ribosomes.

On the basis of these properties, the heterodisperse RNA of the ribosome pellet was equated with mRNA. 50% of the mRNA from 1hr labelled tissue failed to bind to poly U Sepharose, whereas only 6% of the total precipitable poly A was recovered in the unbound fraction (Table III). This suggested that some of the mRNA lacked poly A or contained very short poly A. Thus mRNA not bound to the column is referred to as poly A(-)mRNA.

8. Purification of mRNP

In order to study the kinetic and other properties of poly A(-) mRNA in the absence of rRNA, which is the major component of cytoplasmic poly A(-)RNA, it was necessary to devise a method of purification of mRNA which was independent of the poly A content. The high protein : RNA ratio of animal mRNP compared to ribosomes (e.g. Schoechtman and Perry, 1972), was likely to be a useful property in this respect.

The buoyant density in CsCl of RNP is dependent on the protein : RNA ratio, as proteins have a density of 1.26g cm^{-3} and RNA a density of >1.9g cm^{-3}. Artichoke ribosomes were dissociated, fixed and centrifuged to equilibrium in CsCl in order to demonstrate whether the mRNP had a similar protein : RNA ratio and therefore consequent buoyant density properties to animal mRNP. The mRNP in the ribosome pellet prepared from 1 hr pulse labelled explants were identified as high specific activity components on the CsCl gradients. Figure 14a shows a puromycin dissociated ribosome pellet centrifuged to equilibrium in CsCl and demonstrates that mRNP banded heterogenously between 1.33 and 1.44g cm^{-3} with a component denser than 1.60g cm^{-3}. The ribosome subunits were identified as the UV absorbing component which is a homogenous band of density 1.50g cm^{-3}. Comparison of fig. 14a with the CsCl gradient profile of an undissociated ribosome pellet (fig. 12) shows that release of the high specific activity component from the main band was dependent on puromycin -KCl treatment and supports the proposed mRNP status of this component.

The EDTA dissociated ribosomes showed a completely different buoyant density profile in CsCl. The UV absorbing ribosome subunits formed a broad peak between 1.45g cm^{-3} and 1.6g cm^{-3}. There was no separate high specific activity component, but the denser side of the main band had a higher specific activity, suggesting that EDTA released mRNP had a lower protein : RNA ratio than EDTA dissociated ribosome subunits. Centrifugation to equilibrium in CsCl is not, however, useful as a preparative procedure for the isolation of mRNP, since aldehyde fixation of RNP is essential for maintenance of nucleoprotein integrity in CsCl. However, Wilt et al (1973) have reported that unfixed hnRNP from sea urchin would band isopycnically in Cs_2SO_4. Nucleic acids are more hydrated in Cs_2SO_4 than in CsCl, and therefore band at lower densities. Furthermore, the molarity of the Cs_2SO_4 gradient is less than that of CsCl, so that isopycnically banded nucleoprotein is subject to lower ionic conditions than in CsCl. Rather than allow the Cs_2SO_4 gradients to form during centrifugation, and to avoid exposure of RNP to high salt molarity, the unfixed artichoke RNP preparations were layered over preformed Cs_2SO_4 gradients. Figure 15a shows the result of centrifugation to equilibrium in Cs_2SO_4 of unfixed undissociated artichoke ribosomes. The tissue had been incubated in ^3H uridine for 24hr and ^{32}P phosphate for 1hr immediately before extraction, so that fractions enriched for mRNA would be detected by a low $^3H/^{32}P$ ratio. The UV absorbing material with a density of approximately 1.3g cm^{-3} was not associated with radioactivity, varied between experiments and was therefore probably not nucleic acid. The prominent UV peaks of density 1.50 to 1.54g cm^{-3} contained most of the radioactivity. A small peak of density 1.61g cm^{-3} was the same density

FIGURE 14 : Buoyant density centrifugation in CsCl of dissociated ribosomes from artichoke explants

2 day old cultured explants were labelled for 1hr with ^{32}P phosphate ($50\mu Ci\ ml^{-1}$) and the ribosome pellet was prepared (65000rpm 2hr). After dissociation in triethanolamine containing buffer the samples were fixed in 6% formaldehyde and centrifuged in CsCl gradients.

a) puromycin dissociated ribosome pellet centrifuged for 3 days at 4^{o} and 35000rpm in MSE 6 x 14 rotor

b) EDTA dissociated ribosome pellet centrifuged in a preformed gradient for 40hr at 35000rpm and 4^{o} in an MSE 6 x 5.5 rotor

——— : E_{254}

•———• : ^{32}Pcpm

X———X : density gradient

FIGURE 15 : Buoyant density centrifugation of unfixed artichoke RNP in $Cs_2\ SO_4$

48hr cultures of artichoke explants were pulse labelled with ^{3}H uridine ($10\mu Ci\ ml^{-1}$) for 24hr and ^{32}P phosphate ($50\mu Ci\ ml^{-1}$) for 1hr before extraction. Ribosome pellets were prepared (65000rpm 2hr) and centrifuged in preformed 1.35 to $1.65 cm^{-3}$ Cs_2SO_4 gradients for 40hr at 35000rpm at $0^{o}C$.

a) undissociated, in TKM buffer

b) puromycin dissociated, in 10mM Tris-HCl (pH 8.5 $0^{o}C$), 500mM KCl, 10mM $Mg\ Cl_2$ buffer

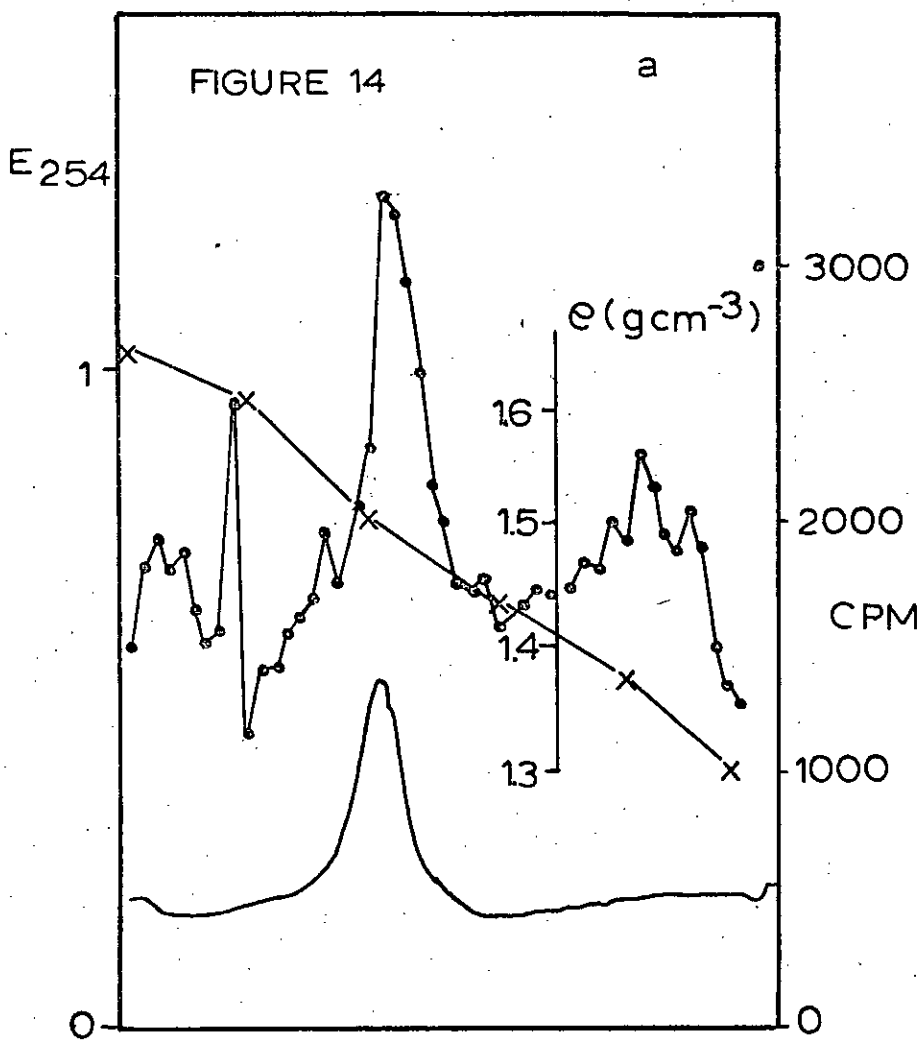
c) EDTA dissociated, in 100mM Tris-HCl, 50mM KCl, 15mM EDTA (pH 8.5, $0^{o}C$)

d) pulse labelled ribosome pellet RNA in TKM

⌣ : E_{254nm} 0———0 : ^{32}P cpm Δ———Δ : ^{3}H cpm

●● : $^{3}H/^{32}P$ X———X : density gradient g cm^{-3}

FIGURE 14


a
E254
1
0
3000
2000
1000
0
CPM
ρ(gcm-3)
1.6
1.5
1.4
1.3

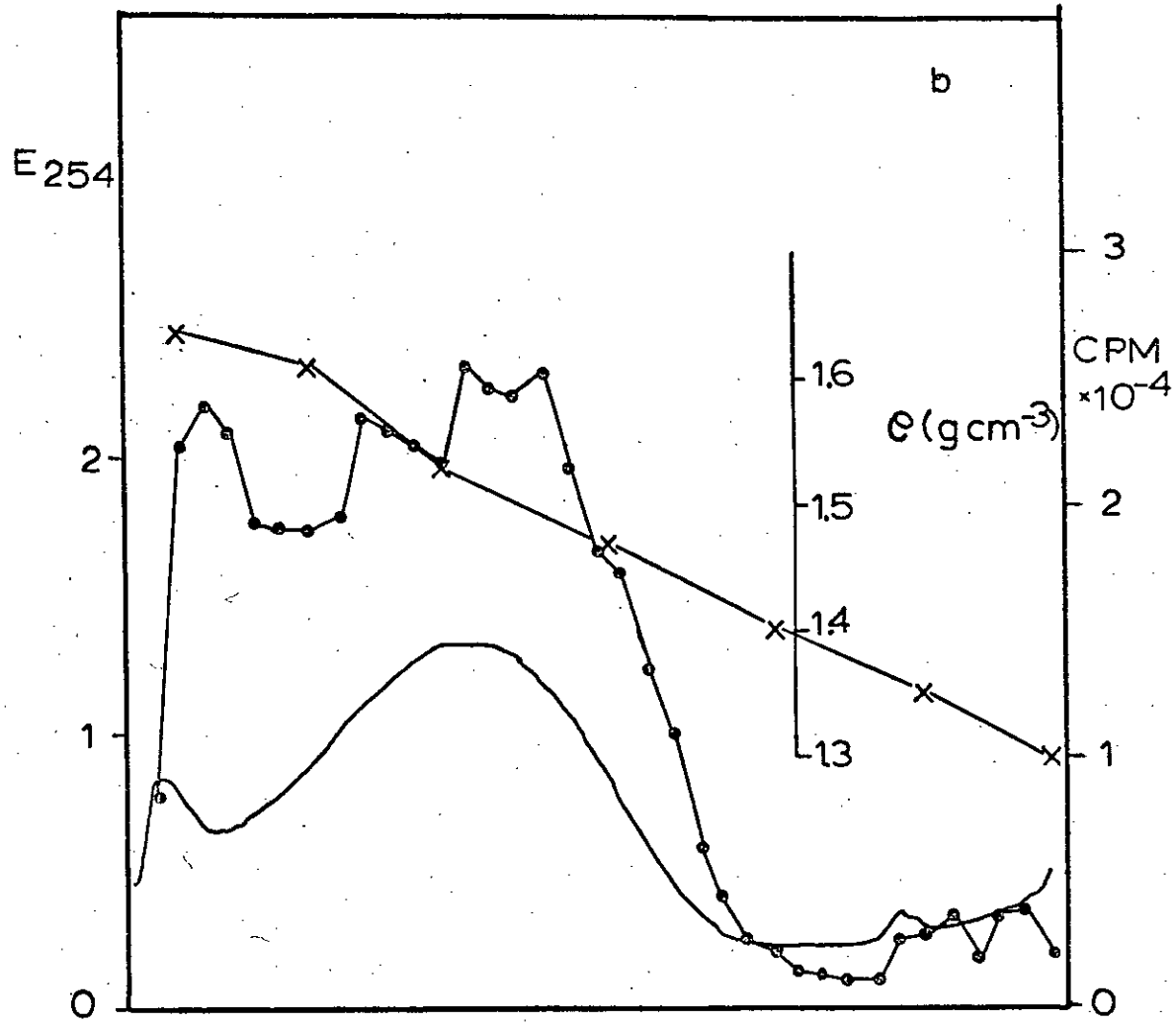
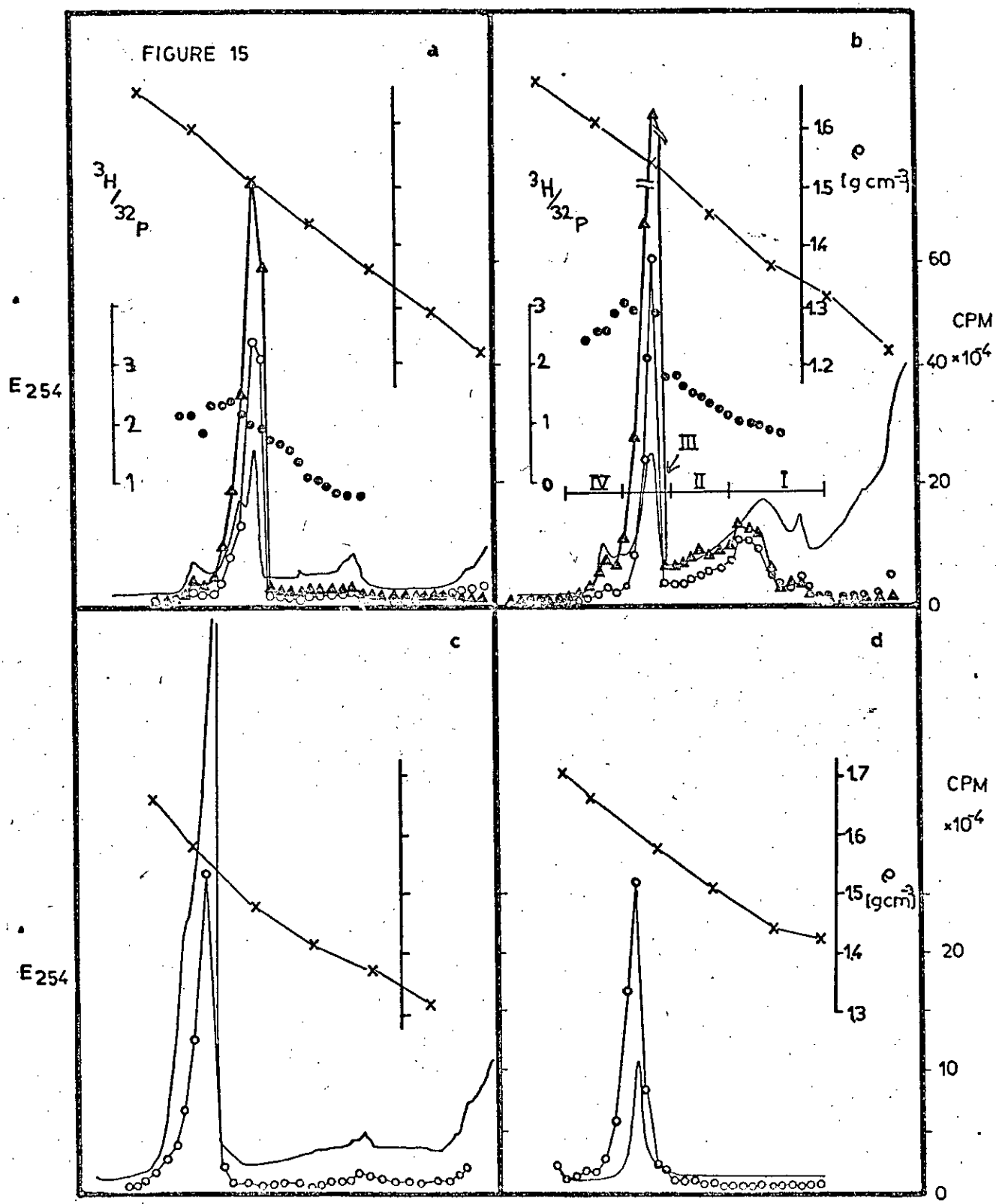

b
E254
2
1
0
3
2
1
0
CPM
×10-4
ρ(gcm-3)
1.6
1.5
1.4
1.3

FIGURE 15



as purified pulse (1hr) labelled cytoplasmic artichoke RNA (fig. 15d) and was therefore identified as free RNA. All RNP preparations showed evidence of a 1.60 to 1.61g cm^{-3} component, suggesting that, despite precautions, salt disruption of nucleoprotein was occurring. A puromycin dissociated ribosome pellet contained three components in the Cs_2SO_4 gradient (fig. 15b). In addition to the main peak (P = 1.54g cm^{-3}) and the 'RNA' peak (P = 1.61g cm^{-3}) there was also a heterogenous less dense component (P = 1.35 to >1.50g cm^{-3}). The $^3H/^{32}P$ ratio of this less dense component was only 1, compared with a ratio of 3 for the main UV absorbing ribosome peak, which suggested that the less dense region was not pure mRNP. Messenger RNA comprised approximately 50% of the ^{32}P radioactivity in a ribosome pellet after a 1hr pulse, while constituting only about 2% of the RNA, so the difference in $^3H/^{32}P$ ratio between ribosomes and pure mRNP should approach fifty told. The RNAs prepared from fractions I to IV (fig. 15b) were analysed electrophoretically in order to determine the extent of mRNP fractionation on Cs_2SO_4. The results, summarised in table IV, show that no fraction was sufficiently enriched in mRNA for this to be a useful method of purifying mRNP. Figure 15c shows that an EDTA dissociated ribosome pellet formed a single peak (P = 1.57g cm^{-3}) after centrifugation to equilibrium in Cs_2SO_4, with a shoulder of free RNA at 1.61g cm^{-3}, and indicates that mRNP had not been separated from ribosomes.

Solutions of Metrizamide are non ionic and relatively non-viscous (compared to sucrose) and form a gradient in a centrifugal field (Rickwood and Birnie, 1975). The factors determining the density of a nucleoprotein are complex, being dependent on the protein : nucleic acid ratio and also the ionic conditions of the centrifugation buffer (Rickwood and Birnie, 1975), (Buckingham and Gros, 1975). The undissociated ribosome pellet in TKM buffer formed a heterogenous band with a buoyant density about 1.3g cm^{-3} (fig. 16a). The composition

TABLE IV - The RNA content of cytoplasmic nucleoprotein after Cs_2SO_4 fractionation

Fraction	1.3×10^6 rRNA (%)	0.7×10^6 rRNA (%)	mRNA (%)
I	21	14	64
II	16	6	78
III	28	17	56
IV	14	28	58

Fractions I to IV from a gradient run parallel to that illustrated in figure 15b were collected, diluted and the RNP collected by centrifugation at 65000rpm (MSE 10 x 10 Ti rotor) for 4hr. RNA was prepared from the pellets, collected by ethanol precipitation with carrier RNA and electrophoresed on 2.3% polyacrylamide gels. The content of heterodisperse mRNA in each fraction was estimated from the ^{32}P profile of the gels.

of RNA in fractions I to III (Table V) shows that mRNA and rRNA were distributed throughout the gradient. By comparison, the puromycin dissociated ribosome pellet in dissociation buffer produced a main peak at 1.21g cm^{-3} with a heavy shoulder (P = 1.24g cm^{-3}) (fig. 16b) when centrifuged in Metrizamide. Pulse labelled rRNA and mRNA were distributed in the light and heavy regions of this peak (table V). Since RNP density in Metrizamide depends on the ionic conditions (Rickwood and Birnie, 1975), I investigated the effect of changing the K^+/Mg^{++} ratio. The results in figure 17a-c show that there was no obvious effect of increasing the K^+/Mg^{++} ratio from 25 (fig. 17a), through 50 (fig. 17b) to 75 (fig. 17c) and in all cases the main peak banded at 1. 25g cm^{-3} with a heavy shoulder.

An EDTA dissociated ribosome pellet, when centrifuged in Metrizamide in EDTA dissociation buffer, produced a heterogenous peak of density 1.17 to 1.22g cm^{-3} (fig. 18a). The distribution of RNA species through the peak, shown by electrophoresis of nucleic acid from fractions 1, 2 and 3 of figure 18a suggested that there was real fractionation of ribosomal subunits and mRNP (table V and fig.19). Messenger RNA, which was identified on the basis of a heterodisperse electrophoretic profile, was the major component of pulse labelled RNA in fraction 2 (table V and figure 19b). Fraction 2 also contained 0.7×10^6 rRNA. The ribosome subunits were more prominent labelled RNP components of the fractions 1 and 3 (fig. 19a and c and table V). The effect of including Mg^{++} in the centrifugation buffer (10mM $MgCl_2$) and omitting EDTA was to increase the density of the main peak from 1.23 to 1.25g cm^{-3} (fig. 18b). The 1.3×10^6 rRNA was the main RNA component of the dense side of the peak (fraction 6, table V) whereas in the EDTA gradient (fig. 18a) it was the main component of the light side (fraction 1). Messenger RNA was still the main component

TABLE V - The RNA content of cytoplasmic nucleoprotein after Metrizamide fractionation

Fraction	1.3×10^6 rRNA (%)	0.7×10^6 rRNA (%)	mRNA (%)
Total ribosome pellet	16	21	63
Undissociated ribosome pellet (fig. 16a)			
I	24	28	48
II	14	17	69
III	19	22	59
Puromycin dissociated ribosome pellet (fig.16b)			
IV	22	17	61
V	12	10	78
EDTA dissociated ribosome pellet (fig. 18a, 19)			
1	22	5	73
2	0	8	92
3	20	18	62
EDTA dissociated ribosome pellet (fig.18b)			
4	9	16	75
5	9	5	86
6	32	20	48

Gradients which were run parallel to those indicated were fractionated and the fractions corresponding to those indicated were collected. The RNA was prepared from these fractions and the RNA was analysed on polyacrylamide gels. The composition of these fractions was determined from the radioactivity profile of these gels.

FIGURE 16 : Buoyant density centrifugation of unfixed artichoke RNP in Metrizamide

48hr old cultures of artichoke explants were labelled for 1hr in ^{32}P phosphate (100μCi ml^{-1}) and harvested. The ribosome pellet (65000rpm 2hr) was resuspended and added to stock Metrizamide either with or without dissociation to make a solution of density 1.22g cm^{-3}, then centrifuged at 35000rpm for 70hr at $0^{o}C$.

a) undissociated sample. Centrifugation buffer was TKM

b) puromycin dissociated sample in 10mM Tris-HCl (pH 8.5) 50mM KCl, 10mM $MgCl_2$.

●——● : ^{32}P cpm

X——X : density gradient

FIGURE 17 : The effect of KCl concentration on the buoyant density of puromycin dissociated artichoke RNP in Metrizamide

48hr old cultures of artichoke explants were labelled for 1hr in ^{32}P phosphate (100μCi ml^{-1}) and extracted. The ribosome pellet (65000rpm 2hr) was dissociated with puromycin-KCl and centrifuged at 35000rpm for 70hr in Metrizamide containing 10mM Tris-HCl (pH 8.5) 10mM Mg Cl_2 and

a) 250mM KCl,

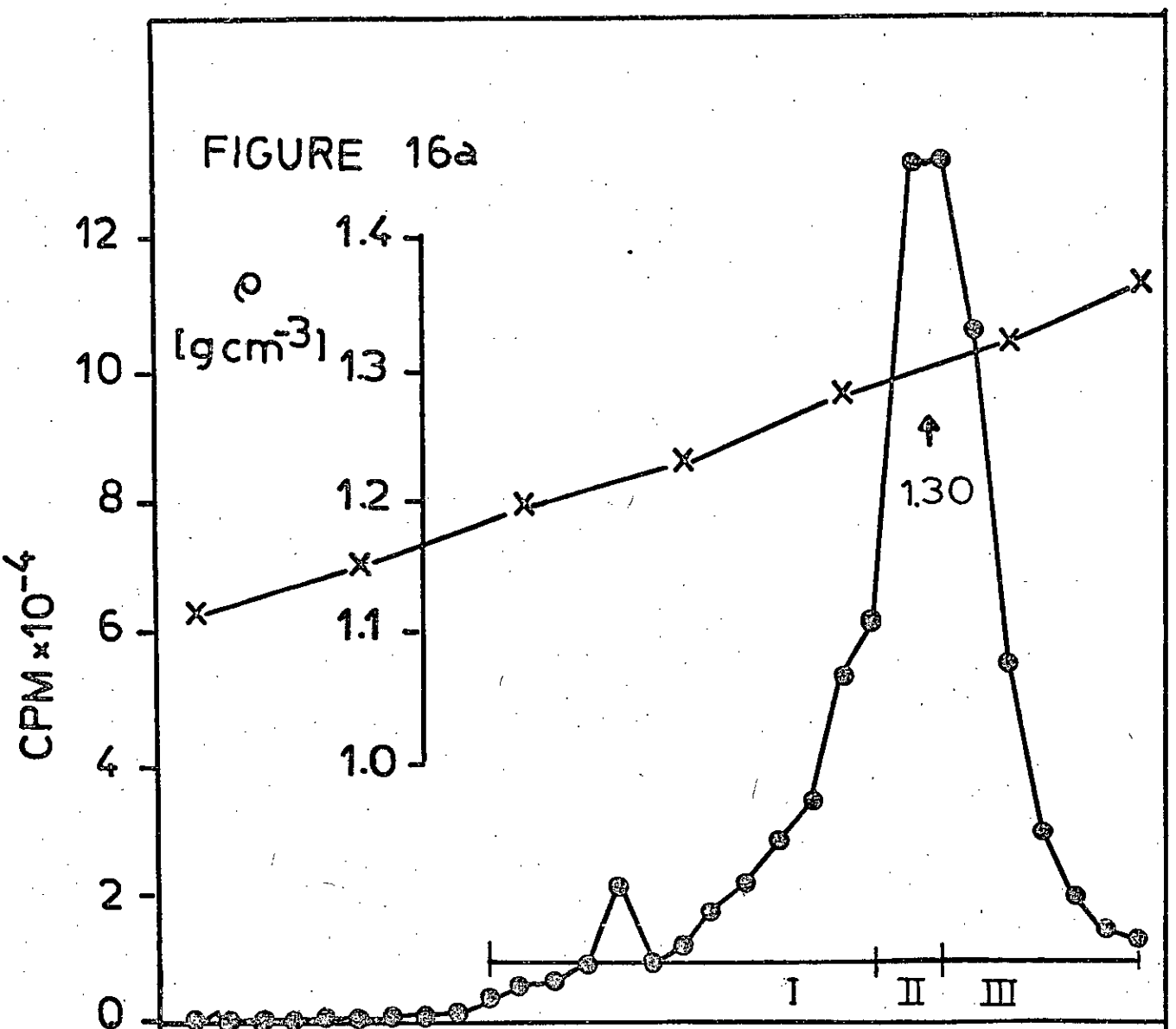
b) 500mM KCl,

c) 750mM KCl

●——● : ^{32}P cpm

X——X : density gradient

FIGURE 16a


ρ [g cm⁻³]
1.4
1.3
1.2
1.1
1.0
CPM ×10⁻⁴
12
10
8
6
4
2
0
1.30
I
II
III

b

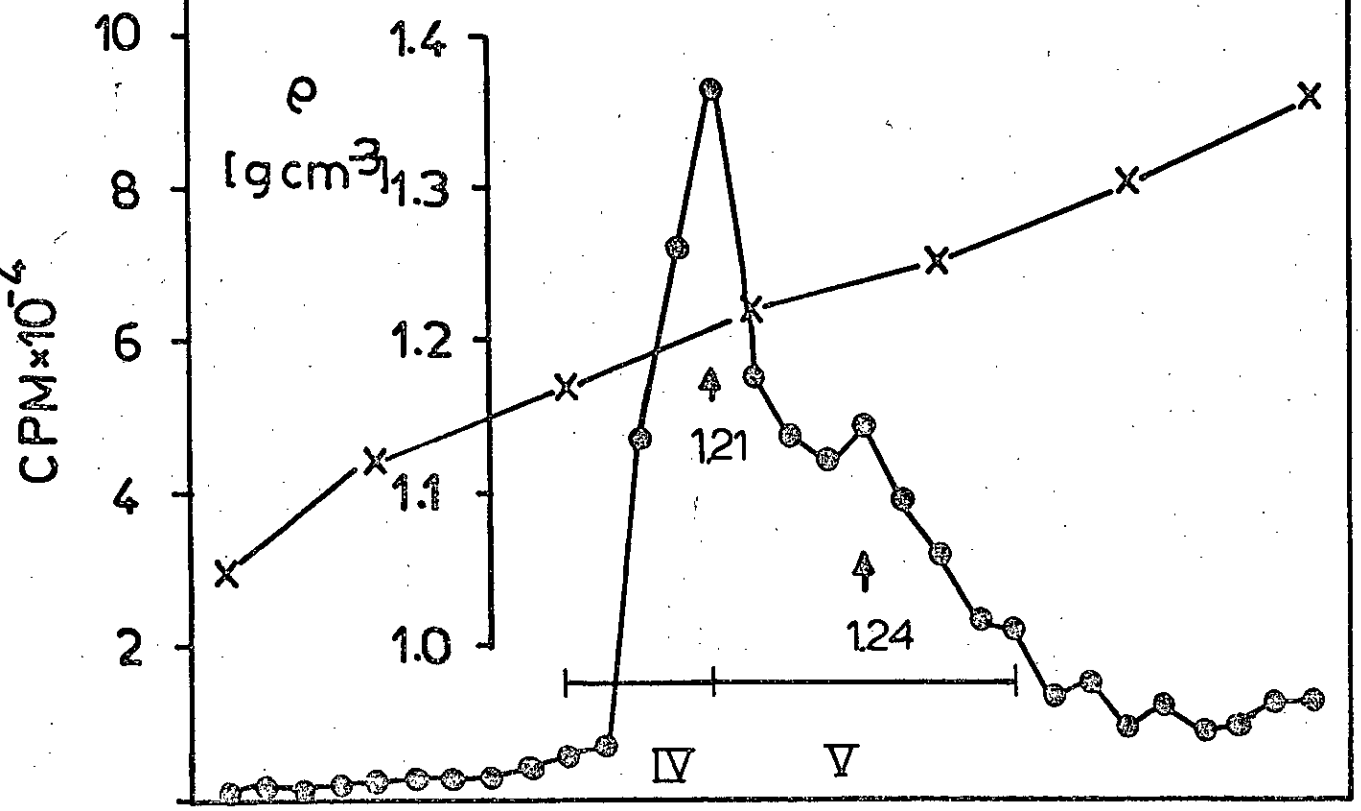

ρ [g cm⁻³]
1.4
1.3
1.2
1.1
1.0
CPM ×10⁻⁴
10
8
6
4
2
1.21
1.24
IV
V

FIGURE 17

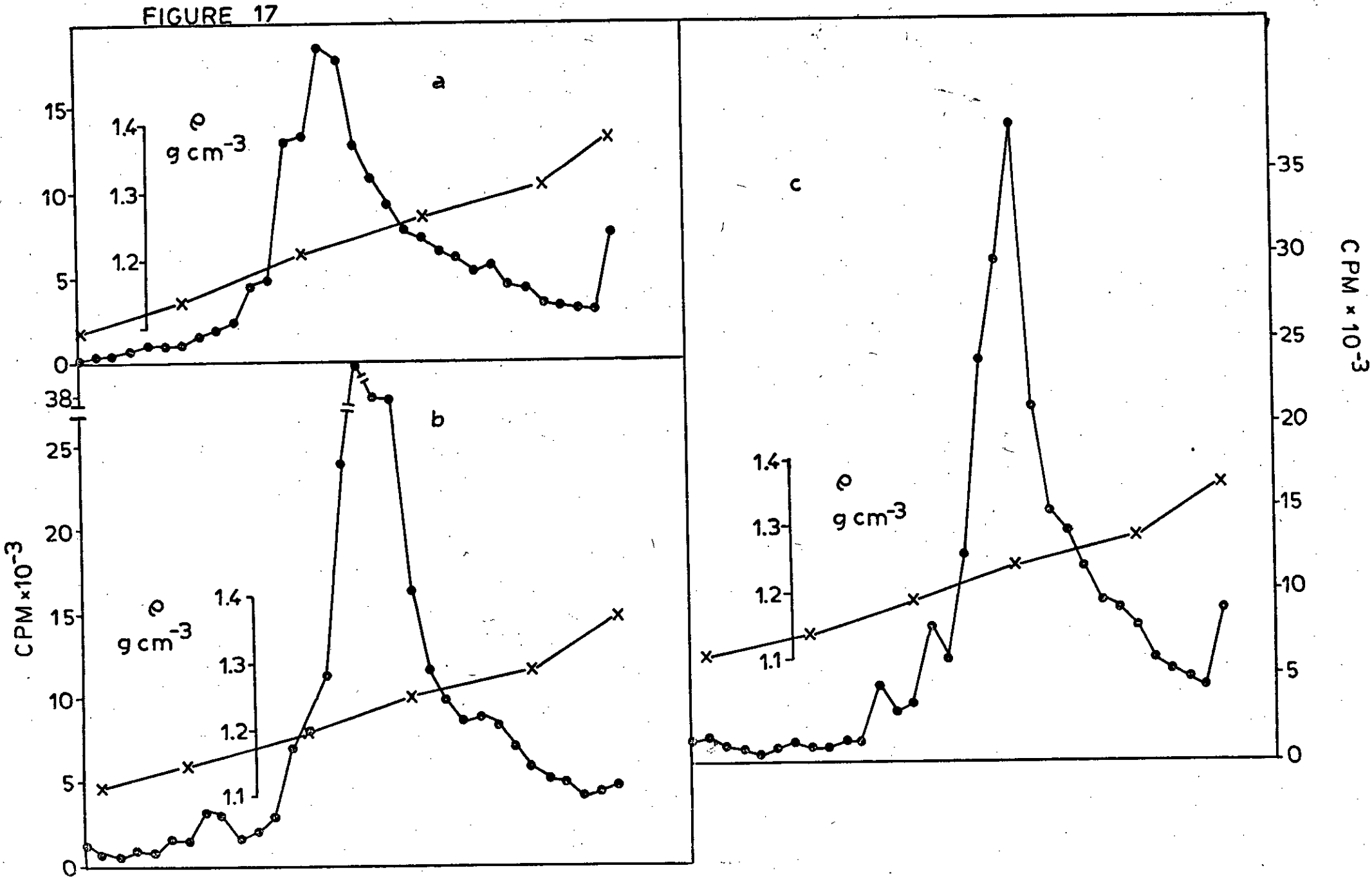


FIGURE 18 : Buoyant density centrifugation in Metrizamide of EDTA dissociated artichoke RNP. The effect of Mg^{++}

48hr old cultures of artichoke explants were labelled for 1hr in ^{32}P phosphate ($100\mu Ci\ ml^{-1}$) and harvested. The ribosome pellet (65000rpm 2hr) was resuspended in 100mM Tris-HCl (pH 8.5 $0^{o}C$), 50mM KCl, 15mM EDTA, 0.2% diethylpyrocarbonate and centrifuged in a Metrizamide solution ($P=1.22g\ cm^{-3}$) for 40hr at 35000rpm. The gradients were fractionated and fractions halved, one half being used for radioactivity determination and the other for RNA analysis (figure 19).

a) centrifugation buffer was 100mM Tris-HCl KCl, 15mM EDTA (pH 8.5 $0^{o}C$)

b) centrifugation buffer was 100mM Tris (pH 8.5 $0^{o}C$) 50mM KCl and 10mM $MgCl_2$

•——• : ^{32}P cpm

X——X : radioactivity

FIGURE 19 : The rapidly labelled RNA from a Metrizamide gradient of EDTA dissociated artichoke RNP

RNA was extracted from fractions 1 (a), 2 (b) and 3 (c) of figure 18a by the addition of 0.1% SLS and 0.15M sodium acetate, and collected by ethanol precipitation with carrier wheat RNA. The RNA was analysed by electrophoresis on 2.3% polyacrylamide gels (50V) for 3hr.

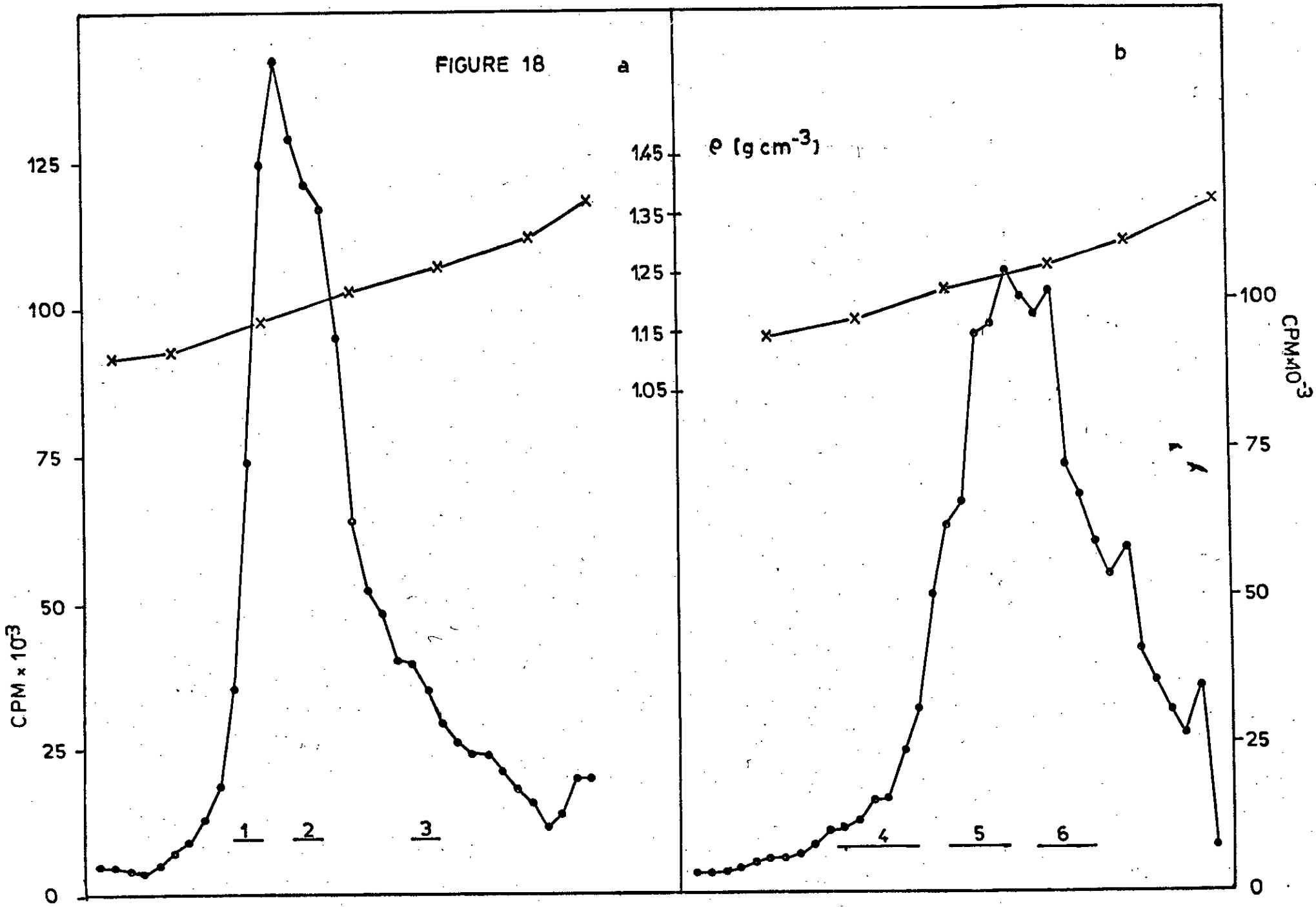


FIGURE 18
a
b
125
100
75
50
25
0
CPM x 10^3
1.45
1.35
1.25
1.15
1.05
ρ (g cm^-3)
100
75
50
25
0
CPM×10^-3
1
2
3
4
5
6

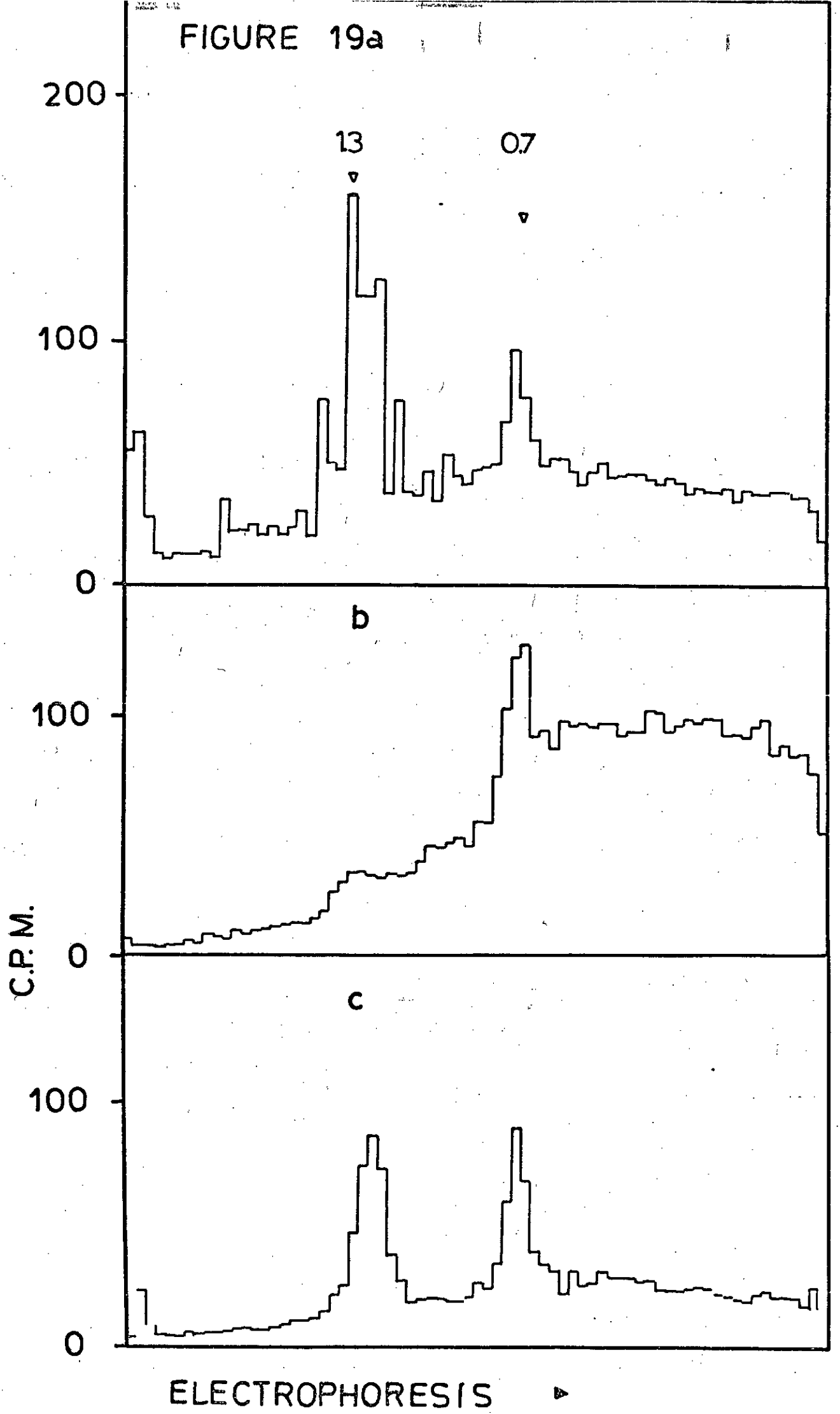


FIGURE 19a
200
100
0
1.3
0.7
b
100
0
C.P.M.
c
100
0
ELECTROPHORESIS

of the central region of the peak (fraction 5 table V). However, despite the use of RNAase inhibitor (DEP 0.2%), the mRNA from both gradients (fig. 18a and b) appeared degraded relative to poly A(+) mRNA (Fig. 11c) and relative to mRNA from puromycin dissociated ribosomes after fractionation on Metrizamide. This observation was repeated twice, so was unlikely to have been due to exogenous RNAase. Fractionation of mRNA by methods which allow degradation of the RNA is of little use since subsequent separation into poly A(+) and (-) RNA depends on molecular integrity. Some degradation of rRNA must also have occurred, since there was insufficient rRNA in the fractions 1 to 6 of figure 18 to account for the rRNA in the total ribosome pellet (table V).

As these data suggested that the properties of mRNP in Metrizamide may depend on the method of ribosome dissociation, the properties of mRNP released by in vivo run off of ribosomes were studied in the hope that the mRNP would be separable from ribosomes. Ribosomes were run off mRNP in vivo by adding MDMP, which is a specific inhibitor of initiation on 80S ribosomes (Weeks and Baxter, 1972), to the cultures 10 min before harvesting. The double label technique (^{32}P phosphate 1hr, ^{3}H uridine 20hr) was employed to enable detection of fractions enriched for mRNP. Released mRNP would probably sediment as slowly as 40S or even slower (e.g. Ajtkhozhin and Akhanov, 1974) so the post mitochondrial supernatant was centrifuged for 6hr to ensure recovery of such RNPs (fig. 6). Figures 20a and b show the results of centrifuging this 6hr ribosome pellet in a sucrose gradient. The polysome structures were absent from the MDMP treated explants, showing that MDMP had effectively 'run off' the polysomes. The Metrizamide gradient profiles of ribosome pellets from MDMP treated and control tissue were quite similar with a main component of density 1.28 to 1.31g cm^{-3}. There was no evidence for a major component (50%

FIGURE 20 : The effect of MDMP treatment of explants on cytoplasmic RNP

44hr cultures were labelled for 20hr with 10 Ci ml^{-1} ^{3}H uridine and for 1hr with 100 Ci ml^{-1} of ^{32}P phosphate. MDMP (10^{-3}M) was added to the culture for 10 minutes immediately before harvesting. RNP were pelleted from the postmitochondrial supernatant by centrifugation (65000rpm 6 hr) through 1M sucrose, and resuspended in 20mM Hepes (pH 8.0 $0^{o}C$). An aliquot of the suspension was analysed by centrifugation through 10-60% sucrose (40000rpm 70 mins, MSE 6 x 14 rotor) and the remainder was layered over preformed Metrizamide gradients and centrifuged for 40hr at 40000rpm at $0^{o}C$ (MSE 6 x 5.5 rotor). The centrifugation buffer was TKM.

a) Control - sucrose gradient centrifugation

b) MDMP treated tissue - sucrose gradient centrifugation

c) Metrizamide gradient of preparation from control tissue

d) Metrizamide gradient of preparation from MDMP treated tissue

——— . ——— : ^{32}P cpm

+ ——————— + : ^{3}H cpm

 : E_{254nm}

X——————— X : density gradient g cm^{-3}

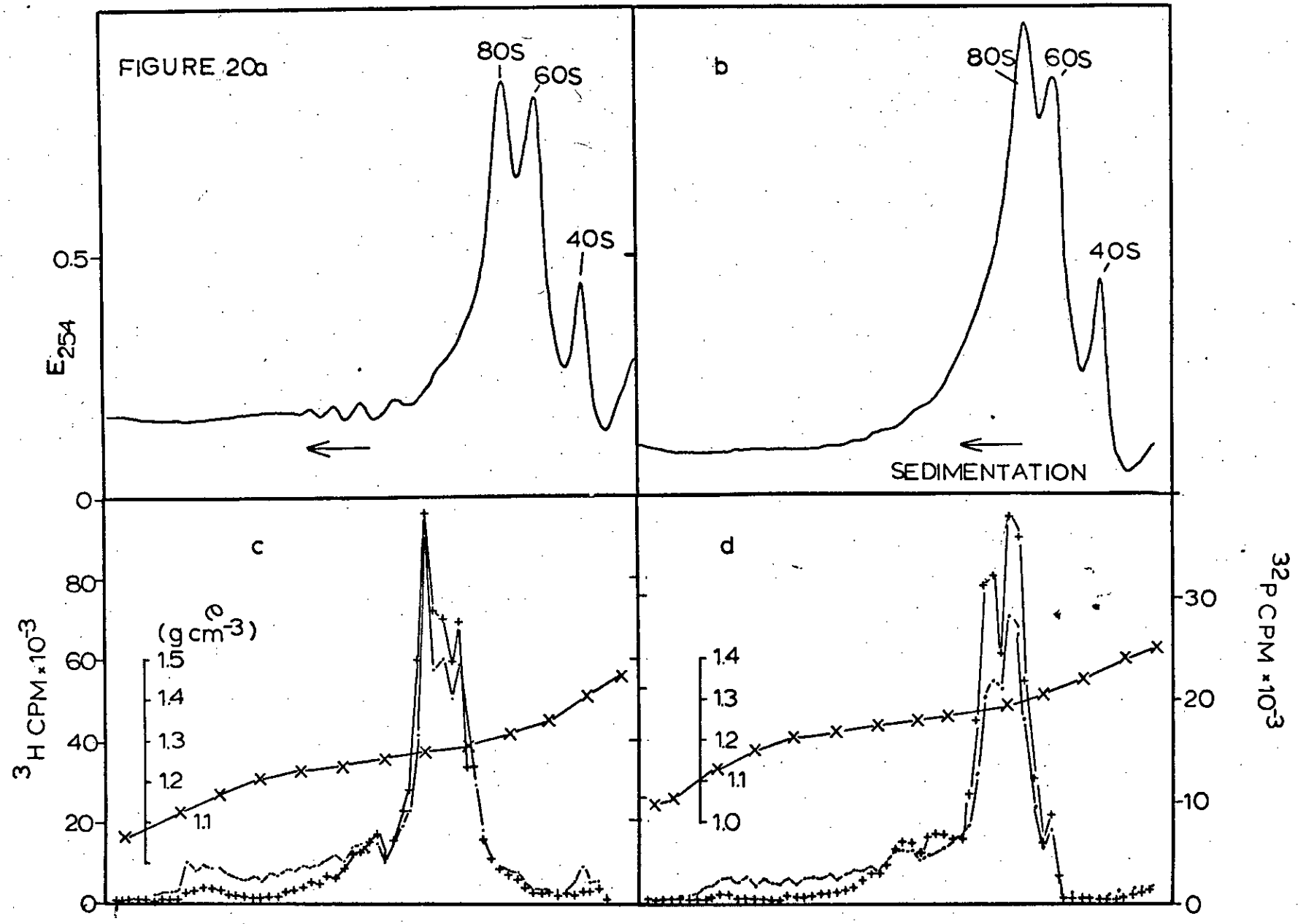


FIGURE 20a
80S
60S
40S
0.5
0
E_{254}
b
80S
60S
40S
SEDIMENTATION
c
d
80
60
40
20
0
^{3}H CPM ×10^{-3}
(g cm^{-3})
1.5
1.4
1.3
1.2
1.1
1.0
30
20
10
0
^{32}P CPM ×10^{-3}

of ^{32}P radioactivity) with a low $^{3}H/^{32}P$ ratio which may be a mRNP component. The mRNP may therefore, either have remained associated with the ribosomes (Schoechtman and Perry, 1972) or may have the same buoyant density as the ribosomes and ribosome subunits.

Goldberg et al (1973) were able to isolate mRNA from sea urchin embryos by isolating and then dissociating the polysomes and collecting the fraction which sedimented more slowly than the small ribosome subunit in a sucrose gradient. As all of the rapidly labelled heterodisperse RNA in the artichoke ribosome pellet was thought to be mRNA, it was not necessary to prefractionate the polysomes. When the ribosome pellet from explants labelled for 1hr in ^{32}P phosphate was dissociated with puromycin and centrifuged in a sucrose gradient made in the dissociation buffer, the mRNP, which were identified as the high specific activity component, sedimented heterogenously across the gradient with a substantial fraction sedimenting more rapidly than the large ribosome subunit (fig. 21a). However, the sedimentation profile of puromycin dissociated subunits was variable due to the propensity of RNP to aggregate in the high salt used in this method (500mM) and the high specific activity component (>60S) shown in figure 21a was not a consistent feature of these profiles.

The sedimentation profile of EDTA dissociated ribosomes which were centrifuged in sucrose gradients containing EDTA dissociation buffer is shown in figure 21b. The high specific activity mRNP (fraction 'X') sedimented more slowly than the ribosome subunits and contained mRNA with a modal size of 0.3×10^{6} daltons. The overall size and yield of mRNA could be increased by centrifuging the dissociated ribosome preparation through a modified sucrose gradient (fig. 22a). Thus the mRNA prepared from fraction 'X' of the gradient shown in figure 22a had a modal molecular weight, determined by co-electro phoresis with artichoke ribosomal RNA (fig. 22b) of

FIGURE 21 : Rate zonal centrifugation of artichoke RNP in sucrose gradients

48hr cultures were labelled for 1hr with 100μCi ml^{-1} of ^{32}P phosphate, harvested and a ribosome pellet was prepared (65000rpm 2 hr). After resuspension with or without dissociation the samples were layered over 10-35% sucrose gradients and centrifuged at 25000rpm at $0^{o}C$ for 16hr (MSE 3 x 25 rotor). Sedimentation values are arbitrary, the '60S' subunit containing 1.3×10^{6} rRNA and the '40S' subunit being the RNP form with 0.7×10^{6} rRNA.

a) puromycin dissociated ribosome pellet centrifuged in 10mM Tris-HCl (pH 8.5 $0^{o}C$), 500mM KCl and 10mM $MgCl_2$ buffer

b) EDTA dissociated ribosome pellet centrifuged in 100mM Tris-HCl, 50mM KCl, 15mM EDTA (pH 8.5 $0^{o}C$)

c) undissociated ribosome pellet centrifuged in TKM buffer

—— : E_{254nm}

●——● : ^{32}P cpm

FIGURE 21

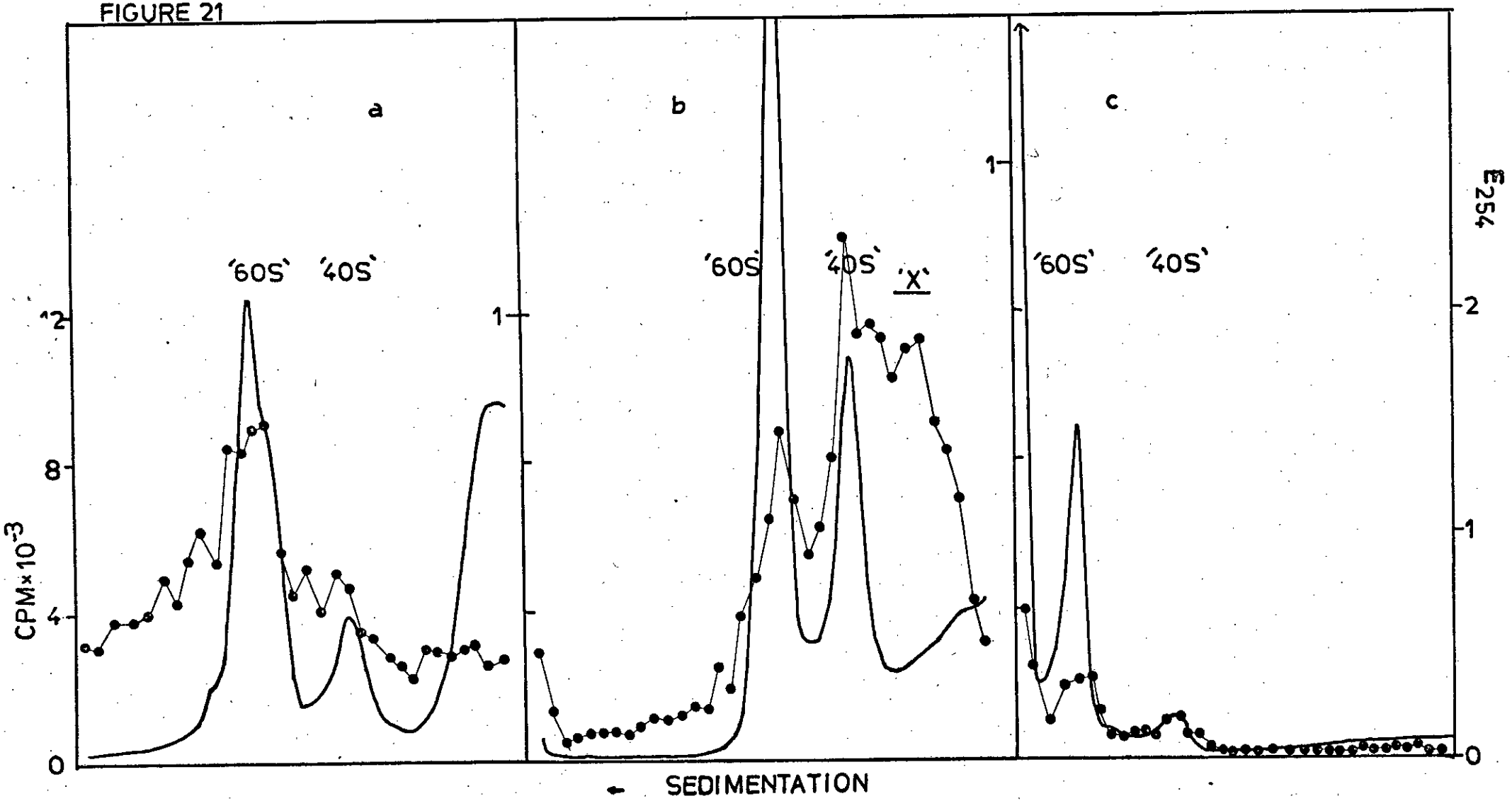


FIGURE 22 : Isolation and properties of EDTA released mRNP from artichokes

60hr cultures of artichoke explants were labelled for 1hr with 100μCi ml^{-1} of ^{32}P phosphate, harvested and the ribosome pellet (65000rpm 2hr) prepared. The pellet was resuspended in 100mM Tris-HCl, 50mM KCl, 15mM EDTA (pH 8.5, $0^{o}C$)(TKE) containing 0.2% DEP and centrifuged for 16hr through a 10 to 50% sucrose gradient in TKE (a). The region 'X' was collected, diluted and mRNP collected by centrifugation at 65000rpm for 6hr. The pellet was either phenol extracted or fixed overnight in TKE with 6% formaldehyde. The phenol prepared RNA was analysed on 2.3% polyacrylamide gels (2hr 15min 50V) (b). The fixed RNP were centrifuged into a preformed 1.35 to 1.65g cm^{-3} CsCl gradient for 40hr at 40000rpm in an MSE 6 x 5.5 rotor at $0^{o}C$ (c)

(a) ——— : E_{254nm}

(b) ⊓⊔ : ^{32}P cpm

(c) ●——● : ^{32}P cpm

——— : E_{254nm}

FIGURE 23 : Long labelled 'mRNP-mRNA'

mRNP-mRNA was prepared from 18hr labelled cultures of artichoke explants (^{32}P 50μCi ml^{-1}, 1 Ci mg^{-1} phosphate) as described in figure 22. The mRNA was fractionated on poly U Sepharose and the unbound (a) and bound (b) RNA was analysed by electrophoresis on 2.3% polyacrylamide gels for 2hr 15 min (50V). 1.3, 0.7 and sRNA denote the positions of marker RNA which was co-electrophoresed in the same gels.

⊓⊔ : ^{32}P cpm

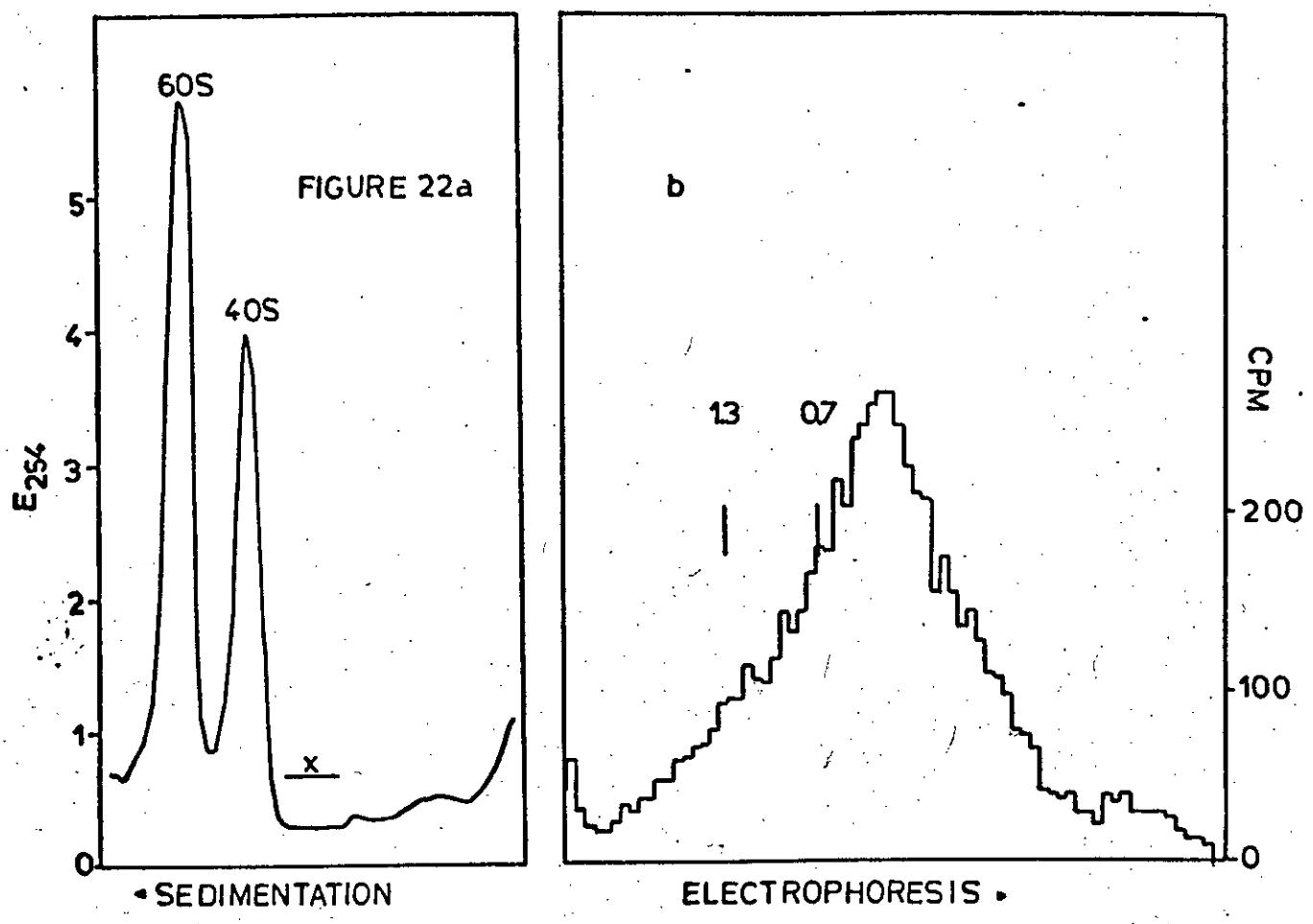
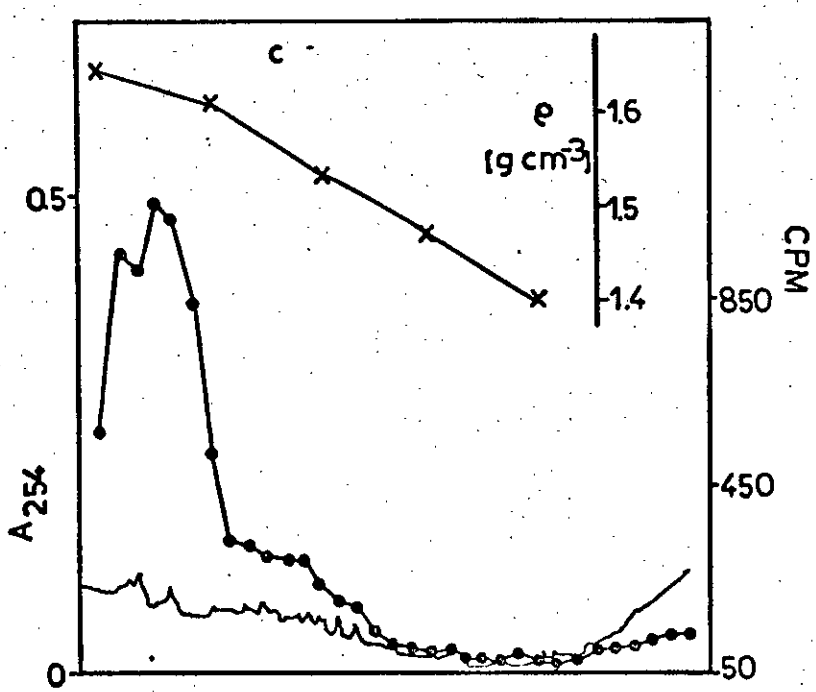

60S
40S
x
5
4
3
2
1
0
E_{254}
SEDIMENTATION
b
1.3
0.7
CPM
200
100
0
ELECTROPHORESIS

FIGURE 22a


c
ρ
(g cm^{-3})
1.6
1.5
1.4
0.5
0
A_{254}
850
450
50
CPM

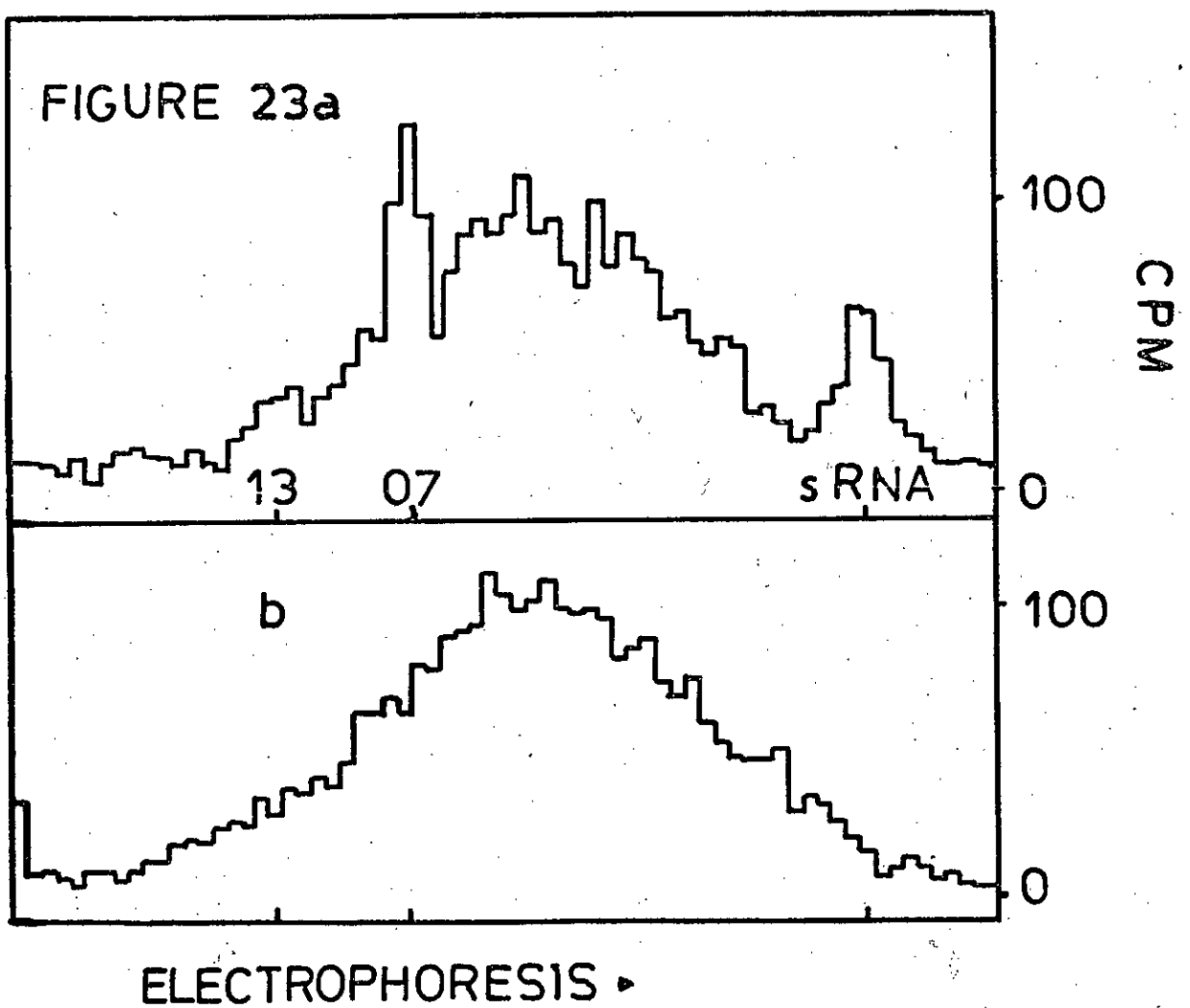


FIGURE 23a
100
0
CPM
13
07
sRNA
b
100
0
ELECTROPHORESIS

0.48×10^6 dalton. From an artichoke ribosome pellet which contained 32250 cpm of mRNA, 9352 cpm (29%) was recovered as mRNA prepared from mRNP (mRNP-mRNA). In order to establish whether any nuclease degradation of RNA was occurring during the centrifugation, a proportion of the total ribosome pellet RNA prepared from explants labelled for 1hr was examined electrophoretically. This profile was compared with that of total RNA recovered off a sucrose gradient after centrifugation an EDTA dissociated ribosome pellet. The two were identical, implying that DEP effectively inactivated ribosome associated RNAase activity during the 16hr centrifugation of the sucrose gradient, butwas not effective during the longer centrifugation (40hr) of Metrizamide gradients (fig. 19). The fact that these 'mRNP' were released by EDTA is further confirmation of their messenger status (Penman et al, 1968). A control gradient (fig. 21c) loaded with undissociated ribosomes extracted from 1hr pulse labelled explants, contained no high specific activity material in the<40S region of the gradient.

The buoyant density of the <40S EDTA released mRNP was 1.60 to 1.64g cm^{-3} (fig. 22c). This is in agreement with the results shown in figure 14b which demonstrate that the EDTA dissociated total ribosome pellet had a higher specific activity in the >1.6g cm^{-3} region of a CsCl gradient than in the <1.6g cm^{-3} region.

The 1hr pulse labelled mRNP-mRNA (fig. 22a) was not contaminated with radioactive rRNA or sRNA (fig. 22b). However, using 18hr pulse labelled mRNP-mRNA, it was possible to detect 0.7×10^6 rRNA and sRNA contaminants on the electrophoretic profile of poly A(-) mRNP-mRNA (fig. 23a). The contaminants amounted to 20% of the total radioactivity. The poly A(+) fraction of 18hr pulse labelled mRNP-mRNA contained heterodisperse mRNA only. In order to establish whether the distribution of poly A(+) and poly A(-) mRNA in mRNP-mRNA was typical of total mRNA, the binding to poly U Sepharose of total ribosome pellet and mRNP-mRNA

both prepared from the same batch of 1hr pulse labelled explants, was compared. In this experiment, 40% of the total polysomal mRNA bound to poly U Sepharose compared with 36% of the mRNP-mRNA. The mRNP-mRNA was therefore considered representative of total mRNA in this respect.

The mRNP fraction was free of impurities which interfered with poly U Sepharose binding. This was shown by assaying the poly A content of poly U Sepharose bound and unbound mRNP-mRNA by digestion with RNAases A and T_1 in high salt. The bound mRNP-mRNA contained 96% of the recovered poly A and was 7.5% poly A, compared with the unbound mRNP-mRNA which was 0.09% poly A and contained 4% of the recovered poly A. Total recovery of poly A was 83%.

9) Kinetics of turnover of poly A(+) and poly A(-) mRNA

Marbaix et al (1974) have suggested that poly A stabilized mRNA which had been introduced into a heterologous living system, the Xenopus oocyte, and Hieter et al (1976) have shown that polyadenylation of RNA increased the RNAase resistance of RNA in vitro. By analysing the labelling kinetics of the mRNP-mRNA, it was possible to compare the stabilities of poly A(+) and poly A(-) mRNA in vivo in a homologous living system.

The experimental approach was to measure the kinetics of the approach to steady state labelling of mRNA (Greenberg, 1973). Explants were incubated in ^{32}P phosphate with 100 x carrier phosphate (1 Ci mg^{-1} phosphate) and mRNP-mRNA was extracted and fractionated by poly U Sepharose into poly A(+) and poly A(-) components. Samples of each preparation were analysed electrophoretically to allow correction of radioactivity in poly A(-) mRNP-mRNA for rRNA and sRNA contamination. The values of radioactivity in poly A(+) and poly A(-) mRNP-mRNA were further corrected for the slight variation in recovery of ribosome

subunits. For exponentially dividing cells in which the stochastic decay of mRNA follows first order kinetics, the radioactivity in mRNA is a function of time (Greenberg, 1973):

$$At/A\infty = 1 - e^{\ln 2 \left(1/T_D + 1/T_{\frac{1}{2}}\right) t} \quad -(1$$

where At = radioactivity per cell of mRNA component at time t

$A\infty$ = radioactivity per cell of mRNA when $t = \infty$

T_D is the cell doubling time (15hr) and $T_{\frac{1}{2}}$ is the half life of the mRNA component

At was approximated as radioactivity of mRNA per unit of ribosome subunit. Figure 24 shows the results of two experiments (• and o) in which the value of $A\infty$ (see below) for each component has in each experiment been normalised to 100% to enable the results of the two experiments to be compared directly. The poly(-) mRNP-mRNA (fig. 24b) reached maximal levels of labelling much earlier than the poly A(+) mRNP-mRNA (fig. 24c) which suggested a more rapid turnover rate of the poly A(-) species. The total mRNP-mRNA curve (fig. 24a) was a hybrid of these two curves. The relationship between At and (t) was found easier to analyse as a log plot (Brandhorst and McKonkey, 1974) in which:

$$\ln (A\infty - At) = \ln A\infty - \ln 2 \left(1/T_D + 1/T_{\frac{1}{2}}\right) t \quad -(2$$

Because of the considerable variation in the 24hr values it was not possible to analyse turnover of any long lived components and approximation to $A\infty$ was difficult. My approach to this has involved analysing turnover kinetics of mRNA as a single component and was to consider that the maximum value of At(cpm) before 24hr was n% of $A\infty$. The line of log ($A\infty$ - At) versus t was calculated for values of 'n' between 80% and 100% for the data of each mRNP-mRNA component in each experiment. The value of n which yielded ($A\infty$ - At) values giving the best correlation coefficient was used for subsequent analysis of data. The lines drawn in figure 25 are the best fit lines to the data from the two experiments. From the slopes of these lines, which equal :

FIGURE 24 : The kinetics of incorporation of ^{32}P phosphate into mRNA

'mRNP-mRNA' was prepared as described in figure 22 from 3 day cultures of artichoke explants which had been labelled for the indicated times in $50\mu Ciml^{-1}$ ^{32}P phosphate ($1Ci\ mg^{-1}$ phosphate). A sample of the RNA was analysed electrophoretically to assess non mRNA contamination. The amount of the labelled mRNA (At) which bound to poly U Sepharose (●) or oligo dT cellulose (O) was estimated by TCA precipitation of total, bound (poly A(+)) and unbound (poly A(-)) RNA. The steady state level of cpm (A∞) was determined by extrapolation as described in the text and the radioactivity expressed as a percentage of A∞.

(a) total mRNP-mRNA, (b) poly A(-) mRNP-mRNA, (c) poly A(+) mRNP-mRNA

FIGURE 25 : A first order decay analysis of the kinetics of incorporation of ^{32}P phosphate into mRNP-mRNA

The results plotted in figure 24 are replotted as log (A∞ -At) versus t. The half life of the mRNA was calculated from the slope of the line which is equal to : log 2 ($^{1}/T_D + {}^{1}/T_{\frac{1}{2}}$) (see text)

(a) total mRNP-mRNA (correlation coefficient, r = -0.9753)

(b) poly A(-) mRNP-mRNA (r = -0.9928)

(c) poly A(+) mRNP-mRNA (r = -0.9875)

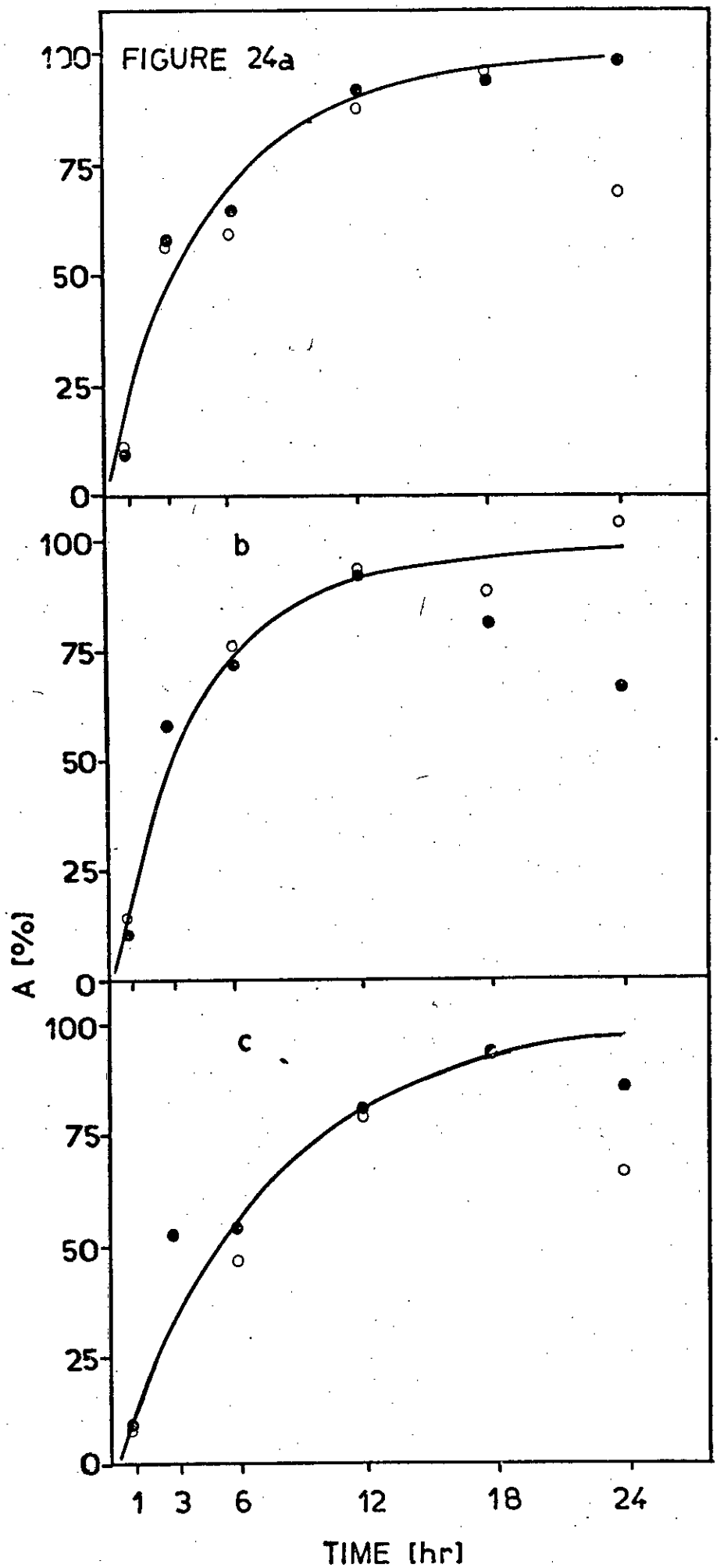


FIGURE 24a
100
75
50
25
0
b
100
75
50
25
0
A [%]
c
100
75
50
25
0
1
3
6
12
18
24
TIME [hr]

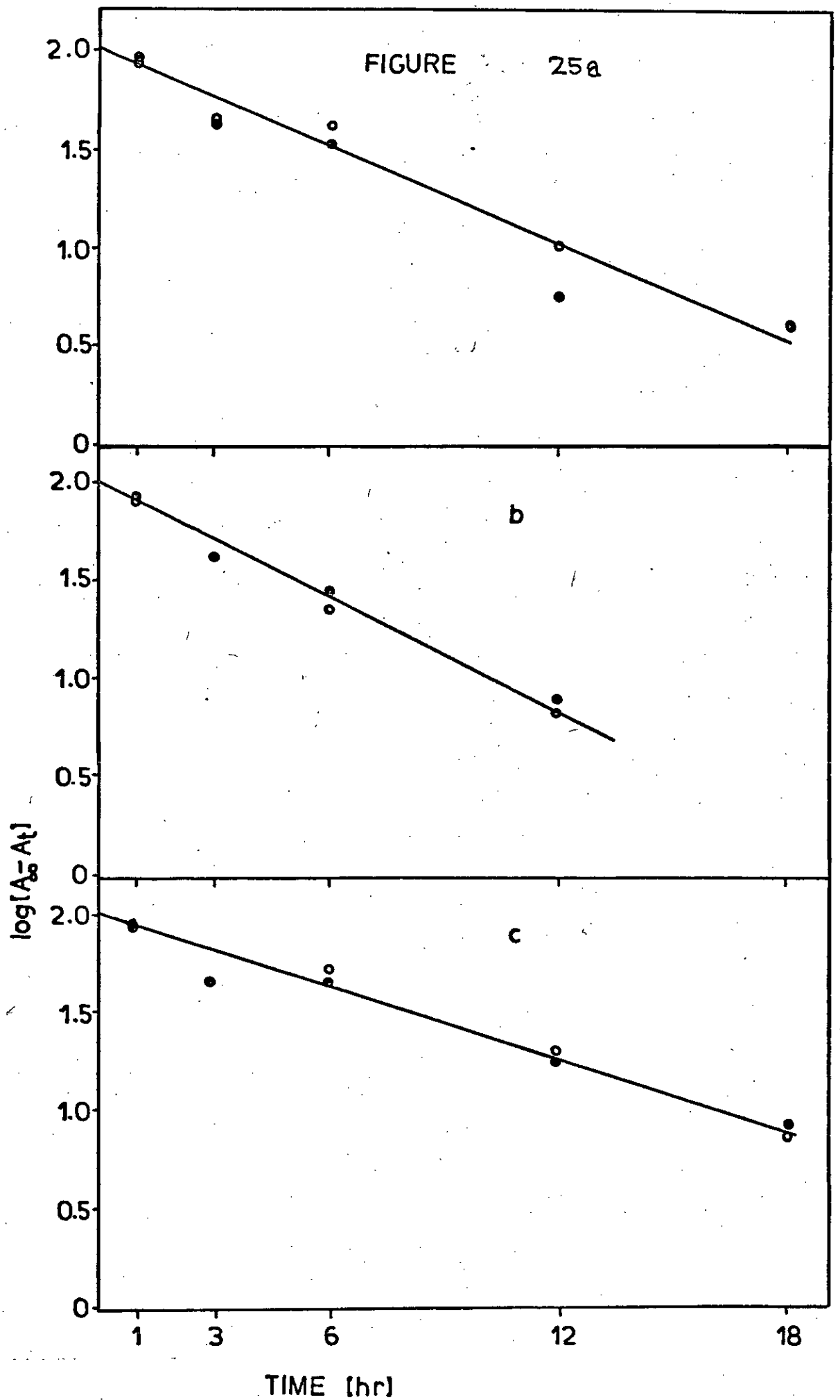


FIGURE 25a
2.0
1.5
1.0
0.5
0
b
c
log[A∞-At]
1
3
6
12
18
TIME [hr]

log 2 ($^1/T_D$ + $^1/T_{\frac{1}{2}}$) average half lives of total, poly A(+) and poly A(-) mRNA were calculated as 5.2, 7.0 and 4.0hr respectively. Comparison of the regression coefficients by the method of Brownlee (1953) showed that the poly A(+) and poly A(-) mRNA half lives were different at the 0.1% level of significance. Knowing that, for each mRNA the measured value of A max (cpm) was n% of A_∞, the relative percentages of poly A(+) and poly A(-) mRNA were calculated as 70% and 30% respectively.

Determination of the mRNA half life from equation 2 required that the rNTP pool was at constant specific activity over the duration of the time course, and that the cells were dividing exponentially. The data in figure 26a and b show the incorporation of radioactivity into rRNA in the artichoke explant cultures used in the kinetic analysis of mRNA turnover (figs. 24 and 25), and the first order decay analysis of this incorporation data. The solid lines in figure 26a and b show the theoretical values for an infinitely stable component in an exponentially dividing culture with a cell division time of 15hr. The experimental data for rRNA in artichoke explants were in good agreement with this and suggested that the rNTP pool used for RNA synthesis was, in fact constant over the duration of the time course. The dotted line shows the theoretical effect if rNTP specific activity in the 18 to 24hr period were only 70% of the value in the 12 to 16hr interval. The deviation from the solid line is considered to be appreciable, and it is concluded that depletions in the average specific activity of rNTP of greater than 30% over a single 6hr period would have been detected by this method.

The data in figure 26c are the cell number data from figure 2 replotted on a log scale and indicate exponential growth of the artichoke explant cultures over the time period of the experiment, which was 48 to 72 hr.

FIGURE 26 : The kinetics of incorporation of ^{32}P phosphate into rRNA

The specific activity of rRNA from tissue used in the experiments shown in Figure 24 was determined from the electrophoretic profiles of total cytoplasmic RNA (b). A∞rRNA was calculated from equation (1 knowing any value of A_t and assuming an infinite half life for rRNA. The data of (b) is replotted as log (A∞-At) in (a). The dashed line shows the theoretical line if the average specific activity of the rNTP pool in the 18 to 24hr period were 70% of the specific activity in the 12 to 24hr period.
c) the cell number data of figure 2 (+24D cultures) is replotted on a log scale to show that there was exponential celldivision in the artichoke cultures

FIGURE 27 : The Kinetics of incorporation of ^{32}P phosphate into RNA of non dividing artichoke explants

Explants were cultured without 2,4,D and mRNA was prepared from mRNP, as described in figure 22, from 3 day cultures which had been incubated in ^{32}P phosphate containing medium (1 Ci mg phosphate) for the indicated time periods. The mRNP-mRNA was fractionated into poly A(+) and polyA(-) components on Poly U Sepharose and At calculated after correction for non-mRNA components.

a) incorporation into rRNA (X——X)

b) incorporation into mRNA (O——O poly A(+) mRNA)

(●——● poly A(-) mRNA)

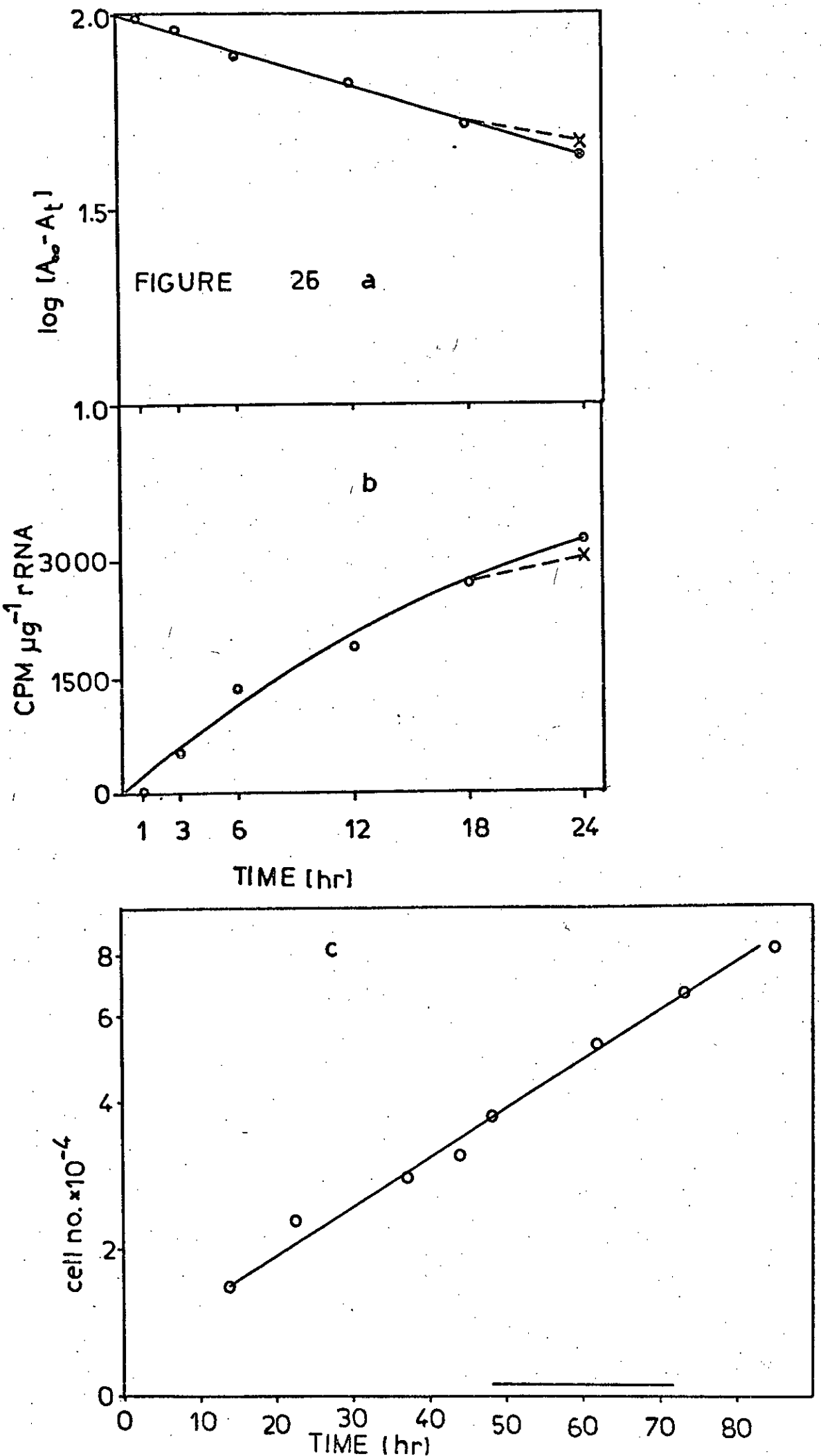
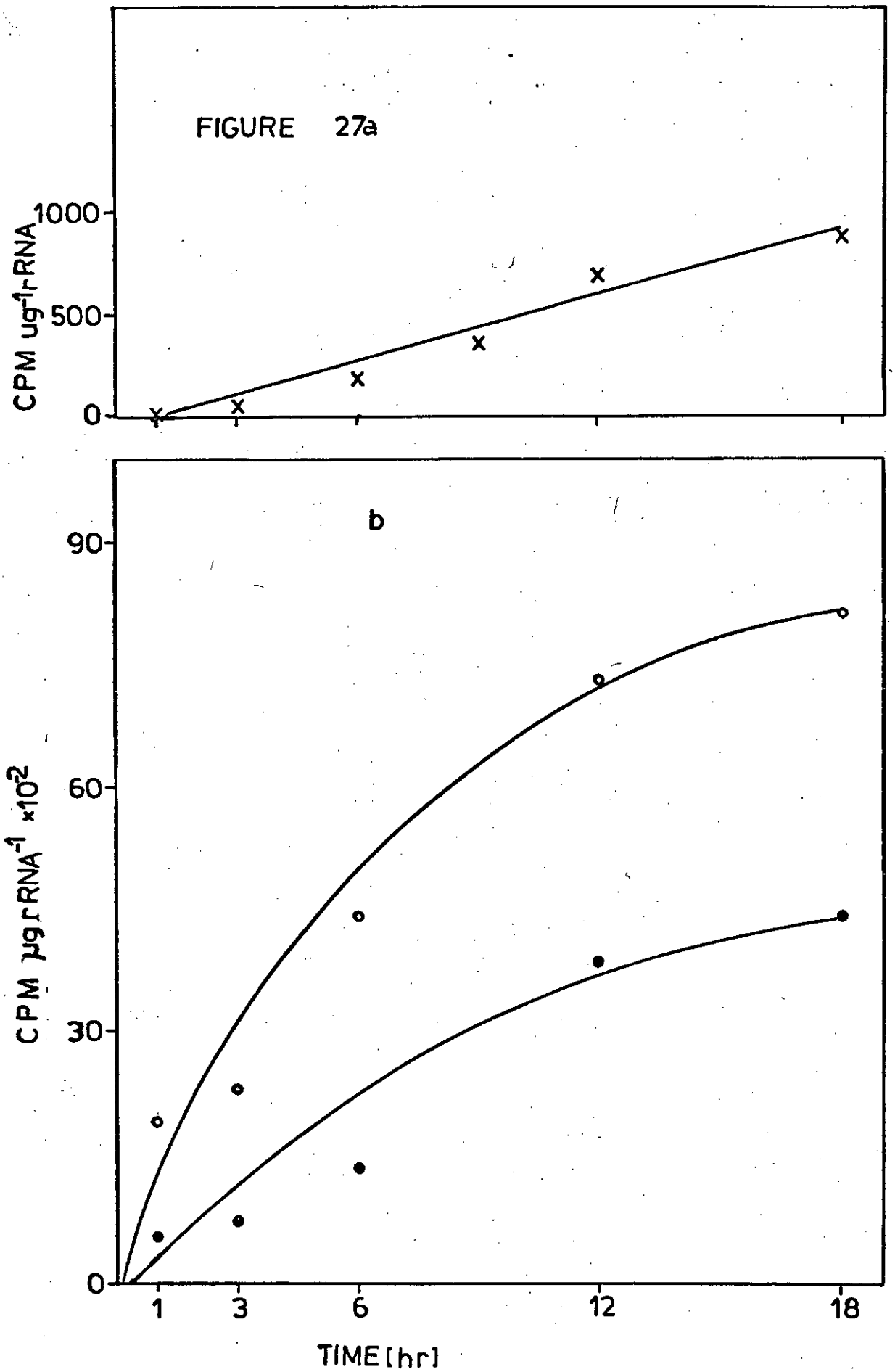

2.0
1.5
1.0
log [A∞-At]
FIGURE 25 a
b
CPM µg⁻¹ rRNA
3000
1500
0
1 3 6 12 18 24
TIME [hr]
c
8
6
4
2
0
cell no. x10⁻⁴
0 10 20 30 40 50 60 70 80
TIME (hr)

FIGURE 27a


1000
500
0
CPM ug⁻¹rRNA
b
90
60
30
0
CPM µgrRNA⁻¹ ×10²
1
3
6
12
18
TIME [hr]

The only identified poly A(-) mRNA to date, is histone mRNA (Adesnik and Darnell, 1972) which in HeLa cells is present in polysomes only during the DNA synthesis stage of the cell cycle ('S' phase) (J. Stein et al, 1975). In order to investigate whether other poly A(-) mRNAs code specifically for proteins synthesised only during 'S' phase, the kinetics of turnover of poly A(+) and poly A(-) mRNP-mRNA were compared in non dividing explants. It would be predicted that the amount of mRNA coding for proteins involved in cell division (e.g. histone, acidic chromatin proteins) would be depleted in non dividing cells. Figure 27 shows the accumulation of radioactivity into RNA from non dividing cultures. In addition to the corrections of mRNA radioactivity made for the values obtained from dividing cells, the results in this experiment were also corrected for failure to recover poly A(+) mRNA from the poly U Sepharose (see Methods Section 7) In non dividing cultures, $T_D = \infty$, so incorporation of radioactivity into stable rRNA was linear (fig. 27a) since t max (18hr) was short relative to $T_{\frac{1}{2}}$ rRNA. Significant deviation of the rRNA values from linearity would have suggested that the rNTP pools were decreasing in specific activity. The incorporation into both poly A(+) and poly A(-) mRNA (fig. 27b) was approaching the steady state level by 18hr.

The half lives of poly A(+) and poly A(-) mRNP-mRNA were determined from a first order decay analysis of the results in figure 27b (fig. 28). The slopes of the lines were quite similar and the half lives of poly A(+) and poly A(-) mRNA were calculated as 5.9 and 5.0hr respectively. Comparison of the respective values of A_∞ which were calculated by extrapolation, as described for the dividing tissue data, showed the steady state levels of poly A(+) and poly A(-) mRNP-mRNA to have been in the same ratio (7:3) as in dividing tissue. In the light of this, I consider it unlikely that poly A(-) mRNA codes specifically for cell division proteins.

FIGURE 28 : A first order decay analysis of the kinetics of incorporation of ^{32}P phosphate into mRNP-mRNA from non dividing artichoke explants

Results from figure 27b are replotted as log (A∞-At) versus t. A∞ was estimated by extrapolation as described in the text. The half life of the mRNA was calculated from the slope of the line which is equal to $\log 2(^{1}/T_D + {}^{1}/T_{\frac{1}{2}})$

0——0 : poly A(+) mRNA,

●——● : poly A(-) mRNA

FIGURE 29 : The association of poly A(+) and poly A(-) mRNA with different sized polysomes and ribosomes

Three day old cultures were labelled for 1hr with 100μCi ml^{-1} of ^{32}P phosphate and harvested. The ribosome pellet was prepared from the post mitochondrial supernatant (65000rpm 2hr) resuspended in 20mM Hepes (pH 8.0, $0^{o}C$) and centrifuged through a 10-60% sucrose gradient at 40000rpm for 70min (MSE 6 x 14 rotor). The gradient was fractionated (a) and RNA prepared from each fraction was further fractionated on oligo dT cellulose. The bound and unbound RNA was analysed on poly acrylamide gels.

b) the composition of each fraction in terms of poly A(-) mRNA as a percentage of total mRNA in that fraction

c) the percent distribution of the poly A(+) mRNA (clear histogram), poly A(-) mRNA (shaded histogram) and total mRNA (dark histogram) between the fractions of the sucrose gradient.

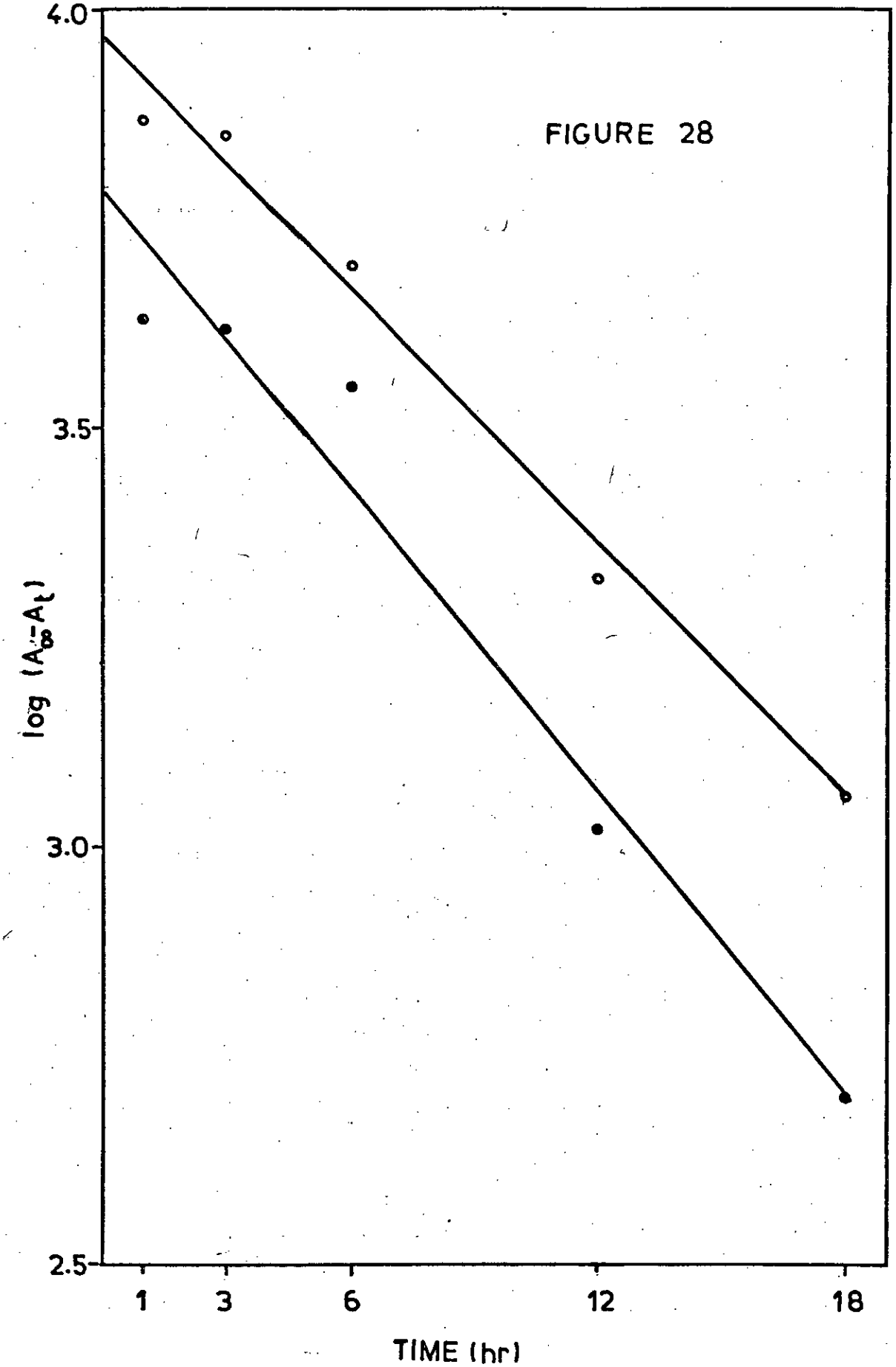


FIGURE 28
4.0
3.5
3.0
2.5
log (A∞-At)
1
3
6
12
18
TIME (hr)

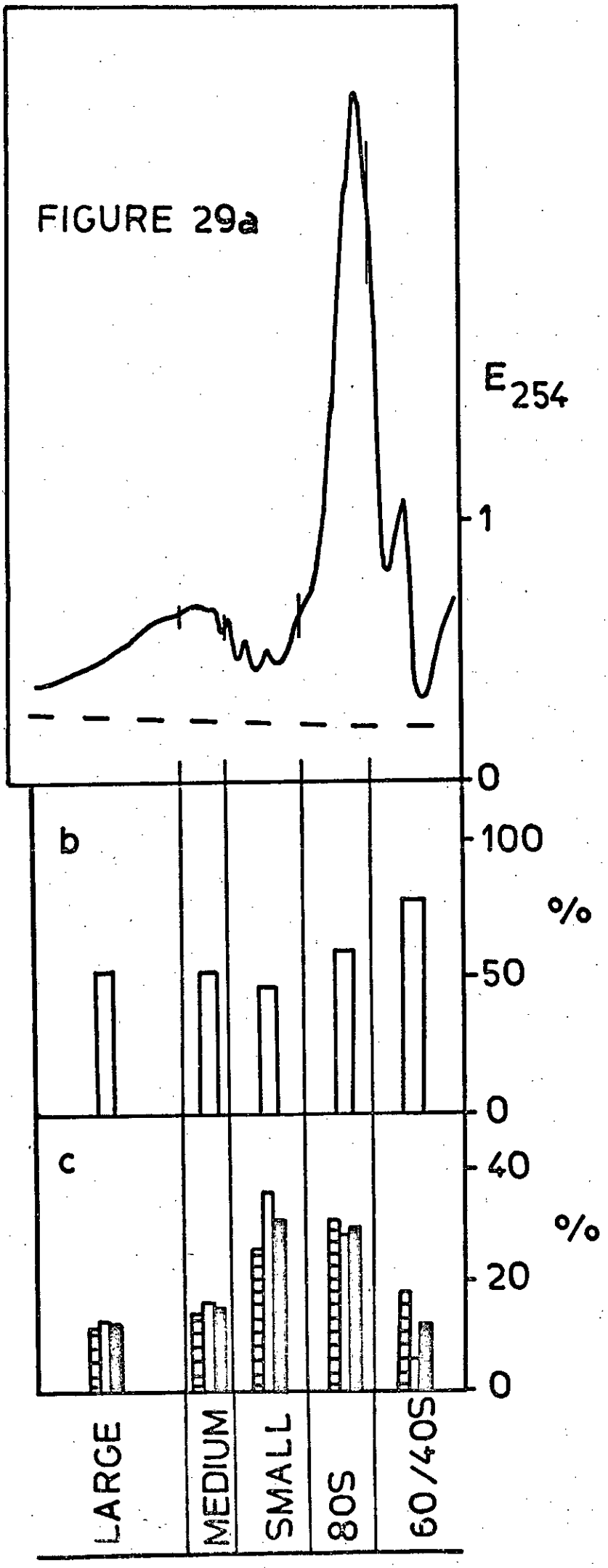


FIGURE 29a
E_{254}
1
0
b
100
%
50
0
c
40
%
20
0
LARGE
MEDIUM
SMALL
80S
60/40S

10) Translation Properties of mRNA

a) in vivo

The ribosome pellet prepared from 1hr pulse labelled explants was fractionated on a sucrose gradient (fig. 29a). RNA was prepared from polysomes of different sizes, ribosomes and ribosome subunits and fractionated on oligo dT cellulose. The total, bound and unbound RNA from each fraction was analysed on polyacrylamide gels in order to detect any enrichment for either poly A(+) or poly A(-) mRNA in these fractions.

The ribosome subunit fraction was highly enriched for poly A(-) heterodisperse RNA (fig. 29b), but accounted for only 12% of the total heterodisperse RNA on the gradient. The ribosome and polysome fractions contained approximately equal amounts of poly A(+) and poly A(-) mRNA. There may have been some cross contamination between poly A(+) and poly A(-) mRNA, as oligo dT cellulose was inefficient in separating poly A(+) and poly A(-) mRNA (see page 47). Electrophoresis of the poly A(+) RNA also revealed some rRNA contamination (fig. 30), such that the possibility of minor contamination of poly A(+) RNA with poly A(-) RNA cannot be eliminated. Nearly 70% of the total mRNA was present in the 80S monomers and small (2 to 4 ribosomes) polysomes (fig. 29c). The modal size of the poly A(+) mRNA (fig. 30b, c) on these fractions was about 0.5×10^6 daltons, a size which may potentially contain up to 15 or 20 ribosomes (Nemer et al, 1975). Much of the poly A(+) mRNA must therefore have been deficiently loaded with ribosomes. Large ($>1.3 \times 10^6$ dalton) mRNA molecules were observed in both the poly A(-) mRNA (fig. 31b, c) and poly A(+) mRNA (fig. 30b, c) from small polysomes and ribosomes, showing that in vivo in artichoke cells there was no correlation between the degree of ribosome loading on mRNA and poly A content. 30% of the mRNA was accounted for in large and medium polysome fractions (fig. 29c). Electrophoretic analysis

showed that small mRNA with a molecular weight lower than 0.4×10^6 daltons was detected even in the large polysomes (fig. 30d, e and fig. 31d, e) As this small mRNA must have been nearly fully loaded with ribosomes and was found in both the poly A(-) and poly A(+) fractions of mRNA on medium and large polysomes, this further suggested no correlation between poly A content of mRNA and ribosome loading capacity.

b) in vitro

A purified post-mitochondrial supernatant (S.30) prepared from ungerminated wheatgerm is an efficient and accurate system for translating exogenously added mRNA (Roberts and Paterson, 1973; Marcu and Dudock, 1974). RNA prepared from the ribosome pellet isolated from artichoke explants was incubated with a wheatgerm S.30 (kindly provided by Dr C.J.Leaver) in order to demonstrate any messenger activity. Significant stimulation of ^{35}S methionine incorporation was detected in all fractions tested. The most efficient fractions, the poly A(+) RNA and the mRNP-mRNA components (Table VI) were those in which heterodisperse RNA had been fractionated from rRNA. Although the results (Table VI) suggest that poly A(+) mRNP-mRNA was more efficient than poly A(-) mRNP-mRNA (11 fold stimulation over background compared with 7 fold stimulation) as a template for in vitro protein synthesis, it should be noted that the system had not been optimised for the different mRNAs. This was reflected in the different response of the two fractions to increasing the RNA input into the S.30. Poly A(+) mRNP-mRNA was equally efficient at 0.8 and 1.6µg per 50µl incubation and was therefore probably at optimal concentration at less than 0.8µg input. In contrast, the poly A(-) mRNP-mRNA was not near optimal concentration at 1.3µg per 50µl incubation, as increasing the concentration to 2.6µg per 50µl incubation doubled the efficiency of translation perµg of RNA.

FIGURE 30 : The poly A(+) RNA from different polysome and ribosome regions of the sucrose gradient

RNA from gradient fractions of figure 29a which bound to oligo dT cellulose was collected by ethanol precipitatation and electrophoresed on 2.3% poly acrylamide gels for 3.0hr (50V). The position of 1.3 and 0.7 x 10^6 rRNA markers is shown.

a) 60/40S fraction (figure 29a), b) 80S, c) small polysomes, d) 'medium sized' polysomes, e) large polysomes.

FIGURE 31 : The size of poly A(-) RNA from different sized artichoke polysomes and ribosome regions of the sucrose gradient

RNA from gradient fractions of figure 29a which failed to bind to oligo dT cellulose was collected by ethanol precipitation and analysed on 2.3% polyacrylamide gels for 3.0hr (50V). The 1.3 and 0.7 x 10^6 rRNA peaks are indicated.

a) 60/40S fraction of figure 29a

b) 80S fraction

c) small polysomes

d) 'medium sized' polysomes

e) large polysomes

FIGURE 30

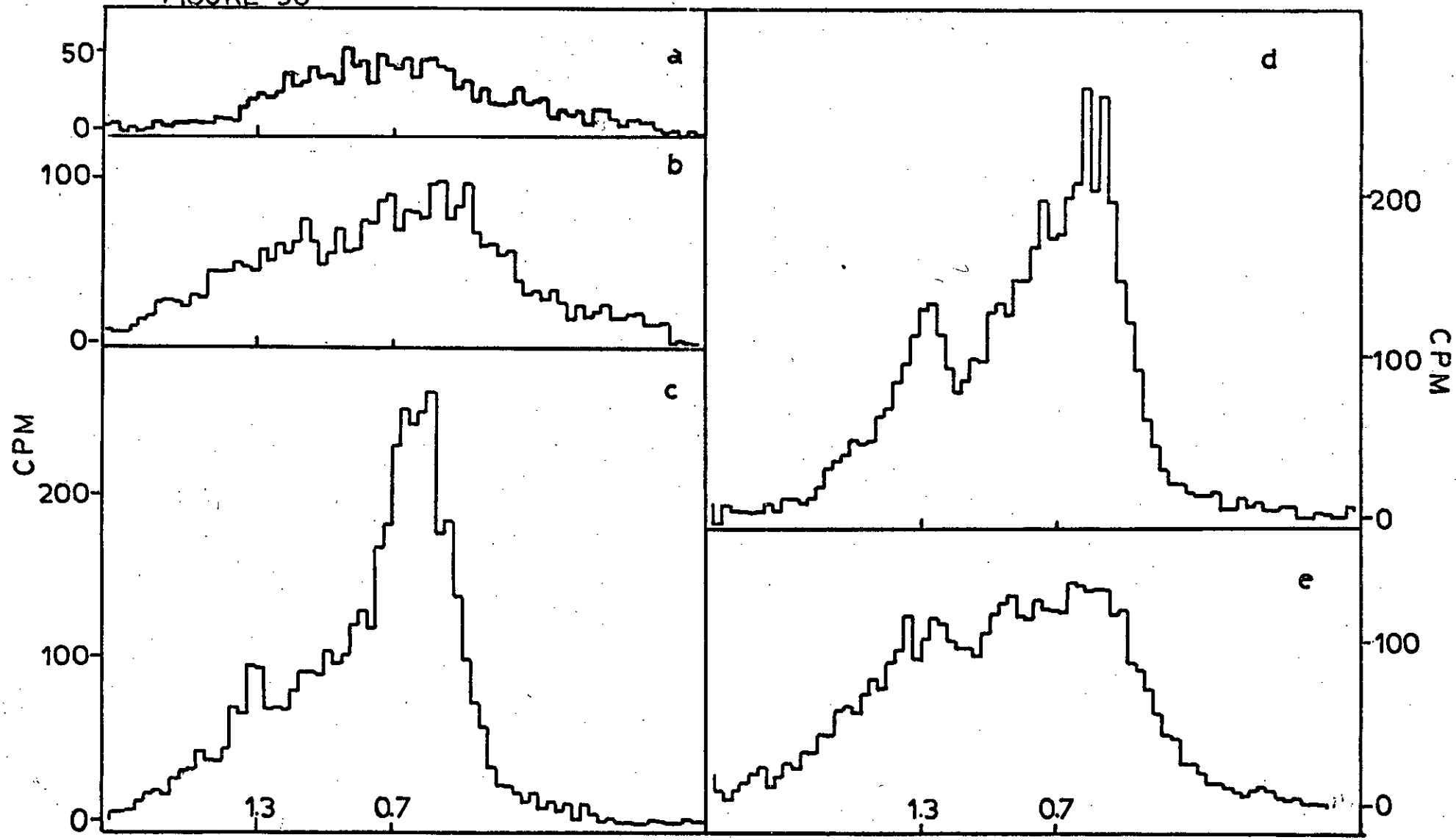


FIGURE 31

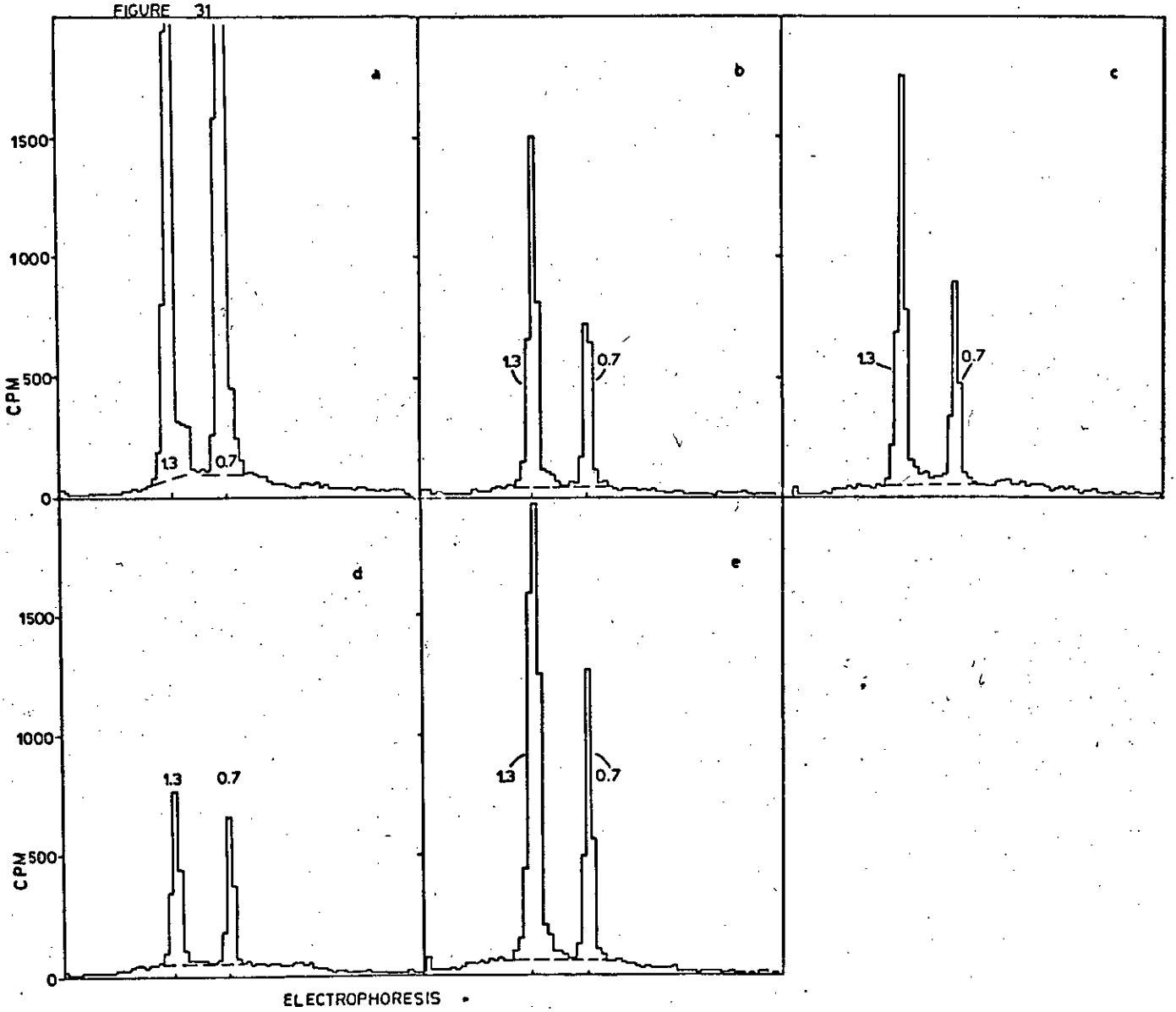


TABLE VI - ^{35}S methionine incorporation in a Wheatgerm S30 protein synthesising system directed by artichoke RNA

RNA	input of RNA (µg)	^{35}S methionine incorporation (cpm µg^{-1} RNA input)	Fold stimulation over background*
Total ribosome pellet	4.2	5552	3.4
	3.4	3870	4.4
Poly A(-) ribosome pellet	5.6	2851	2.7
	11.2	2954	4.4
Poly A(+) ribosome pellet	1.2	61742	8.7
	2.4	87191	22.7
Total mRNP-mRNA	1.3	38850	6.2
	2.6	68450	19.3
Poly A(-) mRNP-mRNA	1.3	11838	2.6
	2.6	22358	7.0
Poly A(+) mRNP-mRNA	0.8	61800	6.1
	1.6	61612	11.2

* Stimulation over background =

$$1 - \frac{^{35}S \text{ methionine incorporation } \mu l^{-1} \text{ incubation mix containing exogenous RNA}}{^{35}S \text{ methionine incorporation } \mu l^{-1} \text{ incubation mix lacking exogenous RNA}}$$

The ribosome pellet from explants which had been cultured for 60hr was prepared and used for either direct RNA preparation or isolation of ribosome subunit free, EDTA released mRNP (fraction 'X', fig. 22). The mRNA was deproteinised by phenol extraction. Samples of both RNA fractions were passed through poly U Sepharose, to yield poly A(+) and poly A(-) subfractions. The indicated amounts of total or subfractionated RNA were added to 50µl incubation mixtures of a wheatgerm S30 protein synthesising system and the incorporation of ^{35}S methionine into acid insoluble radioactivity was determined as described. A control incubation lacked exogenous RNA which was replaced with water.

The products of protein synthesis in vitro in a wheatgerm S.30 primed by artichoke ribosome pellet RNA were compared by electrophoresis and autoradiography of the radioactivity profile. These results are illustrated in figure 32. Although there was extensive homology between the products of poly A(+) mRNA and poly A(-) RNA from poly U Sepharose, which was almost completely depleted of poly A containing RNA, several differences were also detected. These differences are indicated in figure 32. Band 'a' appeared relatively enriched in the poly A(-) mRNA products (slot 1, fig. 32) whereas bands c to e were more enriched in the poly A(+) mRNA products (slot 3, fig 32). Slot 4 (fig. 32) shows the products of in vitro protein synthesis in a wheatgerm S.30 which was primed by cucumber RNA. It is noted that several features are common to the artichoke RNA and cucumber RNA products.

The products of protein synthesis primed by RNA which flowed through oligo dT cellulose (slot 2, fig. 32) were intermediate between the poly U Sepharose poly A (-) RNA products and the poly A(+) mRNA products. This is consistent with the finding that oligo dT cellulose only partially bound artichoke poly A containing RNA (Table III).

11) A Comparison of Kinetics of Labelling of Nuclear and Cytoplasmic RNA

In some preliminary experiments using 1975 artichokes, the kinetics of labelling of total mRNA were compared with those of hnRNA. The results are shown in figure 33. All the results have been normalised to cpm μg^{-1} DNA and so are directly comparable. The kinetics of incorporation of ^{32}P phosphate into the 2.4×10^6 pre-rRNA and 0.7×10^6 rRNA are also included. After 6.5hr labelling, the nuclear RNAs were labelled to steady state, mRNA was approaching steady state labelling and the 0.7×10^6 dalton rRNA was still rapidly increasing in specific activity. Analysis of these curves as single component first order decay plots indicated that the half lives of 2.4×10^6 pre rRNA, hnRNA and mRNA were

FIGURE 32 : A comparison of the in vitro protein synthesis products on poly A(+) and poly A(-) templates

RNA prepared from the ribosome pellet which had been isolated from 3 day old cultures of explants was fractionated into poly A(+) and poly A(-) fractions on oligo dT cellulose or poly U Sepharose. This RNA was then used to stimulate protein synthesis in a wheatgerm S30 system and the ^{35}S methionine labelled products were analysed on 15% polyacrylamide gels which were autoradiographed.

1) poly U Sepharose unbound RNA - poly A(-) RNA
2) oligo dT cellulose unbound RNA - poly A(-) RNA
3) poly U Sepharose bound RNA - poly A(+) RNA
4) Cucumber RNA

FIGURE 33 : The kinetics of incorporation of ^{32}P phosphate into nuclear and cytoplasmic RNA

Artichoke explants were cultured for 2 days and pulse labelled for the indicated time periods. On harvesting, one half of the culture was used to prepare cytoplasmic RNA from a ribosome pellet and the remainder to prepare nuclear RNA (Chapman and Ingle, 1976). RNA from each subcellular fraction was electrophoresed on 2.3% polyacrylamide gels for 3hr (50V) to enable measurement of radioactive incorporation into each RNA component. The values were corrected to cpm μg^{-1} DNA.

a) nuclear RNA (O——O: hnRNA, ●——● 2.4×10^6 pre rRNA)
b) cytoplasmic RNA (O——O: mRNA, X——X rRNA)

This data was obtained in collaboration with Dr Keith Chapman.

FIGURE 32

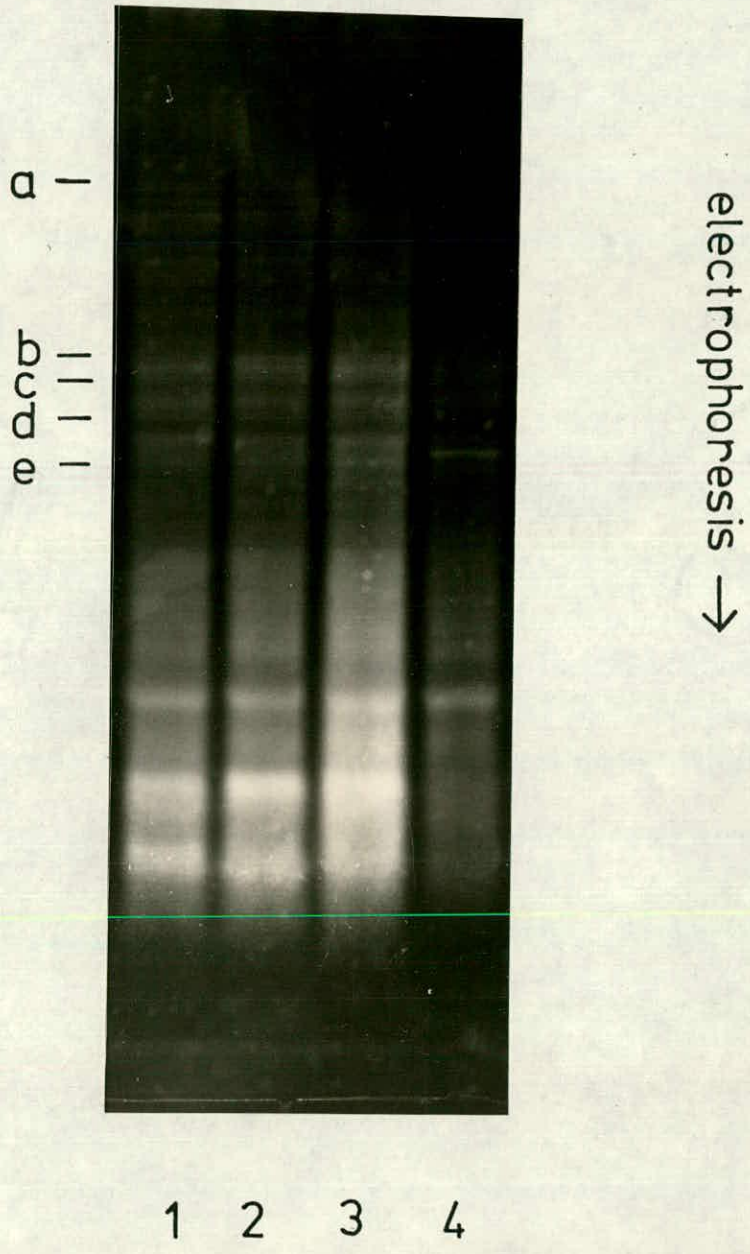
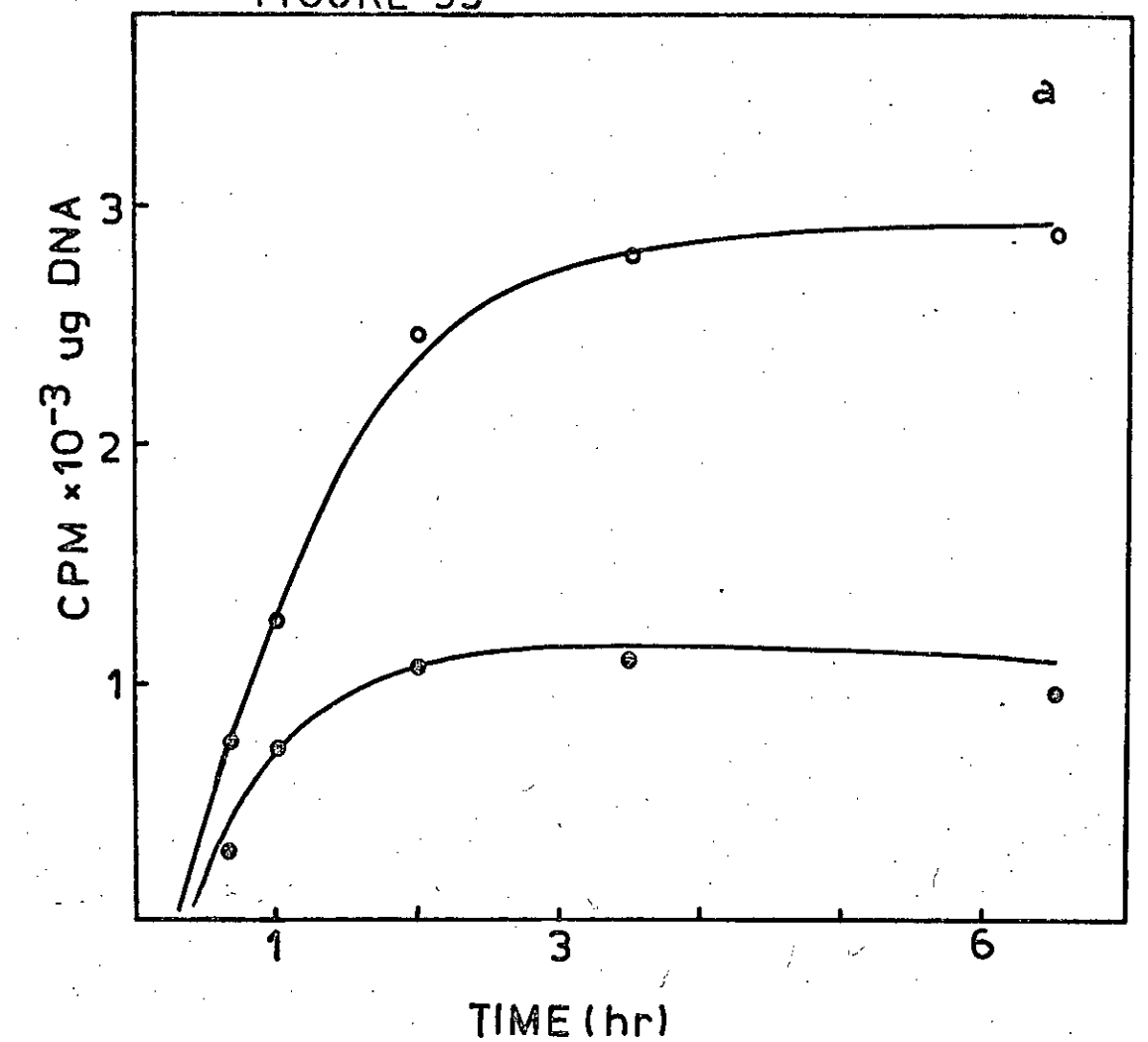
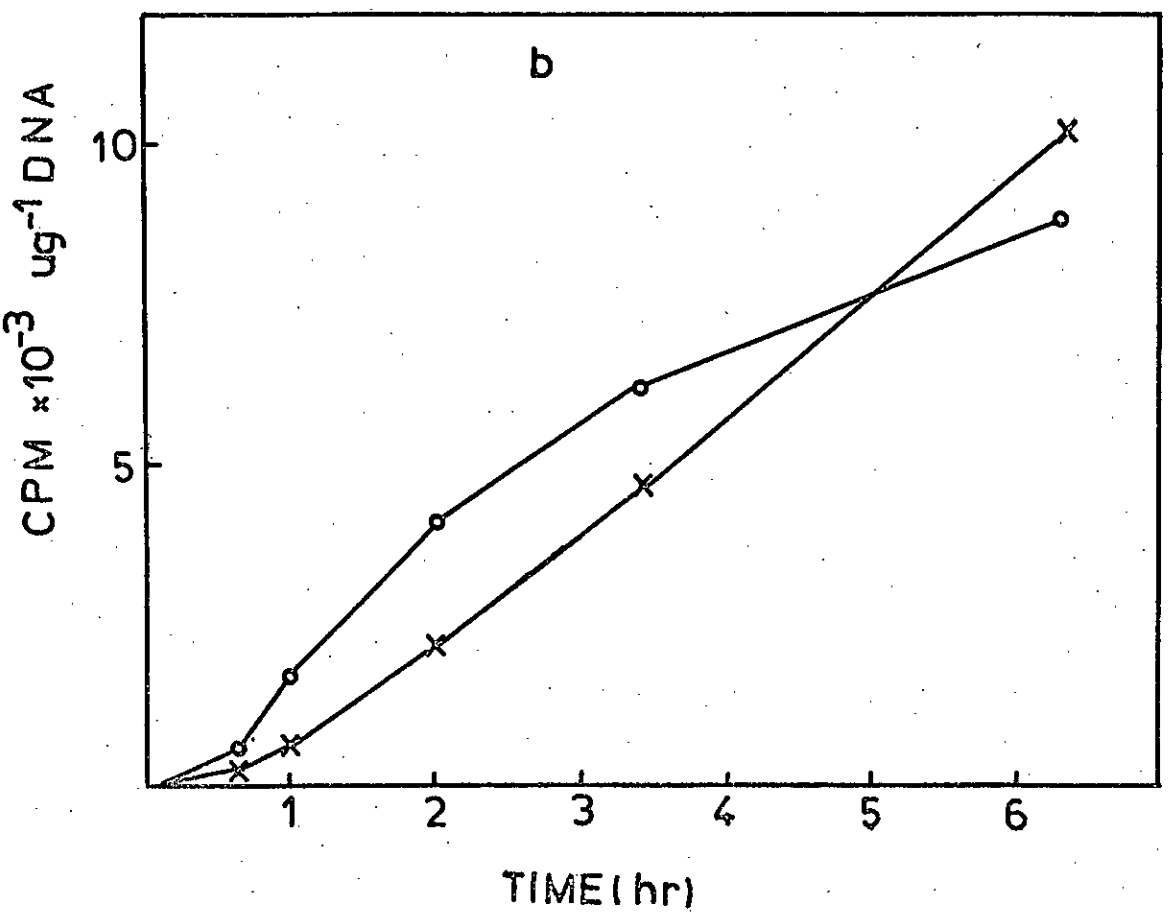


FIGURE 33


a
CPM ×10⁻³ ug DNA
3
2
1
1
3
6
TIME (hr)


b
CPM ×10⁻³ ug⁻¹ DNA
10
5
1
2
3
4
5
6
TIME (hr)

24, 38 and 127 min respectively (fig. 34). If the half life ($T_{\frac{1}{2}}$) and the steady state labelling level per cell (A_{∞}) of an RNA species are known it is possible to calculate the rate of synthesis of a molecule from the differential of equation - (2 (Brandhorst and McConkey, 1974):

$$\frac{dA}{dT} = A_{\infty} \ln 2 \left({}^{1}/T_{D} + {}^{1}/T_{\frac{1}{2}}\right). \quad - (3$$

In a steady state, the rate of synthesis of a molecule per cell is equal to the rate of degredation. Thus the rate of synthesis of 2.4×10^6 pre rRNA should have accounted for the rate of synthesis of 0.7×10^6 rRNA. By assuming that 0.7×10^6 rRNA was infinitely stable, the steady state label level (A_{∞} 0.7×10^6 rRNA) was calculated from equn.-(1 to have been 42248 cpm μg^{-1} DNA hr^{-1} and then from equation -(3 the rate of synthesis was calculated to have been 1951 cpm μg^{-1} DNA hr^{-1}. In order to account for this, the rate of synthesis of 2.4×10^6 pre rRNA would have to have been 6689 cpm μg^{-1} DNA hr^{-1} ($^{2.4}/0.7 \times 1951$) and the half life, 6.3 min. This assumed conservative processing between 2.4×10^6pre rRNA and 0.7×10^6 rRNA. The experimentally determined half life of 2.4×10^6 pre rRNA therefore appears to have been inaccurate by a factor of at least four. This error is thought to have been due to the lag before maximal rNTP specific activity was attained. From the lag observed before rRNA specific activity increased at a maximal rate, this lag was estimated as 30 to 60 mins (fig. 33),(fig. 25b). The calculated $T_{\frac{1}{2}}$ of hnRNA was similar to the uncorrected 2.4×10^6 pre rRNA half life and was probably subject to the same degree of error. If the half life of hnRNA was 10 min ($^{38}/4$) the rate of synthesis would have been 12613 cpm μg^{-1} DNA hr^{-1}.

Messenger RNA turned over much more slowly ($T_{\frac{1}{2}}$ = 126 min) than hnRNA such that the half life estimate would hardly have been affected by the delay before rNTP specific activity was maximal. From the half life of mRNA and A_{∞} mRNA, the rate of synthesis of mRNA (i.e. nucleocytoplasmic

transport of mRNA) was calculated as 3927 cpm μg^{-1} DNA hr^{-1}. Comparison of this figure with the rate of synthesis of hnRNA, suggested substantial intranuclear turnover of hnRNA.

In these experiments which used 1975 artichokes, the half life of mRNA was 2hr, compared to 5hr half life of mRNA in 1976 material. Nuclear RNAs were also more stable in cultures of 1976 explants (K.Chapman personal communication). This increased stability of nucleic acid in explants of 1976 artichokes may be another example of the annual variation in the properties of the tissue.

FIGURE 34 : A first order decay analysis of the incorporation of ^{32}P phosphate into nuclear and cytoplasmic RNA

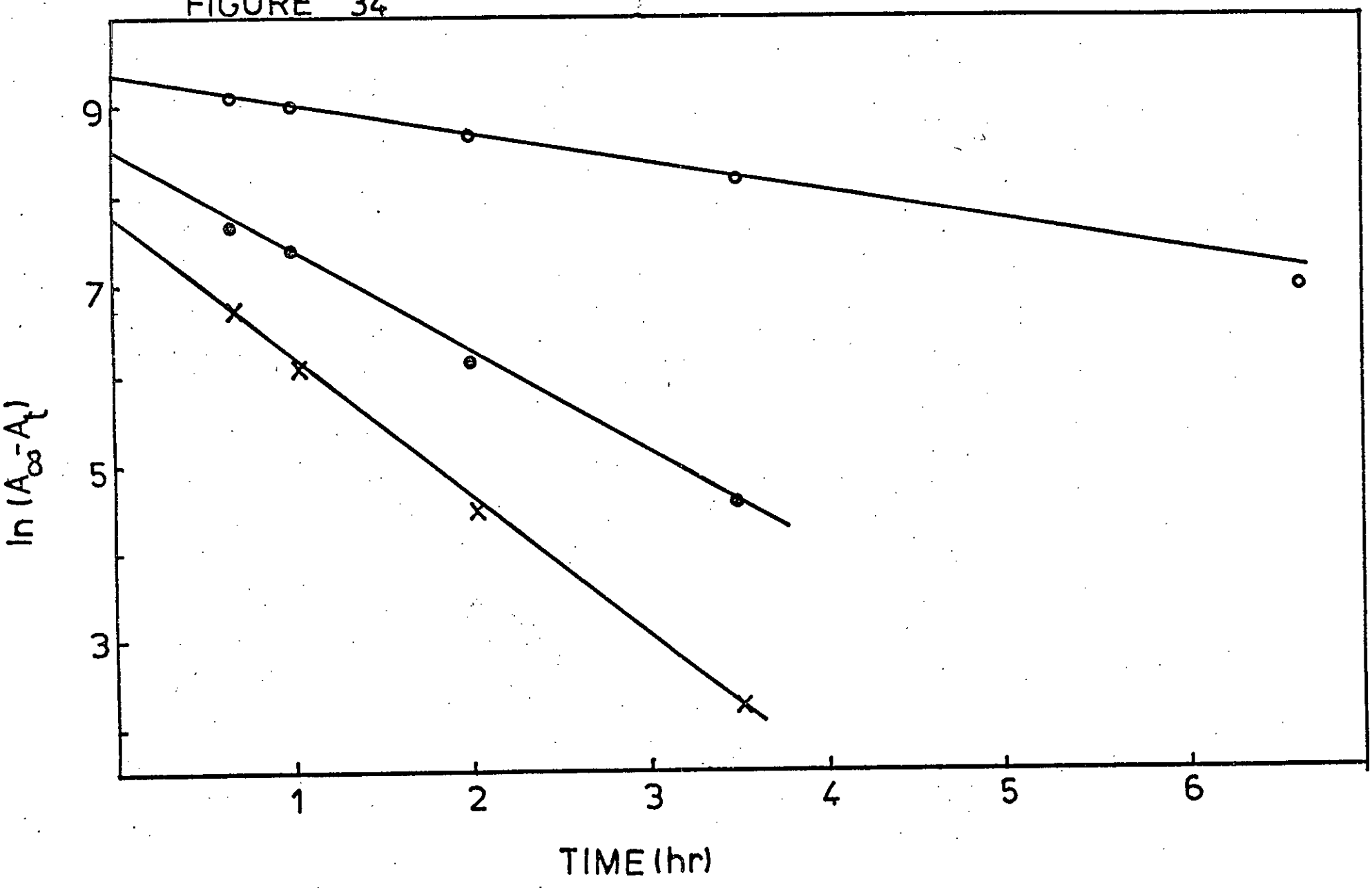
The results from figure 33 were recalculated as ln (A_∞ - At) versus t. The half life of the RNA was calculated from the slope the line which is equal to ln 2 ($^1/TD + {}^1/T_{\frac{1}{2}}$)

O——O : mRNA (A_∞ = 10^4 cpm μg^{-1} DNA)

X——X : 2.4 x 10^6 pre rRNA (A_∞ = 10^3 cpm μg^{-1} DNA)

●——● : hnRNA (A_∞ = 3×10^3 cpm μg^{-1} DNA)

FIGURE 34



DISCUSSION

Key et al (1972) showed that rapidly labelled RNA of soybean could be fractionated into two fractions, on methylated albumin Kieselguhr columns, both of which were rich in AMP. More recently, Key and Silflow (1975) and Schmid et al (1975) established that the basis of the high AMP content of these fractions was a 3' poly A sequence similar to that present on eukaryotic mRNA (e.g. Brawerman 1974). Poly A-containing RNA from plants has directed the synthesis *in vitro*, in a wheatgerm S30 system of leghaemaglobin (Verma et al, 1974), cellulase (Verma et al, 1975) and α amylase (Higgins et al, 1976). The aim of the work described here was to gain more insight into the rôle of poly A on plant RNA.

The method of choice for fractionation of poly A(+)RNA for these studies was affinity chromatography of RNA on poly U Sepharose (Firtel and Lodish, 1973) since essentially all of the poly A(+)RNA was bound after one passage. With the conditions described, oligo dT cellulose bound only 60% of the recovered poly A after two passages. This was probably due to the failure of oligo dT cellulose to bind mRNA containing short poly A tracts (Groner et al, 1974). Sagher et al (1976) have reported that binding of RNA to oligo dT cellulose was improved if carried out at 4°C. A disadvantage of this method, however, may then be that the non specific binding of (poly A(-)) rRNA to oligo dT cellulose, which I have found to be a problem in some preparations even at room temperature, would be increased. Electrophoresis of the poly U Sepharose bound RNA from a ribosome pellet prepared from cultured artichoke explants showed it to be heterodisperse with a modal size of 0.8×10^6 daltons and a number average molecular weight of 0.31×10^6 daltons, which corresponds to a length of about 1000 nucleotides. However, not all of the heterodisperse RNA bound to poly U

Sepharose. 50% of the heterodisperse RNA, prepared from a ribosome pellet of 1hr pulse labelled explants, flowed through the column. The analysis of the poly A content of this unbound RNA by nuclease digestion, suggested that it lacked poly A, and comparison of the size of total poly A and poly A of RNA which bound to poly U Sepharose suggested that the poly U Sepharose column was effective in binding RNA with poly A tracts longer than about 10 bases long. However, neither of these methods would detect smaller sequences accurately and it cannot be stated with confidence that the unbound RNA completely lacked short poly A tracts. Poly A(-)RNA is therefore a practical definition, referring to RNA without poly A or with a poly A sequence which was too short to effect binding to poly U Sepharose.

In order to prove mRNA status of an RNA, it is essential to show that the RNA directed synthesis of high molecular weight protein in vitro and that it was structurally associated with ribosomes in vivo. Demonstration of stimulation of protein synthesis in vitro was not adequate in itself, since messenger RNA precursors are translatable. For example, Stevens and Williamson (1972) have shown that nuclear RNA from myeloma cells directed immunoglobulin synthesis when injected into Xenopus oocytes, and Zahringer et al (1976) have shown that free cytoplasmic RNA from rat liver cells contained ferritin messenger activity when translated in vitro. Both poly A(+) and poly A(-) RNA from the artichoke ribosome pellet did direct synthesis of proteins in a wheatgerm S30 system. However, messenger like RNA in the ribosome pellet could conceivably have been derived from nuclei, mitochondria or plastids, by contamination during the preparative procedure, or from free cytoplasmic informosome like particles (Ajtkozhin et al, 1973). Equilibrium centrifugation of a fixed ribosome pellet in CsCl or an unfixed preparation in Metrizamide failed to show any ^{32}P labelled RNA

components not associated with the ribosomes, and sucrose density gradient centrifugation showed that 90% of the heterodisperse RNA in the ribosome pellet was associated with ribosomes or polysomes. HnRNP or informosomes would have been evident as buoyant density components separate from the ribosome band (Ajtkhozhin et al, 1973; Chapman and Ingle, 1976) and would have largely co-sedimented with the 60/40S region of a sucrose gradient (Ajtkhozhin et al, 1973; Pederson, 1974i). The possibility of plastid origin of this heterodisperse RNA is considered unlikely. Plastid structures are not prominent in this non-green tissue (Bagshaw, 1968), and synthesis of 1.1 and 0.6 x 10^6 mol. wt. plastid rRNA was not detected. Mitochondrial RNA would have at most have comprised <1% of the cellular RNA (Leaver and Harmey, 1973) so would not have been a significant contaminant of the ribosome pellet even if all mitochondria had lysed and their ribosomes not pelleted during the 13000rpm centrifugation. It is therefore postulated that the heterodisperse RNA and the messenger activity were one and the same thing, i.e. mRNA. An implication of this would be that part of the mRNA was polyadenylated (poly A(+)mRNA) and that part contained no poly A or very short (<10 bases) poly A tracts (poly A(-)mRNA).

Histone mRNA has been shown to lack poly A (Adesnik and Darnell, 1972) but was considered as a 'special case'; a cell cycle specific molecule (J. Stein et al, 1975) transcribed on highly repeated genes (Weinberg et al, 1972) possibly subject to extraordinary regulatory processes. However, Milcarek et al (1974) showed that 30% of HeLa cell mRNA failed to bind to oligo dT cellulose and contained no detectable poly A sequences. Poly A(-)mRNA has now been shown to exist in sea urchin (Nemer et al, 1974) mouse L cells (Greenberg, 1976) and artichoke (this thesis). Key and Silflow (1975) concluded tentatively

that part of soybean hypocotyl mRNA lacked poly A. Poly A(-)mRNA may well be of general occurrence.

The main technical difficulty associated with the study of poly A(-)mRNA arises from the large amount of rRNA which copurified with poly A(-)mRNA through oligo dT cellulose or poly U Sepharose. Nemer et al (1974, 1975) studied labelled poly A(-)mRNA from sea urchin blastulae which were not synthesising rRNA, and Milcarek et al (1974) overcame the difficulty of estimating radioactive poly A(-) mRNA by inhibiting rRNA synthesis with 5-flurouracil. In both of these papers, only the radioactive poly A(-)mRNA was studied as the non-radioactive poly A(-)mRNA was not separated from the rRNA. It was clear therefore that a method of preparation of mRNA which was independent of poly A content was an essential prerequisite to a study of the properties of poly A(-)mRNA. Buckingham and Gros (1975) used isopycnic centrifugation in Metrizamide to isolate the 80S RNP, containing free cytoplasmic myoglobin mRNA, from ribosomes. Metrizamide has not, however, been useful as a density gradient medium for purification of mRNP released after dissociation of polysomes. In rat liver, Dissous et al (1976) found that the EDTA released large and small ribosome subunits banded in Metrizamide at 1.20 and 1.23g cm^{-3} respectively, and that mRNP banded over a similar density range. From the distribution of radioactive rRNA in fractions from a Metrizamide gradient of EDTA dissociated artichoke ribosomes, it was concluded that artichoke ribosome subunits were partially separated in Metrizamide (fig. 19) and that the banding properties depended on the magnesium content of the centrifugation buffer. A region between the subunit peaks was found to contain mRNA only, but the mRNA was degraded and would not have been useful for fractionation of poly A(+) and poly A(-)mRNA. This degredation was due to nuclease activity associated with the ribosome subunits and not the Metrizamide, since RNA from

gradients of undissociated or puromycin dissociated ribosome pellets was not degraded. Equilibrium centrifugation of unfixed RNP in Cs_2SO_4 was also ineffective as a method of isolating mRNP from dissociated artichoke ribosomes. However, Greenberg (1976) has reported that good fractionation of mRNP and ribosomes was obtained in Cs_2SO_4 gradients when dimethylsulphoxide (DMSO) was included in the centrifugation buffer. The effect of DMSO was to prevent the precipitation of mRNP, which were more resistant to salt disruption than ribosome subunits, into the denser ribosome subunit region of the gradient (Greenberg, 1976).

The method of isolation of mRNP used in this study involved fractionation of mRNP released from ribosome subunits by EDTA, using sucrose density gradient centrifugation. The recovery of pure mRNA by this method was low (29% of total mRNA) and the mRNP-mRNA obtained was enriched for small mRNA. There was also non-mRNA fractionation revealed by analysis of mRNP-mRNA prepared from 18hr pulse labelled tissue. Knowing the $T_{\frac{1}{2}}$ and value of A_∞ (equation 2) of poly A(-)mRNP-mRNA, and having calculated A_∞ of rRNA in the poly A(-)mRNP-mRNA preparation assuming no turnover of rRNA, the degree of rRNA contamination of the poly A(-)mRNP-mRNA was estimated as 40%. Nevertheless, the purification of mRNA was substantial and estimation of poly A(-)mRNA, even of preparations from long labelled explants, was straightforward.

Messenger RNA status of the RNA from these 'mRNP' was confirmed by demonstrating stimulation of ^{35}S methionine incorporation in a wheatgerm S.30 system. The poly A(-)mRNP-mRNA was distinct from rRNA, poly A(-)hnRNA and poly A(+)mRNA on the basis of turnover kinetics and so was not likely to have derived from these by breakdown during preparation or by nuclear leakage. The mRNP-mRNA bound to poly U Sepharose to the same extent as total mRNA and was not, on the basis

of electrophoresis enriched for a single mRNA species (e.g. histone mRNA). It was therefore assumed to have been typical of the total mRNA.

The buoyant density in CsCl of the aldehyde fixed EDTA released mRNP was >1.6g cm^{-3} which is denser than the value reported for EDTA released mRNP from wheat embryos and pea (1.4g cm^{-3}, Ajtkhozhin and Akhanov, 1974), or other eukaryotic cells (e.g. Schoenman and Perry, 1972). A possible explanation of this is that DEP, which was included in the dissociation buffer to prevent digestion of RNA by RNAase absorbed onto ribosomes (Dyer and Payne, 1974), may have deproteinised the mRNP (Ehrenberg et al, 1976). As protein is less dense in CsCl than RNA (Hamilton, 1971), the density of RNP would increase if protein was removed.

Experiments in which the kinetics of labelling of nuclear and cytoplasmic RNA were compared, were designed to detect any intranuclear turnover of poly A(+) and poly A(-) hnRNA. There have been several approaches to measuring rates of hnRNA turnover. A classical pulse chase experiment was inappropriate due to the long time required to chase radioactivity out of the large pools which are characteristic of plant cells (Dara Melanson personal communication). Scherrer et al (1970) used actinomycin D to inhibit incorporation of label into the RNA during the chase period. Usage of actinomycin D was avoided in the artichoke experiments because of possible artifactual side effects due to this drug. Actinomycin D has been shown to affect, in addition to RNA synthesis, the stability of RNA (Endo et al, 1973; Singer and Penman, 1973) and, by inhibiting release of RNA from it's DNA template, the transport of RNA into the cytoplasm (Egyhazi, 1974). It was therefore decided to measure artichoke RNA turnover by the kinetics of the approach to steady state labelling (Greenberg, 1972).

The main problem with this method was caused by the finite delay before rNTP pools had attained maximal specific activity. Probably the best solution to this problem is to saturate the rNTP pools with a short pulse of very high specific activity followed by transfer to lower specific activity for the duration of the experiment. Brandhorst and McConkey (1975) using this method, found that the rATP was maximally labelled almost instantly and was constant within 20% of the mean over the duration of the time course. RNA incorporation measurement may be corrected directly for fluctuations in rNTP specific activity (Brandhorst and McConkey, 1975), but only if the interval between measurements is short relative to the half life being measured. In my experiments, the effect of delayed attainment of maximal rNTP specific activity was corrected for indirectly. As the uncorrected data suggested that hnRNA and 2.4×10^6 pre rRNA turned over at similar rates (38 min and 24 min respectively) the error of half life estimation due to changing rNTP specific activity would therefore have been similar for both of these RNAs. The half life estimate of the 2.4×10^6 pre rRNA was corrected by a factor of four to account for the rate of synthesis of 0.7×10^6 rRNA, so the half life estimate for hnRNA was corrected for by a similar amount. This correction was based on unpublished results of Dara Melanson who has shown that 2.4×10^6 pre rRNA was conservatively processed into 0.7×10^6 rRNA in dividing artichokes. The corrected half life of hnRNA allows a rate of synthesis of hnRNA which are more than three times the rate required to produce mRNA. There must, therefore, have been substantial intranuclear turnover of hnRNA sequences. Poly A(+)hnRNA comprised 20% of the total hnRNA and turned over at the same rate as poly A(-)hnRNA (Chapman and Ingle, 1976), and was therefore synthesised and degraded at 20% the rate of total hnRNA. Poly A(+)mRNA was 50% of the rapidly labelled (1hr) mRNA, which suggested that poly A(+) and poly A(-) mRNA were

synthesised at equal rates. Considering this, and also accounting for the difference in number average molecular weight between hnRNA andmRNA (0.6×10^6 daltons (from the data of Chapman and Ingle, 1976) and 0.3×10^6 daltons respectively), it was calculated that the rate of synthesis of poly A(+)hnRNA was hardly sufficient to produce poly A(+)mRNA. If the immediate precursor of poly A(+)mRNA was contained in poly A(+)hnRNA this result suggests that any intranuclear turnover of hnRNA involved poly A(-)hnRNA. This does not necessarily imply a rôle forpoly A in selection of sequences for nucleocytoplasmic transport (Darnell et al, 1973). The same result would have been obtained if polyadenylation occurred on molecules which had been pre-selected for possible export into the cytoplasm (Price et al, 1974). The evidence for intranuclear turnover of poly A(+)hnRNA in animal cells was discussed in the introduction and suggests that in many cell types, the poly A(+)hnRNA was not conservatively processed into mRNA. This is considered to be strong evidence against the hypothesis that addition of poly A to hnRNA has a function in selection of sequences for export into the cytoplasm.

There is on the other hand, considerable evidence concerning the metabolism of poly A, the 'translatability' of poly A(+)mRNA and the stability of poly A(+)mRNA which favours a cytoplasmic role for poly A.

The evidence for the metabolism of poly A on mRNA in artichoke was obtained from a comparison of the length of poly A in newly synthesised and long labelled mRNA, which demonstrated a decrease in the number average length of the poly A tract by 7 to 10 bases over 6hr. This is entirely consistent with the finding of Sheiness and Darnell, (1973) that the poly A tract on HeLa cell mRNA shortened with time in a protein synthesis independent reaction. The length of artichoke poly A on mRNA (approximately 50 nucleotides long) contrasts with the longer 70 to 110 number average base length of cytoplasmic soybean

poly A (Key and Silflow, 1975; Schmid et al, 1975), and the undoubtedly shorter, but undefined, length of maize root poly A (Van de Walle, 1973). In animal cells, the length of poly A varies from 200 bases in newly synthesised HeLa mRNA (Sheiness and Darnell, 1973) down to 100 bases in Xenopus (Rosbash and Ford, 1974) and in yeast the poly A is between 20 and 60 bases long (Groner et al, 1974). Some of this apparent interspecies variation in the length of poly A may derive from the errors inherent in the methods used to estimate it (electrophoresis and AMP/adenosine ratio). Even so, one may reasonably expect interspecies or intertissue variation in poly A length if the steady state length of the poly A tract is the result of an equilibrium between a poly A shortening and a cytoplasmic lengthening reaction (Diez and Brawerman, 1974). There is indirect evidence for such a cytoplasmic lengthening of the poly A tract in artichoke explants. On the basis of the very high radioactive AMP content and poly A content of rapidly labelled (1hr) poly A(+)mRNA, it was proposed that in artichoke, as in other eukaryotic cells (Jelinek et al, 1973), the poly A was added post-transcriptionally. Now the length of 1hr labelled artichoke nuclear poly A was short relative to 1hr labelled cytoplasmic poly A, with a modal size of less than 4S RNA in 7.5% polyacrylamide gels (Chapman and Ingle, 1976). If poly A(+)hnRNA contained the precursor to poly A(+)mRNA, it is necessary, in order to explain the different sizes of cytoplasmic and nuclear 1hr. labelled poly A to invoke either intranuclear turnover of a large proportion of the poly A(+)hnRNA (that with the shorter poly A), or cytoplasmic synthesis of poly A. The former is inconsistent with our data on nucleocytoplasmic transport of poly A(+)RNA and so it is proposed that in artichoke cells, there was cytoplasmic synthesis of poly A during the processing of mRNA. It follows from this proposal that the immediate cytoplasmic product of nucleocytoplasmic transport

of hnRNA should have been depleted in poly A, relative to the total mRNA. Spohr et al (1972) suggested, on the basis of kinetic data, that hnRNA was processed into mRNA via a free cytoplasmic RNP (informosome). Although these components were not identified directly in the ribosome pellet from artichoke explants, 12% of the total cytoplasmic heterodisperse RNA was located in the 60S/40S fraction of a sucrose gradient. This small amount of radioactivity would probably not have been detectable as an RNP component which was distinct from the ribosome subunits even after fixation and equilibrium centrifugation in CsCl of the separated 60S/40S fraction. Nevertheless, the heterodisperse RNA present in the 60S/40S fraction was found to be depleted in poly A containing molecules and as such fulfilled the predicted property of an intermediate in the hnRNA-mRNA processing. Physical compartmentalisation of newly synthesised mRNA could explain why there was an initial net increase in the length of the poly A tract, followed by a slow net decrease in poly A length as the mRNA aged. This would have occurred if either of the enzymes responsible for the metabolism of poly A were also compartmentalised between ribosomes and 'informosomes'. Experiments involving in vitro deadenylation of mRNA followed by in vitro translation of the mRNA in a Krebs Ascites cell free protein synthesising system (Williamson et al, 1974), suggested that a 3' poly A tract does not affect the translation potential of a mRNA molecule. Determination of the ratio of poly A(+):poly A(-) mRNA in different sized artichoke polysomes would tend to support a similar conclusion about the translation potential of artichoke mRNA. However, Nemer et al (1975) showed that non-histone poly A(-)mRNA in sea urchin blastulae was less loaded with ribosomes than poly A(+)mRNA, which implied that the poly A(-)mRNA was translated in vivo at a different rate to poly A(+)mRNA. Measurement of poly A(+):polyA(-)mRNA ratio can only really indicate a difference and cannot prove a similarity

in the 'translatability' of the two mRNA types. If, for example poly A(-) mRNA initiated and elongated protein synthesis more slowly than poly A(+)mRNA, it is possible that the ratio of poly A(+):poly A (-)mRNA would be the same in different sized polysome despite the reduced rate of translation of poly A(-)mRNA. Further evidence that poly A may affect the translation efficiency of mRNA was obtained by Doel and Carey (1976), who used the reticulocyte lysate system to translate intact and deadenylated ovalbumin mRNA. They were able to demonstrate that both elongation and initiation of protein synthesis were less efficient on poly A(-)mRNA. These differences were not detectable in the wheatgerm system, and Doel and Carey suggested that the failure of other workers to demonstrate a difference in translation efficiency between poly A(+) and poly A(-) mRNA using the wheatgerm system (Bard et al, 1973) or Krebs Ascites cell free protein synthesising extracts was due to the inefficiency of those systems relative to the reticulocyte lysate or in vivo protein synthesis.

The analysis of turnover of poly A(+) and poly A(-)mRNP-mRNA in dividing artichoke explants shows clearly that poly A(+) molecules were, on average, more stable than poly A(-)mRNA. There may have been additional long lived components of both poly A(+) and poly A(-) mRNA which were not detected due to the variability in the values of ^{32}P phosphate incorporation into mRNA after incubations of longer than 18hr. Also, any very short lived components would not have been detected due to the delay in attainment of maximal specific activity of r NTP. The incorporation of radioactivity into the mRNA was approximated to cpm per cell by correction for units of ribosome subunits recovered. This correction assumed that the amount of ribosomes per cell remained constant, and that the ratio of mRNA:

rRNA did not change over the duration of the time course. These parameters were not measured for these experiments so the absolute half life values must be considered approximate. This does not however invalidate the finding that the relative half lives of poly A(+) and poly A(-) mRNA were quite different.

The differential turnover of poly A(+) and poly A(-) mRNA is quite consistent with the observations of Nudel et al (1976), that the stability of globin mRNA which had been injected into Xenopus oocytes was increased if the length of the poly A tract was above a minimum of 16 bases, and those of Hieter et al (1976) who reported that a 3' poly A tract increased the endoribonuclease resistance of an RNA molecule. In non dividing artichoke explants, sea urchin blastulae (Nemer et al, 1975) and HeLa cells (Milcarek et al, 1974), the poly A(+) and poly A(-) mRNAs were equally stable. In order to reconcile these conflicting results it is necessary to postulate that certain endoribonucleases show differential affinity for poly A(+) and poly A(-) mRNA, and that these enzymes are absent or inactive in some cell types. Alternatively, the poly A may interact with other cellular components (Milcarek and Penman, 1974; Kwan and Brawerman, 1973) in certain cell types such that it has no effect on mRNA stability.

Unfortunately it is not possible to quantitate the sequence homology of artichoke poly A(+) and poly A(-) mRNA from the data presented here. Although the electrophoretic profile of the proteins synthesised in vitro in a wheatgerm S30 system showed extensive similarity with only a few differences between artichoke poly A(+) and poly A(-) mRNA, it is probable that these features were due primarily to protein synthesis on the more abundant mRNAs. In animal cell types, not always terminal differentiation cells, the poly A(+)mRNA comprised a spectrum of abundance classes (Bishop et

al, 1974ii) and the low abundance class may have contained up to 7600 (Levy and McCarthy, 1975) or even 35000 different mRNAs (Bishop et al, 1974ii). If all of these mRNAs were translated in vitro it would be impossible to resolve the products on a one or even two dimensional gel system. The electrophoretic profile of the in vitro products may have been further complicated by the synthesis of products of the endogenous wheatgerm mRNA. Senger and Gross (1976) have proposed that addition of exogenous mRNA to a wheatgerm S-30 protein synthesising system may stimulate protein synthesis on the endogenous mRNA as well as the added mRNA. This effect may explain the similarity between the products on artichoke mRNA and cucumber RNA (courtesy of Elizabeth Weir).

A more quantitative method of comparing poly A(+) and poly A(-) mRNA sequences would involve hybridisation of cDNA to poly A(+)mRNA with an excess of poly A(-)mRNA. The amount of hybridisation of the probe would indicate the degree of similarity between the RNAs.

Considerable effort was directed at preparing a cDNA copy of artichoke poly A(+)mRNA, but despite several purification procedures (desalting on G50/Chelex, repeated ethanol precipitation, passage through SP-50, preparation in CsCl (Glisin et al, 1974)) an inhibitor of Reverse Transcriptase copurified with the mRNA in 4 different preparations. This inhibitor was also active in the wheatgerm S.30 system which was stimulated only 12 fold by poly A(+) mRNA which had been prepared from the ribosome pellet by the method of Glisin et al (1974), precipitated three times from 0.3M NaCl - 10mM Na Ac (pH 5.5) with 3 volumes of ethanol and passed through Sephadex SP50. Marcu and Dudock (1974) obtained up to 70 fold stimulation above background with moth mRNA in a wheatgerm S.30.

Milcarek et al (1974) and Nemer (1974), hybridised cDNA of

poly A(+)mRNA to poly A(-)RNA and could not detect any sequence homology between the two. However, because the poly A(-)mRNA was only approximately 1% of the poly A(-)RNA the required $Rot_{\frac{1}{2}}$ (RNA concentration x time of hybridisation) value of the reaction between cDNA to poly A(+)RNA and poly A(-)mRNA would be 100 times the value for the cDNA - poly A(+)mRNA reaction if poly A(+) and poly A(-)mRNA were 100% homologous. The $Rot_{\frac{1}{2}}$ would be 1000 that value if poly A(+) and poly A(-) were 10% homologous.

The experiments of Nemer et al (1974) and Milcarek which purport to show no homology between poly A(+) and poly A(-)mRNA would hardly have detected 50% homology. Ryffel (1976) has presented data obtained in the manner described above, using Xenopus liver poly A(+)mRNA, which suggested extensive homology between poly A(+) and poly A(-)mRNA and with Dictyostelium, Lodish et al (1974) have shown that the products of an in vitro protein synthesis on RNA which had been passed several times through oligo dT-cellulose, were strikingly similar to those produced on the bound, poly A(+) RNA. It will be possible, now that polyadenylating enzymes are available (Mans and Huff, 1975; Hell et al, 1976), to prepare cDNA of poly A(-) mRNA (prepared by the method of Greenberg, 1976), to test the reaction of cDNA to poly A(-)mRNA versus poly A(+)mRNA, and to characterise the abundance spectrum and complexity of poly A(-)mRNA (Bishop et al, 1974ii).

The similarity between poly A(+) and poly A(-) mRNA may have derived from the cytoplasmic polyadenylation of poly A(-)mRNA, the deadenylation of poly A(+)mRNA or from the partial polyadenylation of the pre-mRNA population in the nucleus. If poly A(-)mRNA had been the precursor to poly A(+)mRNA, the rate of labelling of poly A(+) mRNA would have increased until the poly A(-)mRNA became fully

labelled. If poly A(+)mRNA had been the precursor to poly A(-) mRNA, the poly A(-)mRNA could not have reached maximal specific activity until after the poly A(+)mRNA. As the rate of labelling of poly A(+)mRNA was maximal long before poly A(-) was fully labelled and as the specific activity of poly A(-)mRNA reached its highest level before poly A(+)mRNA was fully labelled (fig. 24), neither of the first two possibilities existed. The reactions determining the proportion of a mRNA which was poly A(+) must, therefore, have been nuclear.

Humphries et al (1976) found that globin sequences were detectable in the cytoplasm of many non erythropoietic tissues and that the proportion of globin sequences which bound to poly U Sepharose varied between cell types. This suggested that the proportion of poly A(+) globin RNA varied and poses the question as to the relationship between the proportion of a specific mRNA which is polyadenylated, the difference in properties of poly A(+) and poly A(-)mRNA in each cell type and the rôle of the specific protein in each cell type.

The poly A content of mRNA appears to affect the cytoplasmic metabolism of mRNA in a cell specific manner. Thus in HeLa cells, which are a very old cultured cell line and which may have lost many of the regulatory mechanisms, the stability and translation efficiency of poly A(+) and poly A(-)mRNA were similar (Milcarek et al, 1974). In sea urchin blastulae the translation potential of poly A(+)mRNA differed from the non histone poly A(-)mRNA (Nemer et al, 1975) and in dividing artichoke cells the stability of poly A(+)mRNA was greater than poly A(-)mRNA. In non-dividing artichoke cells these two fractions turned over at a similar rate. Such results are all consistent with the notion that polyadenylation may affect the

secondary structure of mRNA. Jeffries and Brawerman (1974) showed that the poly A tract of mouse sarcoma cell mRNA prepared under mild conditions, was associated with another sequence of the molecule and Vournakis et al (1975) found that deadenylated globin or chorion mRNA electrophoresed more homogenously than poly A(+)mRNA. They attributed this to an effect of the poly A tract on the secondary structure of the mRNA. Since poly A is at the 3' terminus of mRNA and message sequences are close to the poly A (Darnell et al, 1973), any interaction between poly A and the non coding region of mRNA at the 5' end may result in a circularisation effect. Circular and spiral polysomes are often observed in electron micrographs of eukaryotic cells including artichoke (Bagshaw, 1968). Of course, any intra molecular associations may affect ribosome attachment and elongation and the ribonuclease sensitivity of mRNA.

In order to explain the variation between cells of the properties associated with polyadenylation of mRNA, I propose that the influence of poly A on the secondary structure may be mediated via poly A associated proteins (Kwan and Brawerman, 1973; Blobel, 1973), RNA molecules (Heywood and Kennedy, 1976) or membrane components (Milcarek and Penman, 1974). The activity of these components may vary between cell types such that, for example, in one cell type poly A may increase the stability of a molecule and not the translation efficiency and in another cell type the sole effect of the poly A - "mediator" - mRNA interaction may only be to increase the translation efficiency of the mRNA. The ultimate effect of changing the stability or rate of translation of a mRNA would ultimately be to change the rate of production of the protein coded for by that mRNA. It may be possible to test this hypothesis by injecting mRNP into Xenopus oocytes and then measuring the stability of the mRNA compared with the stability of deproteinised poly A(+) and poly A(-)mRNA. It is proposed that in

Xenopus oocytes, the poly A content of the exogenous mRNA correlated with mRNA stability (Nudel, 1976) because the poly A interacted with endogenous cellular components which mediated the appropriate poly A -mRNA interaction. If mRNP, from cells in which poly A content does not correlate with mRNA stability, were injected into oocytes then the poly A interaction with these endogenous cellular components may be prevented. In this case, if the hypothesis is correct, the poly A (+)mRNA in mRNP would be no more stable than deproteinised poly A(-)mRNA. Poly A(+)mRNA in mRNP from cells in which poly A content is correlated with mRNA stability should be more stable than deproteinised poly A(-) mRNA in Xenopus oocytes.

This hypothesis is admittedly somewhat speculative. However, it is not speculative to state that the distinction between poly A(+) and poly A(-)mRNA is of some significance to cellular functioning. This distinction would have evolved before the divergence of animals and plants and has persisted in such distantly related organisms as Humans (HeLa cells) and artichoke.

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