THE PROCESSING AND PROPERTIES OF RNA

by

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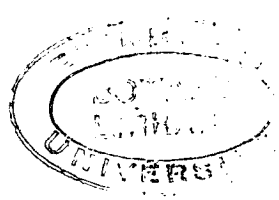
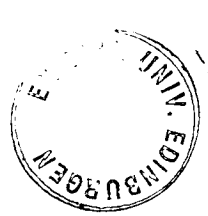




See dying vegetables life sustain,
See life dissolving vegetate again;
All forms that perish other forms supply.
(By turns we catch the vital breath and die)
Like bubbles on the sea of matter borne,
They rise, they break, and to that sea return.

Alexander Pope: Essays on Man.

CONTENTS

ACKNOWLEDGEMENTS

I wish to express my thanks to the Staff, Technicians and Librarian of the Botany Department for their help and encouragement during the course of this work. Particular thanks are due to my Supervisor, Dr. J. Ingle for his invaluable aid and constructive criticism over the last four years.

SUMMARY

The integrity and stability of RNA have been studied using ribosomal RNA (rRNA) as a model system. It has been shown that the two major rRNA components from plants, mammals and Escherichia coli are covalently bonded chains in the mature state. Reports which indicate that they have a subunit structure have been deduced from profiles of rRNA which has been broken down by either nuclease attack or thermal hydrolysis.

The hydrolysis constant has been derived for a phosphodiester bond and shown to be the value for thermal rather than nuclease hydrolysis.

The true sizes of rRNA molecules have been studied by polyacrylamide gel fractionation of native and denatured molecules and it has been concluded that the true molecular weight is given by fractionation of the native molecules.

The 7S rRNA has been shown to be cleaved from the immediate precursor to 25S rRNA at the final processing stage. It has also been demonstrated that the 7S rRNA sequence is located at the 3' end of this precursor.

The artichoke explant culture system has been used for the study of messenger RNA (mRNA). Poly (A) sequences have been shown to constitute 9% of the total, 12% of the nuclear and 6% of the cytoplasmic polydisperse RNA. The size of this poly (A) determined by direct and indirect methods is shown to be up to 250 nucleotides with a mean of 160 nucleotides. 60% of the total polydisperse RNA is shown to be associated with polysomes. 66% of these putative mRNA molecules contain poly (A) sequences, indicating that poly (A) is not an integral part of all mRNA molecules.

The possible role of poly (A) is discussed in the light of these experiments and studies made by other workers on nuclear and cytoplasmic rapidly labelled RNA in animal cells.

INTRODUCTION

Analysis of newly synthesised RNA shows that a large proportion of it is polydisperse in size as opposed to the discrete molecules of ribosomal RNA (rRNA), transfer RNA (tRNA) and rRNA precursors. This polydisperse RNA contains sequences which are restricted to the nucleus and messenger RNA (mRNA) sequences which later appear in the cytoplasm attached to polysomes. Since the majority of analytical techniques available for the study of RNA involve a separation on size, the polydisperse nature of this fraction makes it difficult to study. In finite terms, the size itself is a problem. In the mid-sixties when the so-called giant nuclear RNA was described, many attempts were made to show that its very high molecular weight was not a result of aggregation by means of either thermal denaturation prior to analysis on sucrose density gradients (Attardi, Parnas, Hwang and Attardi, 1966; Houssais and Attardi, 1966) or denaturation in 8.0M urea followed by direct examination under the electron microscope (Scherrer, Marcaud, Zajdela, London and Gross, 1966). Denaturation itself introduces many problems as a result of breakdown of the RNA molecules during treatment. In order to study denaturation and its reversible and irreversible effects on RNA molecules, it is more convenient to examine discrete molecules under denaturing conditions. To this end, the assessment of denaturation techniques was made using rRNA from a range of organisms.

Although it is generally accepted that the rRNAs from the large and small ribosomal sub units of animals and plants are covalently bonded molecules, some groups have claimed that under denaturing conditions, these rRNAs break down to sediment in the 16-18S region of sucrose density gradients, eg Bramwell and Harris (1967) and Higo, Higo and Tanifuji (1971). These workers used high temperatures for varying periods of time followed by sucrose density

gradient analysis to show that the discrete peak of 28 or 25S rRNA is decreased in size comparable to that of the 18S. In all these cases, however, there is some question as to whether the observed effect is the result of an *in vivo* process or an *in vitro* product of extraction and handling. In contrast, Shine and Dalgarno (1973) have clearly shown with a number of insects, that the 26S rRNA exists in the large ribosomal sub unit as two molecules approximately equal in molecular weight. The evidence indicated that one phosphodiester bond was broken, presumably *in vivo* at a specific site in the molecule. Apart from insects, examination of the evidence from animals and plants seems to indicate a reduction in size of rRNA by random cleavage of phosphodiester bonds by either nuclease or thermal hydrolysis. The work presented here examines the conditions of denaturation necessary to remove all secondary structure from rRNA (eg Cox, 1966). Under these conditions, broken portions of polynucleotide chains which have been held together by double helical regions will fall apart. Analysis by polyacrylamide gel electrophoresis thereafter enables the percentage of intact rRNAs to be determined. The role of ribonuclease and thermal hydrolysis in causing the observed breakdown is considered in order to justify the conclusion that the rRNAs in plant and animal ribosomes exist as large covalently bonded chains, the molecular weight of the molecule from the 60S sub unit being two to three times that of the molecule from the 40S sub unit.

Studies on the secondary structure of rRNA have called into question the method of molecular weight determination by polyacrylamide gel electrophoresis (Reijnders, Sloof, Sival and Borst, 1973). The observation was that when rRNA is electrophoresed under continuously denaturing conditions (eg high temperature in the presence of 7.0M urea) the molecular weight of many rRNA molecules changes in relation to *Escherichia coli* rRNA standards. This is

attributed to the molecules losing all secondary structure. If the molecules are denatured and then electrophoresed under non denaturing conditions, the molecules appear to renature very rapidly presumably as a result of the complementary sequences being linked in the phosphodiester chain and thus being in an infinitely high effective concentration. Both denaturation rationales are used here in order to clarify the position with regard to the determination of molecular weight by electrophoresis.

During the processing of the rRNA of the large ribosomal sub unit of eukaryote ribosomes, a small molecule is apparently cleaved from the parent chain (Pene, Knight and Darnell, 1968; Payne and Dyer, 1972). This molecule, the 7S rRNA, has no known function to date. It can be melted from the mature parent molecule by mild denaturation, but its precise mode of cleavage and location within the precursor have not yet been fully described. The use of labelling kinetics in this work has enabled its time of cleavage and position in the rRNA cistron to be determined.

Having confirmed the existence of giant nuclear RNA as covalently bonded chains, many groups tried to show a function for it. It was clear that a large part of it never left the nucleus (Shearer and McCarthy, 1967) and its large size of at least 60S indicated that it could not be simply mRNA, which sedimented as a broad band in the 18S region of a sucrose density gradient (Penman, Rosbash and Penman, 1970). Georgiev, Ryskov, Coutelle, Mantieva and Avakyan (1972) used hybridization techniques to demonstrate that this giant nuclear RNA and mRNA are related in a precursor-product relationship. They showed that giant nuclear RNA completely competed with mRNA but that the reverse competition was only partly effective. However mRNA completely competed with the 3' ends of the giant nuclear RNA.

One characteristic of mRNA molecules has enabled this precursor-product relationship to be studied in more detail. Lim and Canellakis (1970)

reported the presence of an adenine-rich polymer associated with rabbit reticulocyte mRNA. Since then this poly (A) segment has been characterised in some detail and shown to be 30-40 nucleotides in length in pure reticulocyte mRNA (Hunt, 1973).

In conjunction with the characterization of the poly (A) sequences, a number of techniques were developed for enriching and purifying mRNA and putative precursors by selection of those molecules containing poly (A). Poly (A) will specifically bind to poly (dT) cellulose (Nakazato and Edmonds, 1972) poly (U) bound to cellulose or fibreglass (Sheldon, Jurale and Kates, 1972) millipore filters (Lee, Mendecki and Brawerman, 1971) and the lignin contaminant of cellulose (De Larco and Guroff, 1973).

The use of specific cell types and protein characterisation such as tryptic digestion and antibody precipitation has enabled a number of fairly pure mRNAs for specific polypeptides to be identified. Thyroglobulin (Vassart, Brocas, Nokin and Dumont, 1973), collagen (Benveniste, Wilczek and Stern, 1973), ovalbumin (Palacios, Sullivan, Summers, Kiely and Schimke, 1973), immunoglobin (Aviv, Packman, Swan, Ross and Leder, 1973; Cowan and Milstein, 1974), liver albumin (Taylor and Schimke, 1973), myoglobin (Thompson, Buzzart and Heywood, 1973), myosin (Przybyla and Strohman, 1974), calf lens crystallin (Lavers, Chen and Spencer, 1974; Piperno, Bertazzoni, Berns and Bloemendal, 1974), feather keratin (Partington, Kemp and Rogers, 1973), moth chorion (Vournakis, Gelinas and Kafatos, manuscript in preparation), and from plants, leghaemoglobin (Verma, Nash and Schulman, 1974) mRNAs have either been isolated in reasonable purity or been clearly shown to exist in crude mRNA preparations. In all of these, with the exception of collagen and myoglobin mRNA where there are no published poly (A) studies to date, the presence of a segment of poly (A) has been demonstrated. To date, only histone mRNA has been definitely shown to lack poly (A) (Adesnik and Darnell,

1972) although a recent report by Houdebine, Gaye and Favre (1974) shows that only half of the ewe α_S casein mRNA molecules contain poly (A) while Suzuki and Brown (1972) show that the poly (A) of silk fibroin mRNA must be 10 nucleotides or less. Adesnik, Salditt, Thomas and Darnell (1972) have shown that all, or virtually all mammalian mRNAs contain a length of poly (A). However, Johnson, Abelson, Green and Penman (1974) show in fibroblasts that not all polysomal RNA binds to oligo (dT) cellulose. In contrast, Perry, Kelley and La Torre (1972) have shown that poly (A) is absent from E. coli mRNA, although it is present in mitochondrial mRNA (Hirsch, Spradling and Penman, 1974). A great many viral mRNAs have been shown to contain poly (A) sequences (Gallo, 1973), while others not (eg Stoltzfus, Shatkin and Banerjee, 1973). There have been preliminary reports that poly (A) is present in mung bean (Higgins, Mercer and Goodwin, 1973), rice (Manahan, App and Still, 1973), Zea mays (Van de Walle, 1973) and Vicia faba (Sagher, Edelman and Jakob, 1974).

The location of the poly (A) sequence at the 3' end of animal mRNA has been demonstrated by Mendecki, Lee and Brawerman (1972) by 3' OH terminal labelling and by Molloy, Sporn, Kelley and Perry (1972) by exonuclease digestion from the 3' end. This is in contrast to the work of Ryskov, Farashyan and Georgiev (1972) who claimed that the poly (A) of Ehrlich ascites carcinoma cell giant nuclear RNA lay in the middle of the molecule. When this molecule was processed, the poly (A) was revealed at the 5' end of the mRNA. However, these conclusions appear to have been retracted (Samarina, Aitkhozhina and Besson, 1973).

The size of the poly (A) segment varies depending on the mRNA and organism studied. As has been already mentioned, the size of the poly (A) in globin mRNA is 25 (Kaufman and Gross, 1974) or 30-40 nucleotides (Hunt, 1973). The poly (A) from silk moth ovarian follicle chorion mRNA is very

similar at 34-40 nucleotides (Kaufman and Gross, 1974; Vournakis, Gelinas and Kafatos, manuscript in preparation). The size of poly (A) in total polysomal message in yeast is 50 nucleotides long (McLaughlin, Warner, Edmonds, Nakazato and Vaughan, 1973) while that from Dictyostelium discoideum is approximately 100 nucleotides long (Firtel, Jacobson and Lodish, 1972). Mammalian cell poly (A) has been shown to be very much larger at approximately 200 nucleotides (Sheiness and Darnell, 1973). The degree of homogeneity in these poly (A) sequences is high. In Vicia faba, the poly (A) is much more heterogeneous, ranging from 150 to 250 nucleotides (Sagher et al, 1974). Although Higgins et al (1973) conclude that the poly (A) of mung bean polysomes is similar in size to that of yeast, their data indicates that it is much larger. The evidence for the size of the poly (A) in rice presented by Manahan et al (1973) is inconclusive showing no clear peak at all. In maize, the poly (A) appears slightly smaller than tRNA (Van de Walle, 1973), but the size was determined on low concentration polyacrylamide gels where there is no sieving of molecules in the tRNA molecular weight range.

Studies with inhibitors of RNA or poly (A) synthesis indicate that synthesis of poly (A) segments is independent of transcription (Darnell, Philipson, Wall and Adesnik, 1971). Enzymes capable of adding poly (A) have been described in nuclei by Edmonds and Abrams (1960) and in nuclear RNP particles by Niessing and Sekeris (1973). In adenovirus (Philipson, Wall, Glickman and Darnell, 1971) and three RNA viruses (Marshall and Gillespie, 1972) it has been shown that although the mRNAs contain poly (A) sequences, there are no complementary sequences in the genome which could direct its synthesis. Similarly in HeLa DNA there are no poly (dT) stretches long enough or frequent enough to justify a conclusion involving transcriptional synthesis (Birnboim, Mitchel and Straus, 1973). Poly(A) addition appears to

be restricted to the cell nucleus (Jelinek, Adesnik, Salditt, Sheiness, Wall, Molloy, Philipson and Darnell, 1973) although cytoplasmic polyadenylation has been shown to occur in fertilized sea urchin eggs (Slater and Slater, 1974). It appears possible, however, that this polyadenylation is the addition of more poly (A) to molecules already carrying a poly (A) sequence, almost tripling the average size of the poly (A) segment (Slater, Slater and Gillespie, 1974). In addition, viral mRNAs which apparently never enter the nucleus and yet carry non-transcriptionally synthesised poly (A) must also be polyadenylated in the cytoplasm (Marshall and Gillespie, 1972).

The precursor product relationship between giant nuclear and cytoplasmic mRNA has been fully investigated by means of the poly (A) sequences they contain. In HeLa, the preliminary polyadenylation takes place in the nucleus and is then followed by a reduction in size of the poly (A) containing RNA and transport to the cytoplasm (Jelinek et al, 1973). They also show that not all the nuclear RNA molecules carry poly (A) sequences. The experiments of La Torre and Perry (1973) suggest that there is a pool of non-adenylated pre-mRNA which serves as a precursor to the polyadenylated pre-mRNA. They, however, claim that only a quarter of the nuclear poly (A) containing RNA is then chased into the cytoplasm. It is possible that the actinomycin D and cordycepin used in their investigations could be inhibiting part of the mRNA transport system as well as synthesis. However in a related paper, Perry, Kelley and La Torre (1974) provide evidence for a similar conclusion from a detailed examination of the labelling kinetics of the two fractions in the absence of inhibitors.

While the work discussed so far provides circumstantial evidence for a precursor-product relationship between mRNA and the large nuclear RNA, it cannot be demonstrated conclusively until such a relationship can be confirmed for individual mRNAs. Ruiz-Carrillo, Beato, Schutz, Feigelson and Allfrey

(1973) using duck globin mRNA as the substrate for an in vitro protein synthesising system, Stevens and Williamson (1973) using myeloma protein binding to the polypeptide synthesised by polysomes containing immunoglobin heavy chain mRNA, Williamson, Drewienkiewicz and Paul (1973) using micro-injection of mouse globin mRNA into Xenopus oocytes and Macnaughton, Freeman and Bishop (1974) using hybridization studies with duck globin mRNA have all shown that large molecular weight nuclear RNA fractions contain the sequences which appear in the cytoplasm in smaller molecules as the respective mRNAs. In all these cases, the putative precursor was at least twice the size of the mRNA, showing a considerable excess of RNA in the transcription product.

From a consideration of the data presented thus far, it can be seen that the role of poly (A) is far from clear. If it is involved in the selection of pre-mRNA for transport to the cytoplasm, it is difficult to reconcile the evidence of nuclear turnover of poly (A) (Perry et al, 1974). Although it is clear that the bulk of polysomal mRNA contains poly (A), the fact that a small percentage, and in particular the histone mRNA, does not mitigate against any role for poly (A) involving transport or regulation of protein synthesis. It is important to note, however, that in the case of histone mRNA, the genes are reiterated some 1,200 fold in sea urchin embryos (Weinberg, Birnstiel, Purdom and Williamson, 1972) and hence their lack of poly adenylation may represent a special case where multiple copy genes use a different regulatory system. Johnson, Abelson, Green and Penman (1974) have shown in fibroblast polysomes by competitive hybridisation that the molecules which do not contain poly (A) are different from those which do but it is not clear whether the two groups represent different degrees of gene reiteration. In the case of silk fibroin where there are 1 to 3 copies of the gene per haploid genome (Suzuki, Gage and Brown, 1972) the poly (A) segment is probably no more than 10 nucleotides long (Suzuki and Brown,

1972) but this may be adequate for a role of poly (A) in a transport system. All mRNAs which contain poly (A) appear to be transcribed from genes which are present in low numbers. The poly (A) containing mRNA from HeLa cells has been shown by Bishop, Morton, Rosbash and Richardson (1974) to comprise three frequency classes none of which originate from repetitive DNA although the mRNAs contain a 10% repetitive component. Thus about 10,000 mRNA copies originate from about 17 sequences, about 450 copies from about 350 sequences and about 10 copies from about 35,000 sequences. The non poly (A) containing histone mRNA also shows faster transport to the cytoplasm than do those mRNAs containing poly (A) (Adesnik and Darnell, 1972). It is therefore still possible that poly (A) plays a role in transport of particular types of mRNA from the nucleus to the cytoplasm.

The role of poly (A) in protein synthesis has also been investigated. Kwan and Brawerman (1972) and Blobel (1973) have shown the specific association of a protein with the poly (A) segment of mRNA in animal cells. A protein-poly (A) complex has also been demonstrated in nuclear ribonucleoprotein complexes containing pre-mRNA (Samarina et al, 1973). This interaction with a specific protein could indicate a role for poly (A) in the binding of mRNA to ribosomes. However, Bard, Efron, Marcus and Perry (1974) demonstrate that the presence or absence of poly (A) on mRNA has no effect on its translation efficiency in a cell free system. In contrast, Rosenfeld, Abrass, Mendelsohn and Miller (1973) claim that when poly (A) is removed from mRNA by nuclease digestion, the loss of poly (A) is matched by a reduced ability of the mRNA to bind met-tRNA in a cell free system. It is, however, possible that the incubation is producing breakdown of the mRNA itself and thus affecting the binding. Both these pieces of evidence can be challenged on the basis of their use of purified mRNA and cell free systems which do not represent the in vivo state of the mRNA. Some,

as yet, unpublished data from Marbaix and his colleagues using injection of globin mRNA with and without poly (A) into oocytes indicates that the lifetime of the mRNA is considerably increased when it has poly (A) attached. If this is so, it could indicate that in vivo, the poly (A) does have a role in regulating mRNA translation. Sarkar, Goldman and Moscona (1973) have shown using cordycepin and ethidium bromide inhibition in neural retina cells that as the poly (A) synthesis is inhibited, so the induction of glutamine synthetase is suppressed. Once again, however, the inhibition of poly (A) synthesis could be a parallel result of a more general inhibition rather than the direct cause of the supression.

The results of Sheiness and Darnell (1973) and Brawerman (1973) in demonstrating a reduction in the size of the poly (A) segment with translation, suggest that it could be involved in controlling the number of translation events on each message. However, to reconcile these results with the lack of poly (A) in the histone mRNA, once again the existence of more than one regulatory system must be postulated.

In order to investigate the location and function of poly (A) in a plant system, a number of criteria were looked for; sterility, definable growth characteristics and limited cellular differentiation. The artichoke explant culture system (Yeoman, Dyer and Robertson, 1965) proved very suitable for this work. Although the initial growth of the excised explant is probably a wound response, the tissue will continue dividing for up to 5 days in an artificial medium comprising mineral salts, sucrose and auxin. The RNA synthesis occurring in asynchronous cultures - 3 to 4 day old - was characterised, particularly in terms of the poly (A) content, and the distribution of poly (A) containing molecules determined in nuclear and cytoplasmic fractions.

MATERIALS & METHODS

All chemicals used were BDH Analar grade unless otherwise stated. Centrifugal values are for the average g force unless otherwise stated.

1) Source and homogenisation of tissue.

Escherichia coli

Escherichia coli (d +/L strain) was grown in a medium containing 10 gl^{-1} Nutrient Broth (Oxoid), 5 gl^{-1} Yeast extract, (Oxoid), 2.5 gl^{-1} Glucose, 5 gl^{-1} Na Cl, pH 7.5 for a period of 24-36 hr at 37°C with constant shaking. Cells were harvested by centrifugation (10 min, 2000 g) and washed with 50mM NaCl, 10mM Tris-HCl (pH 7.4) (40 ml per litre culture). The harvested, washed cells were lysed by shaking with 30-50 ml/g fwt of 50mM NaCl, 10mM Tris-HCl (pH 7.4) containing 6.0% p-aminosalicylic acid, sodium salt (PAS) and 1.0% tri-iso-propylnaphthalene-sulphonic acid, sodium salt (TNS, Eastman Kodak) with enough phenol mixture (see nucleic acid extraction for composition) to dissolve the PAS (PAS/TNS grinding buffer).

Mouse

Whole livers were removed from freshly killed mice and cooled in ice. Thirty to fifty ml/g fwt of ice cold 50mM NaCl, 10mM tris-HCl (pH 7.4) were added and the tissue homogenised in a Virtis "45" homogeniser for 1 minute at full speed. Solid PAS and 10% aqueous TNS were immediately added to a final concentration of 6.0% and 1.0% respectively, together with enough phenol mixture to dissolve the PAS.

Pea (Pisum sativum var Meteor)

Batches of peas (Carters Tested Seeds Limited) were sorted to remove those with obviously damaged testas and the remainder surface sterilised with methanol (30 sec) followed by 20% sodium hypochlorite (2-3% available chlorine for 20 min). The seeds were washed in distilled water until the

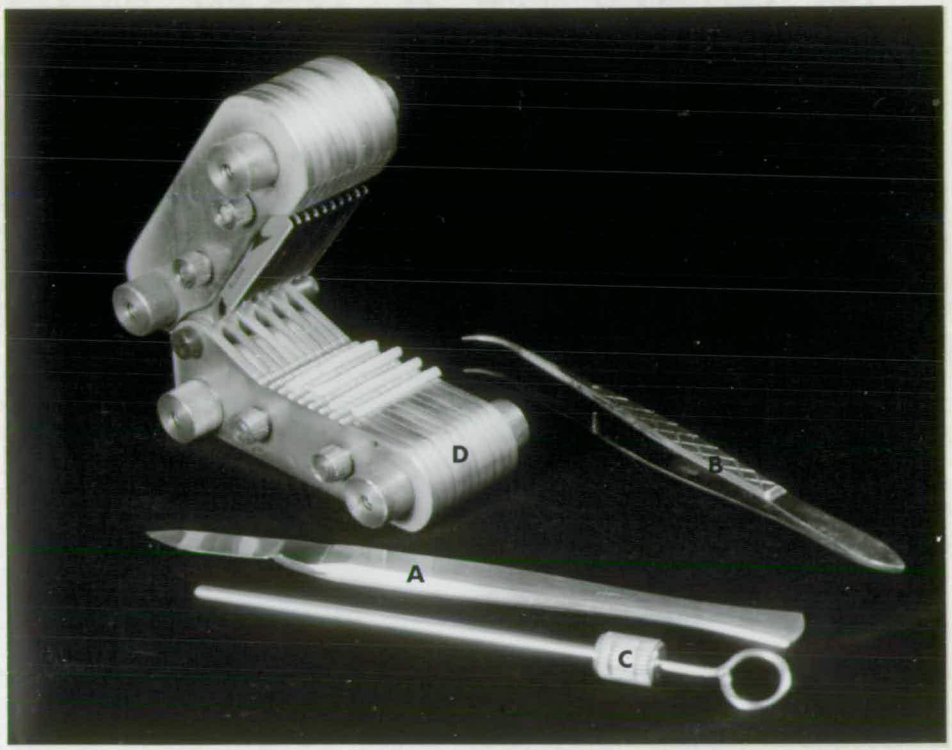
smell of chlorine had been removed and then imbibed for 12 hr prior to planting in soaked vermiculite at 25°C. Roots were excised after 3 days and epicotyls after 5 days growth in the dark. Small amounts of tissue were homogenised in a mortar and pestle in 3-5 ml/g fwt of PAS/TNS grinding buffer while large amounts (> 1 g) were homogenised in ice cold 50mM NaCl, 10mM tris-HCl (pH 7.4) as described earlier for mouse liver except that only 3-5 ml/g fwt of buffer were used.

Artichoke (Helianthus tuberosus var Bunyard's Round)

The Artichoke stock was a single clone of plants maintained in the garden of the Botany Department of Edinburgh University (King's Buildings). Mature dormant tubers were harvested between November and February and stored in damp sand in sealed polythene bags between 0° and 4°C. Although dormancy usually broke during June or July of each year, the tubers could still be used until November or December. Intact healthy tubers showing no signs of surface damage or infection were scrubbed to remove adhering soil and surface sterilised by immersion in 20% sodium hypochlorite solution (2-3% available chlorine) for 25 min. The tubers were then washed several times in sterile distilled water and transferred to a sterile room. Room sterility was maintained by three UV lamps (Philips, tubular 15W) which were switched on at least 30 min before the room was used while a small positive pressure of filtered air reduced the inflow of contaminated air on entry. Conventional flaming techniques were used for routine sterilisation of instruments during use. Instruments and glassware were sterilised in sealed tin boxes at 150°C for 3 hr, while paper tissues and aluminium foil were autoclaved at 15 p.s.i. for 20 min.

Tubers were wiped dry with sterile tissues and the ends cut off. Cores of parenchyma tissue were excised parallel to the tuber axis with a 2 mm diameter stainless steel canula and cut into 2.4 mm lengths by means of a block in which were mounted a number of stainless steel razor blades (Figure 1).

Figure 1.



Artichoke explant preparation equipment.

A: Scalpel
B: Curved forceps
C: 2.0 mm diameter canula for excision of cores
D: Explant cutter with artichoke tuber cores. The razor blades are set 2.4 mm apart.

All operations were carried out in low intensity green light (Ilford bright green safe light, Filter No. 909, less than 1 foot candle) to prevent the inhibitory effect of light on cell division described by Fraser (1968). The tissue explants (10/ml medium) were cultured in 250 ml roller bottles containing 7.5 mls of phosphate free culture medium (Table 1) which had been previously sterilised by autoclaving at 15 p.s.i. for 20 min and the bottle tops sealed with aluminum foil. Where material was to be labelled with radioactive ^{32}P orthophosphate (Amersham, PBSI), the explants were cultured in 1 ml less than the volume required for the number of explants used. Label, in carrier phosphate (pH 6.0), was added during the growth period in 1 ml under sterile conditions. The bottles were turned at 2.5 r.p.m. in the dark

Table 1. Composition of artichoke explant culture medium.

Solution code	Component	Final concentration
A	$Mg\ SO_4\ 7H_2O$	0.146 mM
	KNO_3	0.800 mM
	K Cl	0.880 mM
B	$Fe\ Cl_3$	0.006 mM
	$Ca\ (NO_3)_2$	1.440 mM
2,4-D	2,4-dichlorophenoxyacetic acid	10^{-6} M

All solutions were stored at 100 times final concentrations and diluted for use. Sucrose was added as solid to 4% (0.117 M) final concentration. A and B were aqueous solutions, the 2,4-D was dissolved in ethanol.

at 25°C. After the required growth period, the tissue was homogenised in a mortar and pestle in 5 mls PAS/TNS grinding buffer. Where necessary, growth

of the explants was monitored by determining the cell number, using the technique of Brown and Rickless (1949). Samples of 5 explants were digested in 3 ml of 5% chromium trioxide for one to three days at 0°C. The tissue was macerated by repeated passage through a pasteur pipette and a small volume of the final homogeneous cell suspension was placed beneath the coverslip of a Fuchs Rosenthall haemocytometer slide. The number of cells on six complete grids was determined to ensure statistical significance for the calculated mean cell number per grid. Since the volume of liquid overlying the grid was known (3.2. µl) the cell number per explant could be calculated.

2) Preparation of nucleic acid

Immediately following the initial homogenisation in PAS/TNS grinding medium an equal volume of phenol mixture (phenol containing 15% redistilled m-cresol, 0.1% 8-hydroxy quinoline, saturated with 0.01 M Tris H Cl pH 7.4) was added to effect deproteinisation. After vigorous shaking, the aqueous and phenol phases were separated by centrifugation at 1200 g for 10 min. The phenol layer was discarded, and the aqueous phase together with the interface made 0.5 M with respect to Na Cl and re-extracted with a further equal volume of phenol mixture. After centrifugation, the aqueous phase was removed and extracted a third time with an equal volume of phenol and, after centrifugation the nucleic acids were recovered from the aqueous phase by overnight precipitation with 2 volumes of ethanol at 0°C. The precipitated nucleic acid was collected by centrifugation (1200 g, 10 min) and after washing twice with 5 ml 80% aqueous ethanol containing 0.2% SLS (sodium lauryl sulphate, BDH specially purified), was dissolved in 0.15 M sodium acetate, 0.5% SLS and reprecipitated by the addition of 2 volumes of ethanol for at least 6 hr at 0°C.

Ribosomal RNA (rRNA) was purified from total nucleic acid preparations by precipitation from 2.7 M Na Cl (Parish and Kirby, 1966). The washed total nucleic acid was dissolved in 0.1 M sodium acetate to approximately 1 mg ml^{-1} and solid Na Cl added to 2.7 M final concentration. After precipitation for 24 hr at $0^{o}C$, the rRNA was collected by centrifugation at 1200 g for 20 min. The pellet was washed twice with 3.0 M sodium acetate (pH 6.0) to remove Na Cl, residual DNA and, in the case of mouse liver rRNA, glycogen, and twice with 75% aqueous ethanol, 2.0% sodium acetate. Figure 2 shows normal E buffer gel profiles of mouse liver nucleic acid before and after such a purification. Only trace amounts of DNA and soluble RNA are present with the rRNA. The ratio of 28S to 18S rRNA rises after purification from 2.32 to 2.43 indicating that there is preferential recovery of the 28S component.

Purified 28/25S and 18S rRNA components were isolated from sucrose density gradient fractionations. Total nucleic acid or rRNA from a 2.7 M Na Cl precipitation was dissolved in 0.15 M Lithium acetate, 0.8% SLS and layered onto 20 ml 5-20% linear sucrose gradients made up in the same medium. The gradients were centrifuged for 16 hr at 38,000 g in an MSE 50 (3 x 20 swing out rotor) at $4^{o}C$. The gradients were pumped out from the bottom with 60% sucrose at 46 ml hr^{-1} through a syringe needle inserted in the base of the tube and continuously analysed by an LKB Uvicord II. Figure 3 shows the result of such a fractionation. Mouse liver rRNA was fractionated by gel electrophoresis (Figure 3a) and 400 μg loaded onto a sucrose density gradient (Figure 3b). The fractions marked (S1 and S2 corresponding to 18S and 28S respectively) were precipitated with 2 volumes of ethanol at $0^{o}C$ overnight and collected by centrifugation (2000 g, 10 min). The degree of purification achieved is shown in Figure 3c (S1, 18S) and 3d (S2, 28S). The two major components show very little cross contamination but both show

Figure 2.

2.7 M Na Cl precipitation of mouse liver rRNA

Mouse liver total nucleic acid was dissolved in 0.1 M sodium acetate to approximately 1 mg ml^{-1} and solid Na Cl added to 2.7 M final concentration. After precipitation for 24 hr at 0°C, the rRNA was collected by centrifugation at 1200 g for 20 min. The pellet was washed twice with 3.0 M sodium acetate (pH 6.0) and twice with 75% aqueous ethanol, 2.0% sodium acetate. Samples were fractionated on 2.4% acrylamide E buffer gels at room temperature for 1 hr 50 min. a) total nucleic acid, b) 2.7 M Na Cl precipitate. S-RNA represents soluble-RNA, the combined peak of 4S and 5S RNAs.

Figure 2

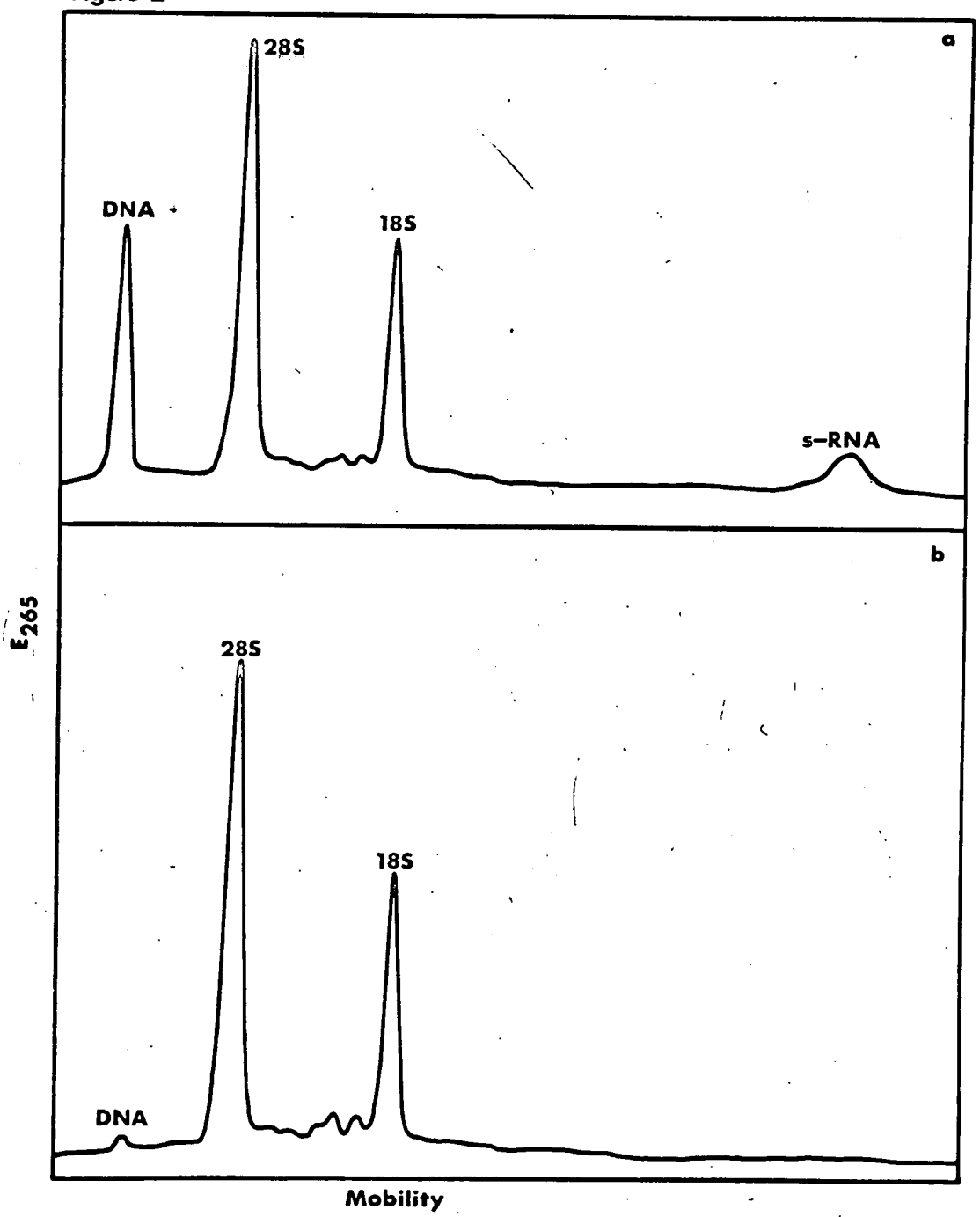
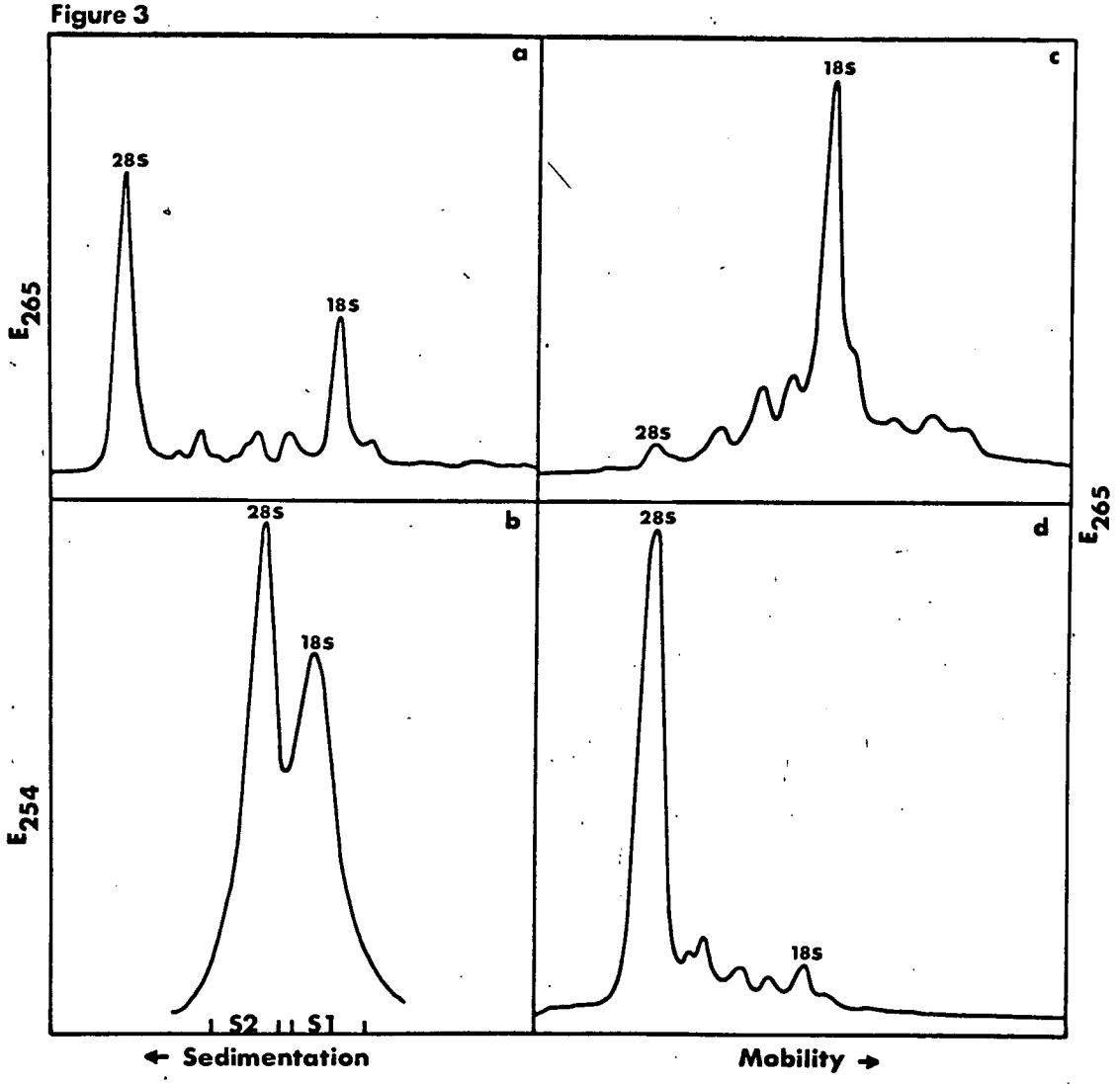


Figure 3.

Sucrose density gradient purification of mouse liver rRNA

Purified mouse liver rRNA was dissolved in 0.15 M lithium acetate, 0.8% SLS and layered onto 20 ml 5-20% linear sucrose gradients made up in the same medium. The gradients were centrifuged for 16 hr at 38,000 g in an MSE 50 (3 x 20 swing out rotor) at 4°C. The 28S (S2) and 18S (S1) rRNA fractions from the gradient were precipitated with two volumes of ethanol at 0°C overnight and collected by centrifugation (2000 g, 10 min). Samples before and after purification were fractionated on 2.4% acrylamide E buffer gels at room temperature for 3 hr. a) initial mouse liver rRNA, c) 18s rRNA, S1, d) 28S rRNA, S2, b) sucrose density gradient of initial sample.

Figure 3


a
28S
18S
E265
c
18S
28S
b
28S
18S
E254
S2
S1
d
28S
18S
E265
← Sedimentation
Mobility →

moderately large amounts of the minor breakdown components always present in mouse liver nucleic acid preparations (see also Figure 2). When plant RNA is used, the contamination from fragments is greatly reduced. If total nucleic acid is fractionated on these sucrose gradients, the DNA spreads throughout the gradient and appears in both fractions.

3) Polyacrylamide gel electrophoresis.

Acrylamide (Eastman Kodak) was recrystalised from chloroform at $0^{o}C$ after dissolving at $50^{o}C$ (70 g l^{-1}) while bis (N, N' - Methylene bisacrylamide was recrystalised at $0^{o}C$ from acetone after dissolving at $50^{o}C$ (12 g l^{-1}). These were stored as a stock solution containing 15% acrylamide and 0.75% bis at $0^{o}C$ in the dark until used. For gel concentrations greater than 5% the bis concentration was reduced to 0.375%. Portions of this solution were mixed with water and 1/5 final volume of 5 x buffer (150 mM Na H_2 PO_4, 180 mM Trizma base, 5mM EDTA-disodium salt, final pH 7.6-7.8) to give the desired final gel concentration. The mixture was degassed for 20-30 sec and 25 µl N,N,N',N' tetramethylethylenediamine (TEMED) added for each 5 ml of stock acrylamide solution used and mixed by gentle swirling. Polymerisation was started by the addition of freshly prepared 10% ammonium persulphate solution (0.25 ml for each 5 ml of stock acrylamide) and gentle swirling to mix. The solution was pipetted into gel tubes (ICI extruded acrylic tube, 9 cm long by 0.6 mm internal diameter closed at the bottom by a PVC support ring stoppered with a short length of 2 mm diameter glass rod), eight cm gels being normally prepared. Water was carefully layered on the surface to ensure a flat top to the gels and polymerisation allowed to proceed at $25^{o}C$ for 2 hr. For 7.5% acrylamide gels, the TEMED and persulphate concentrations were reduced by half to allow time to pipette the solution into gel tubes before polymerisation. After polymerisation the glass plugs were removed and the tubes mounted vertically

between two tanks, each containing 400-500 ml electrophoresis buffer (E buffer: 30 mM Na H_2 PO_4, 1 mM EDTA, 36 mM Trizma base final pH 7.6-7.8, containing 0.2% SLS). The gels were pre-run for 30 min at 50 V (5 to 6 m A per gel) to remove polymerisation catalysts from the top of the gel and to move SLS into this region. Drained nucleic acid precipitates were dissolved in E buffer containing 12% sucrose to a concentration of 1 mg ml^{-1} and up to 50 µg loaded per gel under a small voltage (5-10 volts) to counteract diffusion. The samples were then fractionated at 50 V (5-6 m A per tube) for the times quoted. Under these conditions, gels of less than 3.0% fractionated for less than 2.0 hr contained both high (25S and 18S) and low (4S and 5S) molecular weight RNA species and were scanned immediately on removal from the tubes without washing since the 4S and 5S rRNA molecules diffuse very rapidly from these dilute gels. For all other fractionation conditions, the gels were washed in distilled water for 0.5 to 4 hr prior to scanning. The location of the nucleic acid components was determined by scanning the gel at 7.5 cm length in a quartz cell in a Joyce Loebl polyfrac UV scanner. The light source was a medium pressure mercury vapour lamp with sample wavelength selection (265 nm) by an interference filter and a liquid filter (a freshly prepared solution of p-dimethylaminobenzaldehyde, in methanol, 100 µg ml^{-1}). The E_{265} was recorded by a servoscribe potentiometric recorder geared to give scans of 1x, 2x and 4x the gel length. For gel fractionation of RNA under continuous denaturation as described in Results, gels were polymerised in 7.0 M Urea. The nucleic acid samples were dissolved in E buffer containing 7.0 M Urea (UE buffer) made 12% with respect to sucrose and loaded onto the gels. Electrophoresis was at 50°C.

The scanning system was calibrated by fractionation of known amounts of RNA (determined from the E_{260} measured on a Pye Unican SP 800

recording spectrophotometer with an absorption of 25 OD units per mg RNA). The total peak area was determined in terms of the weight of a tracing and related to the amount of RNA fractionated.

4) Preparation of polysomes.

Artichoke explants were washed under a running tap for 5-10 sec, quickly blotted dry and frozen in aluminium foil packets on solid CO_2: These packets could then be stored on solid CO_2 for up to one week.

The frozen explants (50 to 60) were powdered in a cold mortar and pestle with a small amount of solid CO_2 and the powder transferred to a conical glass in glass homogeniser (Kontes Glass Co., Vineland, New Jersey) to which was added 5 ml grinding medium (50 mM KCl, 10 mM $MgCl_2$, 0.4 M sucrose, 10 mM β mercaptoethanol or 10 mM dithiothreitol [Koch-Light], 1 mg ml^{-1} polyvinylpyrrolidone av. mol. wt. 40,000 [Sigma], 100 mM Tris-HCl, pH 8.5). The temperature was raised to 0°C by warming in the hand and maintained at that value in ice. The powder was homogenised by four twists in the glass in glass homogeniser and collected in an ice cold 15 ml corex centrifuge tube after squeezing through one layer of Miracloth (Chicopee Mills, Inc., New York). The homogeniser was washed out with 2 x 2.5 mls grinding medium which was filtered and added to the initial homogenate. This homogenate was made 2% with respect to Triton X-100 by the addition of 1 ml of 20% Triton X-100 in grinding medium without β mercaptoethanol, dithiothroeitol or polyvinylpyrrolidone. All further manipulations were performed at 0-4°C. Cell wall debris, cell fragments, and nuclei were removed from the homogenate by centrifugation at 10,000 g for 10 min and the supernatant removed. The ribosomes were cleaned and recovered from this supernatant by sedimentation through a 2 ml cushion of 1.0 M sucrose containing 10 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 8.5, at 160,000 g for 3 hr (MSE 50, 10 x 10 fixed angle rotor). The pellet was resuspended in

1 ml 50 mM KCl, 10 mM Mg Cl_2, 10 mM Tris H Cl pH 8.4 (resuspending buffer) and layered onto a 20 ml 10 to 60% linear sucrose gradient (BDH, Aristar) in resuspending buffer. After centrifugation for 3 hr at 70,000 g (MSE 50, 3 x 20 swing out rotor), the gradients were pumped out by piercing the bottom of the tubes with a syringe needle through which 75% sucrose was pumped at 46 ml hr^{-1}, the $E_{254\ nm}$ being continuously monitored with an LKB Uvicord II coupled to a Vitatron potentiometric recorder (UR 401). Fractions were collected using an LKB Ultrorac 7000 fraction collector. Ribosomes were recovered from bulk fractions by the addition of 0.75 volumes of 95% aqueous ethanol, 8 mM Mg Cl_2 and overnight storage at -20°C. The precipitate was collected by centrifugation at 10,000 g for 10 min and RNA prepared from the pellet by adding 5 mls PAS/TNS grinding buffer and extracting in the normal way. Similarly, nucleic acid was prepared from the various stages of the polysome preparation. Where a pellet was used, PAS/TNS grinding buffer was added. Where a supernatant was used, solid PAS and 10% TNS were added to 0.6% and 1.0% respectively.

5) Nuclease digestion

i) Digestion of DNA

Total nucleic acid precipitates were washed twice in 95% aqueous ethanol to remove SLS and dissolved in 2 x SSC (SSC - 0.15 M Na Cl, 0.015 M sodium citrate, pH 7.0) containing 50 mM Mg Cl_2, at 100 µg ml^{-1} Deoxyribonuclease (EC 3.1.4.5., Sigma, DN-EP, electrophoretically purified, ribonuclease free) was added in 0.1 ml 2 x SSC to a final concentration of 30 µg ml^{-1} and the sample was incubated at 25°C for 30 min when a further 70 K units were added and the incubation continued for a further 1 hr. The reaction was stopped by making the mixture 1% with respect to SLS and the nucleic acid deproteinised by shaking with one volume of phenol mixture. The phases were separated by centrifugation and RNA recovered from the

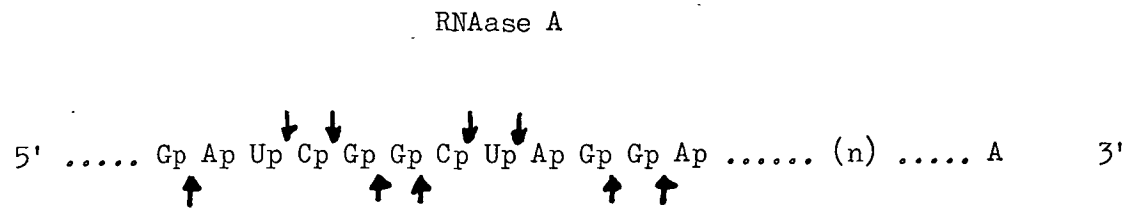
aqueous phase by overnight precipitation at 0°C after the addition of 2 volumes of ethanol.

ii) Digestion of RNA

Nucleic acid precipitates were washed twice with 95% aqueous ethanol to remove SLS and dissolved in NET buffer (0.1 M Na Cl, 0.01 M EDTA, 0.01 M Tris-HCl, pH 7.5) to concentrations up to 100 ug ml^{-1}. The ribonucleases used were RNAase A (EC 2.7.7.16, Sigma type II-A from bovine pancreas), RNAase T1 (EC 2.7.7.26, Boehringer Mannheim, from Aspergillus oryzae) and RNAase T2 (EC 2.7.7.17, Calbiochem 556865, from Aspergillus oryzae). These were stored frozen in NET buffer (0.1 M NaCl, 0.01 M EDTA, sodium salt, 0.01 M Tris-HCl, pH 7.5) as stock solutions of 2 mg ml^{-1}, 10,000 units ml^{-1} and 500 units ml^{-1} respectively and used at final concentrations of 10 µg ml^{-1}, 100 units ml^{-1} and 10 units ml^{-1} at 37°C for 0.5 hr unless otherwise stated. RNAase A specifically attacks RNA endolytically at the 3' phosphate group of pyrimidine nucleotides, cleaving the 5' phosphate linkage to the adjacent nucleotide to give pyrimidine-3'-phosphates and oligonucleotides with a pyrimidine-3'-phosphate end group. The reaction proceeds via the cyclic 2':3'-pyrimidine nucleotide. Both T1 and T2 show the same type of reaction as RNAase A, but with different specificities. RNAase T1, attacks at the 3' side of guanosine nucleotides while RNAase T2 attacks non specifically all RNA phosphodiester bonds showing a preference for the 3' side of adenylic acid bonds, (Egami, Takahashi and Uchida, 1964; Uchida and Egami, 1967). Figure 4 summarizes these specificities in diagrammatic form. Combined digestion with RNAase A and T1 yields Uridine, guanosine and cytidine monophosphates together with intact oligo and poly adenylic acid /oligo and poly (A)/ stretches while digestion as above with the addition of RNAase T2 yields only nucleoside monophosphates. If RNA samples are randomly labelled with ^{32}P orthosphosphate then the difference in counts stable to the

Figure 4.

Ribonuclease digestion products.



RNAase A products

5' .... Gp Ap Up
Cp
Gp Gp Cp
Up
Ap Gp Gp Ap .... (n) ... A 3'

RNAase T1 products

5' ..... Gp
Ap Up Cp Gp
Gp
Cp Up Ap Gp
Gp
Ap ... (n) ... A 3'

combined RNAase A and T1 products

2 x Gp
2 x Cp
Up

Ap Up
Ap Gp

Ap .... (n) .... A

RNAase T2 digestion either alone or with RNAase A and RNAase T1

4 Gp
2 Up
2 Cp

3Ap, nAp, A

where U, A, G and C are the ribonucleosides uridine, adenosine, guanosine and cytidine respectively.

two digestion regimes gives a measure of the amount of poly (A) present in the RNA preparation. The reaction was stopped by the addition of either an equal volume of ice cold 10% trichloroacetic acid, 10 mM KH_2PO_4 (see preparation of samples for radioactive assay) or 0.1 volume of 10% SLS. After the addition of SLS, the mixture was shaken with 1 volume of phenol mixture, the phases separated by centrifugation at 850 g for 10 min and the RNA recovered by precipitation with 2 volumes of ethanol in the presence of unlabelled total nucleic acid.

6) Base compositions

RNA was digested in 1 to 2 ml of 10% piperidine, 10 mM EDTA for 48 hr at 60°C. Where a sample was likely to be contaminated with nucleotides or free radioactive phosphate, it was first precipitated for 1 hr from cold 5% trichloroacetic acid (TCA) and washed as described in the next section. The samples were then dried down, taken up in 2 to 3 drops of 7.5% glacial acetic acid, 0.75% pyridine (pH 3.5-4.0) and loaded as 2 cm streaks 10 cm from one end of 40 cm x 14 cm pieces of Whatman No. 1 chromatography paper (3 samples per sheet). A marker dye comprising 0.5% orange G and 0.5% acid fuschin in 50% aqueous ethanol was also spotted on the base line. The paper was then soaked in pyridine-acetic acid buffer and electrophoresed in the same buffer for approximately 2 hr at 1200 v. Papers were cooled by total immersion in white spirit. Electrophoresis was stopped when the orange of the orange-red pair of spots from the marker dye had just run into the positive buffer tank. The papers were then removed and dried in air.

7) Radioactive analysis

i) Preparation of samples.

Gels containing radioactive nucleic acids were frozen in aluminium

sheet troughs on solid CO_2 to the scanned length and cut into 0.5 or 1.0 mm slices using a Mickle gel slicer. The slices were then dried either onto 16 mm cine film (every 2nd frame, emulsion side) or onto Whatman No. 1 Chromatography paper.

Nucleic acid samples in solution (e.g. after nuclease digestion) were precipitated in the cold for 1 hr by the addition of an equal volume of 10% TCA, 10 mM KH_2PO_4. The precipitate was collected by suction filtration onto Whatman glass fibre paper (GF/C, 2.1 cm) held in a Pyrex filter holder (Millipore). With the suction still applied, the filter was washed with 50 ml cold 5% TCA, 10 mM KH_2PO_4 and 5 ml 95% aqueous ethanol. The filters were then removed and dried under an infra red lamp (275 W, Osram).

Samples for the determination of radioactive uptake were taken as 10 ul aliquots of the final aqueous phase of a phenol extraction and air-dried onto GF/C filters.

ii) Radioactive assay.

Gel slices on cine film were counted in a controlled transport system under a Geiger tube coupled to a programmed scalar unit [Panax or J. & P. Engineering (Reading) Ltd.].

Gel slices on Whatman No. 1 filter paper, 0.5 cm strips from base composition papers and dried GF/C filters were counted in 5 or 10 ml of scintillator [butyl PBD:2-(4'-t-butylphenyl)-5-(4" biphenyl)-1,3,4-oxadiazole in toluene, 4 gl^{-1}] in an Intertechnique SL 31 liquid scintillation spectrometer using the preset ^{32}P window settings for single isotope analysis.

The geiger tube counted gels at approximately 33% of the efficiency at the liquid scintillation system. When RNA was to be recovered from gel slices, samples were counted by Cerenkov radiation. Thawed gel slices were counted in 50 x 9.5 mm rimless tubes in scintillation vials in the Inter-

technique SL31 without any additional liquid. Cerenkov radiation was measured using the preset ^{3}H window settings for single isotope analysis. A comparison of radioactive rRNA dried onto GF/C filters and in water indicated that the Cerenkov technique was 43% as efficient as counting on GF/C filters.

PART 1

RESULTS

Stability and Integrity of RNA.

In order to determine the best conditions for thermal denaturation, rRNA preparations, purified by 2.7M Na Cl precipitation, were melted in E or UE buffer and the hyperchromicity determined as described in the Materials and Methods. The melting profile of pea epicotyl rRNA in E buffer (Figure 5, solid line) shows a gradual increase from 20°C to 90°C which can possibly be resolved into many components. The total hyperchromicity when corrected for thermal expansion is of the order of 21%. When, however, the rRNA is melted in UE buffer (Figure 5, dotted line) there is already considerable hyperchromicity by 20°C. It is not possible to determine the initial part of the melt since both the urea and the SLS precipitate at lower temperatures. However, it is clear from Figure 5 that while complete denaturation, as measured by the hyperchromicity plateauing, is complete in UE buffer by 60°C, temperatures up to 90°C are required to produce the same result in E buffer. In order, therefore, to ensure denaturation during electrophoretic fractionation, 2.4% polyacrylamide gels were made containing 7M urea and electrophoresis was performed at 50°C. This was chosen rather than the 60°C required for complete denaturation as it facilitated handling of the equipment, and preliminary investigation showed little if any qualitative difference between gel profiles at these two temperatures.

The presence of 7.0M urea in the gels alters the electrophoretic mobility of the nucleic acid fractions and Figure 6 shows the distances migrated by the various components in a pea root nucleic acid preparation fractionated at 50°C in 2.4% acrylamide, 7.0M urea gels. As can be seen

Figure 5.

Melting profile of pea epicotyl rRNA in E and UE buffer.

Purified pea epicotyl rRNA in either E (solid line) or UE (dotted line) buffer was progressively heated in an SP 800 recording spectrophotometer and the optical density at 260 mm continuously recorded. The hyperchromicity was calculated with the optical density of fully denatured RNA as 100%.

Figure 5.

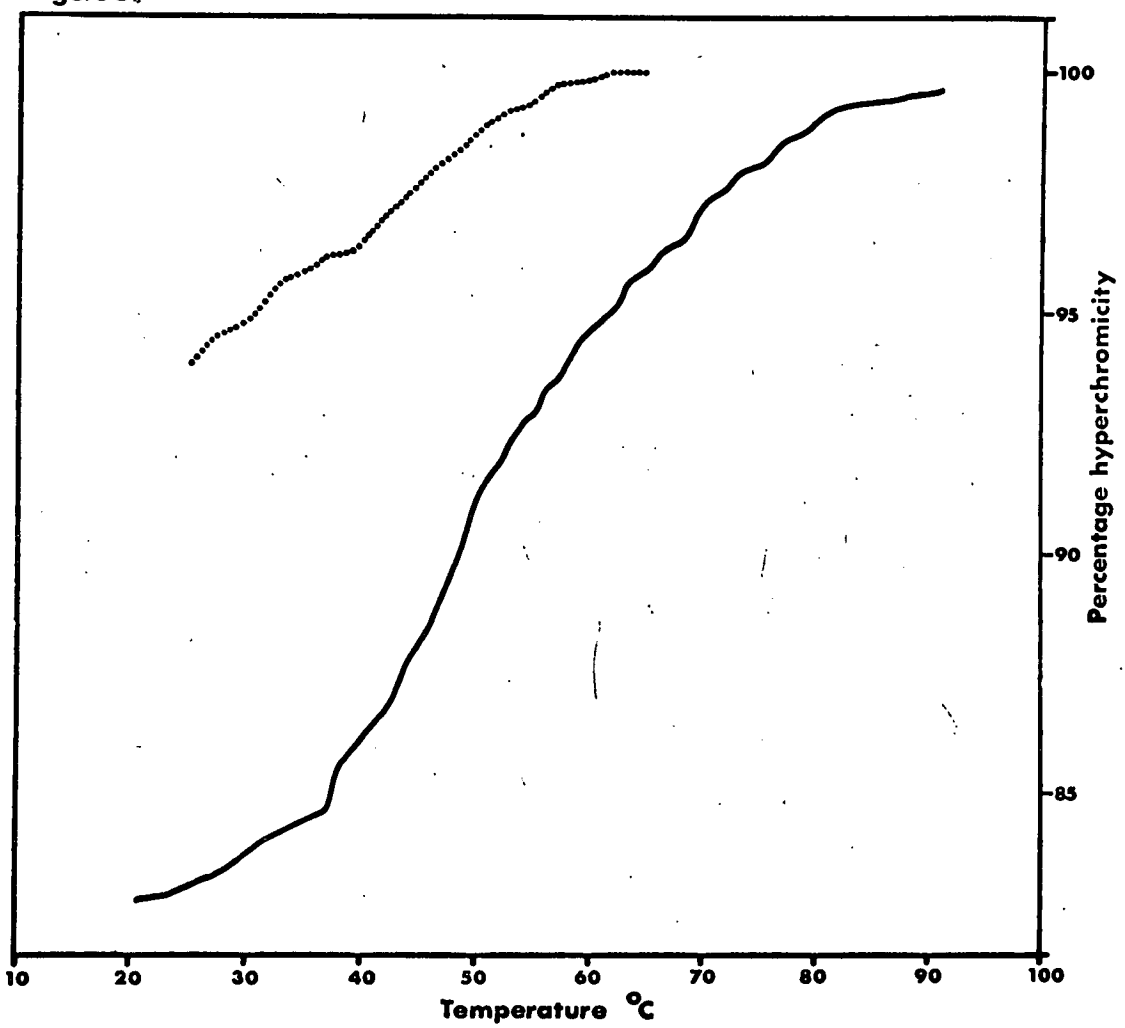

100
95
90
85
Percentage hyperchromicity
10
20
30
40
50
60
70
80
90
100
Temperature °C

Figure 6.

Mobility of pea nucleic acid on 2.4% acrylamide, 7.0 M urea gels at 50°C.

Pea total nucleic acid was dissolved in UE buffer and loaded onto 2.4% acrylamide, 7.0 M urea gels. The samples were fractionated at 50°C for varying times. The arrows mark the mobilities of pea total nucleic acid components dissolved in E buffer and fractionated on 2.4% acrylamide E buffer gels at room temperature.

△——△ : DNA
□——□ : 25S rRNA
○——○ : 18S rRNA
△——△ : 7S rRNA
□——□ : 5S rRNA
○——○ : 4S RNA
—— : ion front

Figure 7.

Mobility of pea nucleic acid on 2.0% acrylamide, 7.0 M urea gels at 50°C.

Pea total nucleic acid was fractionated as in Figure 6 except that 2.0% acrylamide, 7.0 M urea gels were used.

△——△ : DNA
□——□ : 25S rRNA
○——○ : 18S rRNA
△——△ : 7S rRNA
□——□ : 5S rRNA
○——○ : 4S RNA
—— : ion front

Figure 6

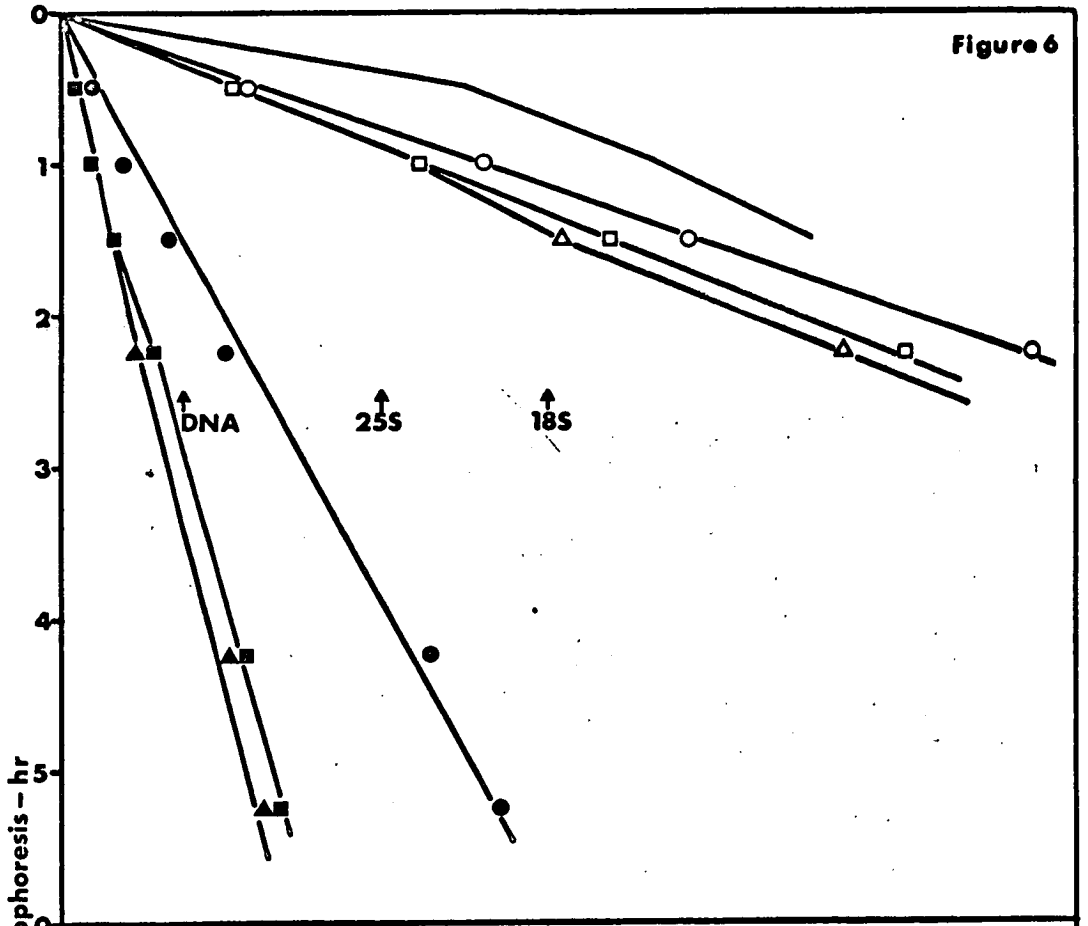
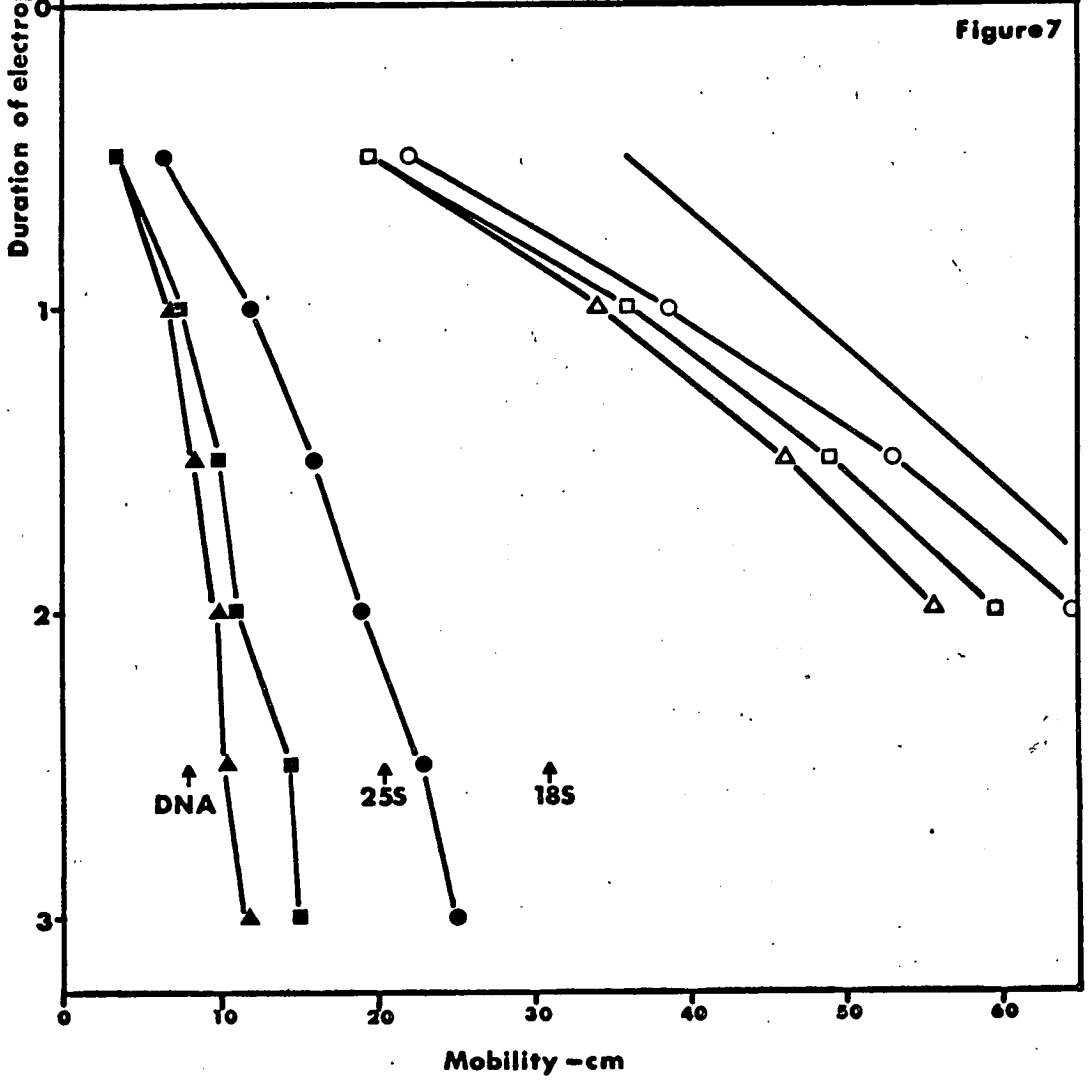


Figure 7



by comparison with the distances migrated by the 25S and 18S after 2.5 hr electrophoresis on a normal 2.4% acrylamide E buffer gel at room temperature (as indicated by arrows on Figure 6), the presence of 7.0M urea in the gel reduces the mobility of the nucleic acid components by more than half. The gross reduction in migration caused by the urea is in fact greater than this, but the elevated temperature, by increasing the ionic mobility, partially compensates. In order to obtain migration rates in 7.0M urea gels at 50°C more comparable with those in E buffer gels at room temperature, the acrylamide concentration was reduced to 2.0%. Although 2.0% acrylamide E buffer gels are too dilute for normal use, when 7.0M urea is present, they have handling qualities at least as good as normal 2.4% acrylamide gels. Figure 7 shows the distances migrated by the pea nucleic acid components on these 2.0% acrylamide, 7.0M urea gels at 50°C. Once again, the distances migrated by similar components during 2.5 hr electrophoresis on a 2.4% acrylamide E buffer gel at room temperature, are indicated by arrows. Although the migrations are still less than those in 2.4% acrylamide E buffer gels at room temperature, a reduction of the acrylamide concentration to 2.0% in the urea gels shows that good separation of the 25S and 18S components can be achieved under these conditions in 3 hr. The various mobilities on Figures 6 and 7 were obtained by measurements made on one gel for each fractionation time and so have an inherent error because of variation between gels. This has been minimised by using only one batch of gels for each acrylamide concentration.

The relationship between electrophoretic mobility and the $\log_{10}$ of molecular weight, as described by Loening (1967) and Bishop, Claybrook and Spiegelman (1967) for E buffer gels, was investigated for 2.0% acrylamide, 7.0M urea gels at 50°C. Table 2 gives the molecular weights of mouse, pea and artichoke rRNA determined by the two fractionation systems. Gel profiles

Table 2. The molecular weights of eukaryote rRNA fractionated on E buffer and hot UE buffer acrylamide gels.

	electrophoresis conditions	molecular weights		
		mouse liver	artichoke tuber	pea epicotyl
25/28S rRNA	2.4% gel E buffer	1.80	1.33	1.27
	corrected*	1.75	1.28	1.22
	2.0% UE buffer gel, 50°C	1.41	1.20	1.25
18S rRNA	2.4% gel E buffer	0.69	0.68	0.69
	2.0% UE buffer gel, 50°C	0.65	0.67	0.66

The molecular weights were determined by co-electrophoresis with E. coli rRNA (1.1 and 0.56 x 10^6 daltons) under electrophoretic conditions described in the text.

*molecular weights corrected for the loss of the 7S which had been melted from the 25/28S rRNA under the denaturing conditions.

from which these molecular weights were determined are shown in Figure 8. In both cases, E. coli rRNA was used as a standard, with the molecular weights of the 23S and 16S components taken as 1.1 x 10^6 and 0.56 x 10^6 daltons respectively (Midgley, 1965a). As a result of the denaturing conditions, the animal 28S and the two plant 25S RNAs fractionated at 50°C in the urea gels lose the 7S RNA component and thus in order to compare more directly the molecular weights obtained by the two fractionation conditions, 45,000 daltons must be subtracted from the weight of the mouse 28S RNA (Pene, Knight and Darnell, 1968) and 50,000 daltons from each of the plant 25S RNAs (Payne and Dyer, 1972) (Table 2 - corrected weights). The results in Table 2 show clearly that no two RNA species respond to denaturation in the same way. Within the limits of experimental error (range as determined from triplicate

Figure 8.

Fractionation of mouse, artichoke and *E. coli* rRNA on room temperature 2.4% acrylamide E buffer or 50°C 2.0% acrylamide UE buffer gels.

Ribosomal RNA from mouse, artichoke and *E. coli* were dissolved in E or UE buffer and mixed in approximately equal amounts. The E buffer sample was fractionated on 2.4% acrylamide E buffer gels for 2.5 hr at room temperature (a) and the UE buffer sample on 2.0% acrylamide, 7.0 M urea gels for 3.0 hr at 50°C (b). The molecular weights were determined relative to *E. coli* rRNA.

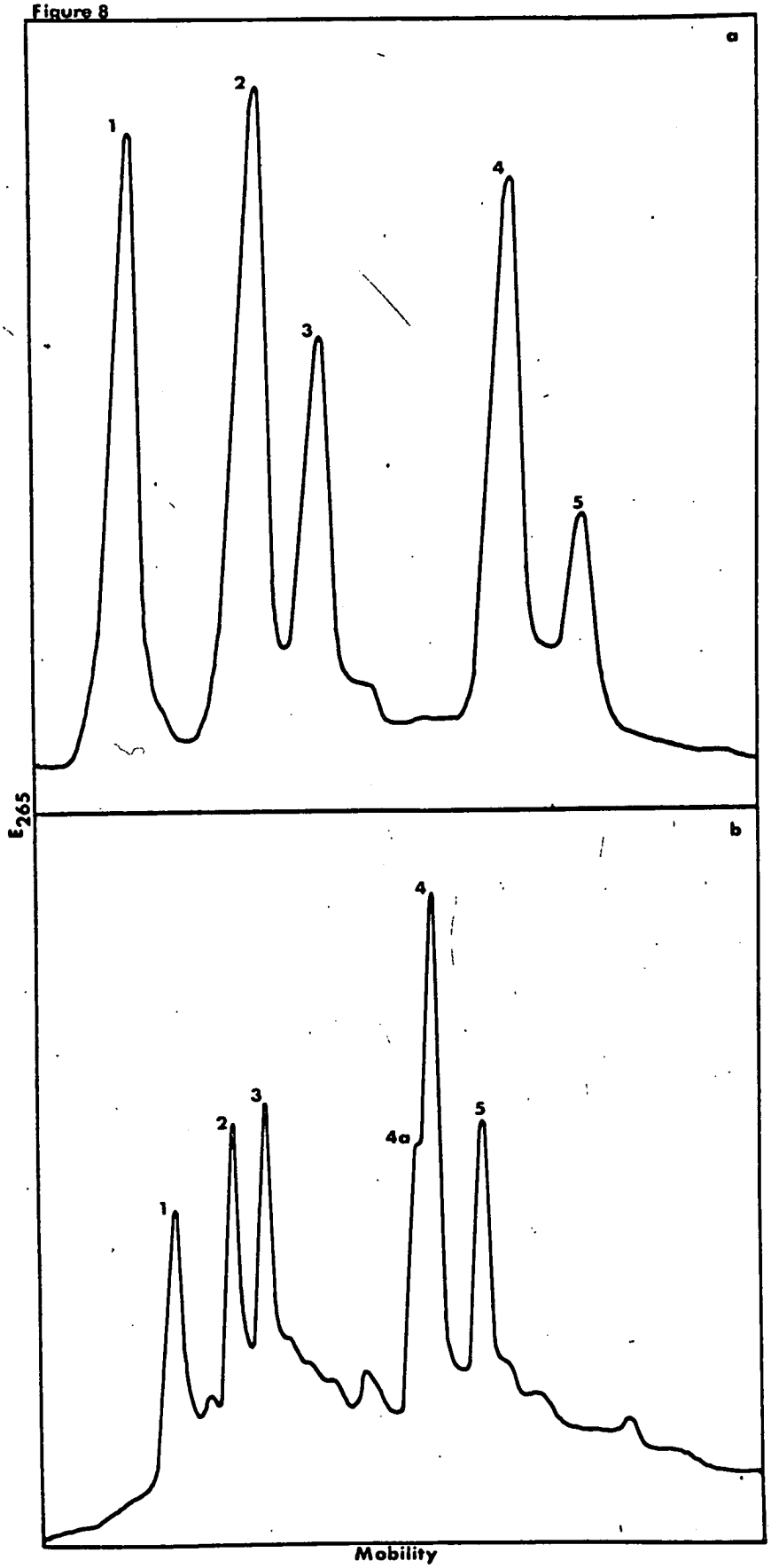
a)
1. Mouse liver 28S rRNA (1.80×10^6 daltons)
2. Artichoke 25S rRNA (1.33×10^6 daltons)
3. *E. coli* 23S rRNA (1.10×10^6 daltons) - standard
4. Mouse liver 18S rRNA (0.69×10^6 daltons)
   Artichoke 18S rRNA (0.68×10^6 daltons)
5. *E. coli* 16S rRNA (0.56×10^6 daltons) - standard

b)
1. Mouse liver 28S rRNA (1.41×10^6 daltons)
2. Artichoke 25S rRNA (1.20×10^6 daltons)
3. *E. coli* 23S rRNA (1.10×10^6 daltons) - standard

4a. Artichoke 18S rRNA (0.67×10^6 daltons)

4. Mouse liver 18S rRNA (0.65×10^6 daltons)
5. *E. coli* 16S rRNA (0.56×10^6 daltons) - standard

Figure 8



measurements of each component = $\pm$ 0.01 x 10^6 daltons), of the 16S molecules, only those from mouse and pea show differences which could be significant. For the 28S molecules, the molecular weight of the pea rRNA shows only a small difference, similar to that of pea 16S rRNA, whereas after denaturation the artichoke is decreased by 6% and mouse by 19%. Apart from the pea 28S where the effect is small, where the apparent molecular weight relative to E. coli changes after denaturation, the result is a decrease. In order to determine whether either of the E. coli 23S or 16S components had changed in effective size as a result of denaturation and fractionation on hot urea gels, 1 mm slices from the 23S and 16S peaks of a 2.0% acrylamide, 7.0M urea gel were rerun on 2.4% acrylamide E buffer gels together with E. coli rRNA as a standard. On all the gels examined, only 2 peaks were visible, but the 23S/16S ratio changed depending on which excised component was rerun with carrier; presumptive 23S causing an increase and presumptive 16S a decrease in the 23S/16S ratio (Table 3). The magnitude of the ratio change depends

Table 3. 23S/16S and 28S/18S ratios of rRNA components from hot urea gels re-electrophoresed with homologous rRNA on normal E buffer gels.

Component rerun	23S/16S ratio	28S/18S ratio
none	2.315	2.121
presumptive 16S/18S	1.615	1.713
presumptive 23S/28S	4.299	2.376

rRNA was fractionated on 2.0% acrylamide, 7.0M Urea gels at 50°C.
The major components were cut from these gels and re-fractioned on 2.4% acrylamid gels at room temperature with homologous rRNA and the ratio of the two major components determined from the peak area.

on the amount of each component carried over in the slice and hence absolute changes are not repeatable although the trends are. When mouse liver rRNA was used as the standard, the molecular weights of the presumptive 23S and 16S could be estimated and shown to be 1.1×10^6 and 0.56×10^6 daltons respectively although the limits of error were rather larger here than for a normal molecular weight determination as the standard and sample could not be premixed. A similar experiment with mouse liver rRNA components isolated from a hot urea gel and refractionated with native mouse liver rRNA on E buffer gels again showed only two major components. The recoveries of the components was considerably less, the 28S/18S ratio showing the same shifts as described above for E. coli albeit with reduced magnitude (Table 3). The molecular weight of the 18S was unchanged while that of the 28S had been reduced when cofractionated with E. coli rRNA as standard. Once again, the range of values was larger than was normal when premixing of sample and standard was possible, but the bulk of the molecular weight loss of the 28S can be attributed to the loss of the 7S fragment on denaturation. The E buffer room temperature system does not resolve the mouse (28S) and mouse (28S-7S) molecules.

For the mouse 28S rRNA then, apart from the loss of 45,000 daltons as a result of the 7S being melted off during denaturation, the difference between the molecular weights obtained by fractionation in the native and denatured states, is solely a reflection of the characteristics of the fractionation systems. By comparing these two extremes with the published values for mammalian rRNA molecular weights, it is concluded that E buffer electrophoresis at room temperature gives the true molecular weight and that fractionation under denaturing conditions does not show the linear relationship between electrophoretic mobility and the $\log_{10}$ of molecular weight. This conclusion is considered in greater detail in the Discussion section.

In order to determine whether it was possible to achieve satisfactory results by denaturing the RNA for a short period prior to fractionation on normal E buffer gels at room temperature, E. coli rRNA was dissolved in E buffer containing 7.0M urea and 12% sucrose and incubated at 50°C for 0.25 hr. The preparation was rapidly cooled in ice and samples fractionated on either 2.4% acrylamide E buffer gels at room temperature or 2.0% acrylamide, 7.0M urea gels at 50°C for 3 hr (Figure 9b and c). The profile of the denatured RNA fractionated on a normal E buffer gel (Figure 9c) is similar to that of the native sample (Figure 9a) while the profile after fractionation under continuous denaturation shows distinct tails of absorbance on the light side of both peaks, a characteristic of the technique (Figure 9b). The ratio of the amount of RNA in the 23S peak to that in the 16S peak can be used as a measure of comparison between treatments since preliminary results indicated that the duration of exposure to denaturing conditions affects RNAs of different sizes to different extents. The 23S/16S ratio (Table 4) is reduced from 2.83 to 2.23 by the 0.25 hr denaturation prior to electropheresis.

Table 4. 23S/16S ratio of native and denatured E. coli rRNA fractionated under native and denaturing electrophoretic conditions.

Prior denaturation of sample	fractionation conditions	mean 23S/16S ratio
None	2.4% acrylamide E buffer gels room temperature	2.83 ± 0.05
0.25 hr		2.23 ± 0.05
0.25 hr	2.0% acrylamide UEbuffer gels, 50°C	2.00 ± 0.10
3.25 hr	2.4% acrylamide E buffer gels room temperature	2.04 ± 0.05

E. coli rRNA was denatured in UE buffer (containing 12% sucrose) at 50°C prior to electrophoresis. The 23S/16S ratio was determined from the peak areas in Figure 9. ± values show the range obtained from triplicate measurements.

Figure 9.

The effect of denaturation prior to and during fractionation on the electrophoretic profile of *E. coli* rRNA.

Purified *E. coli* rRNA was dissolved in UE buffer and incubated at 50°C for 0.25 or 3.25 hr to denature the RNA. Samples were fractionated on 2.4% acrylamide gels at room temperature or 2.0% acrylamide, 7.0 M urea gels at 50°C.

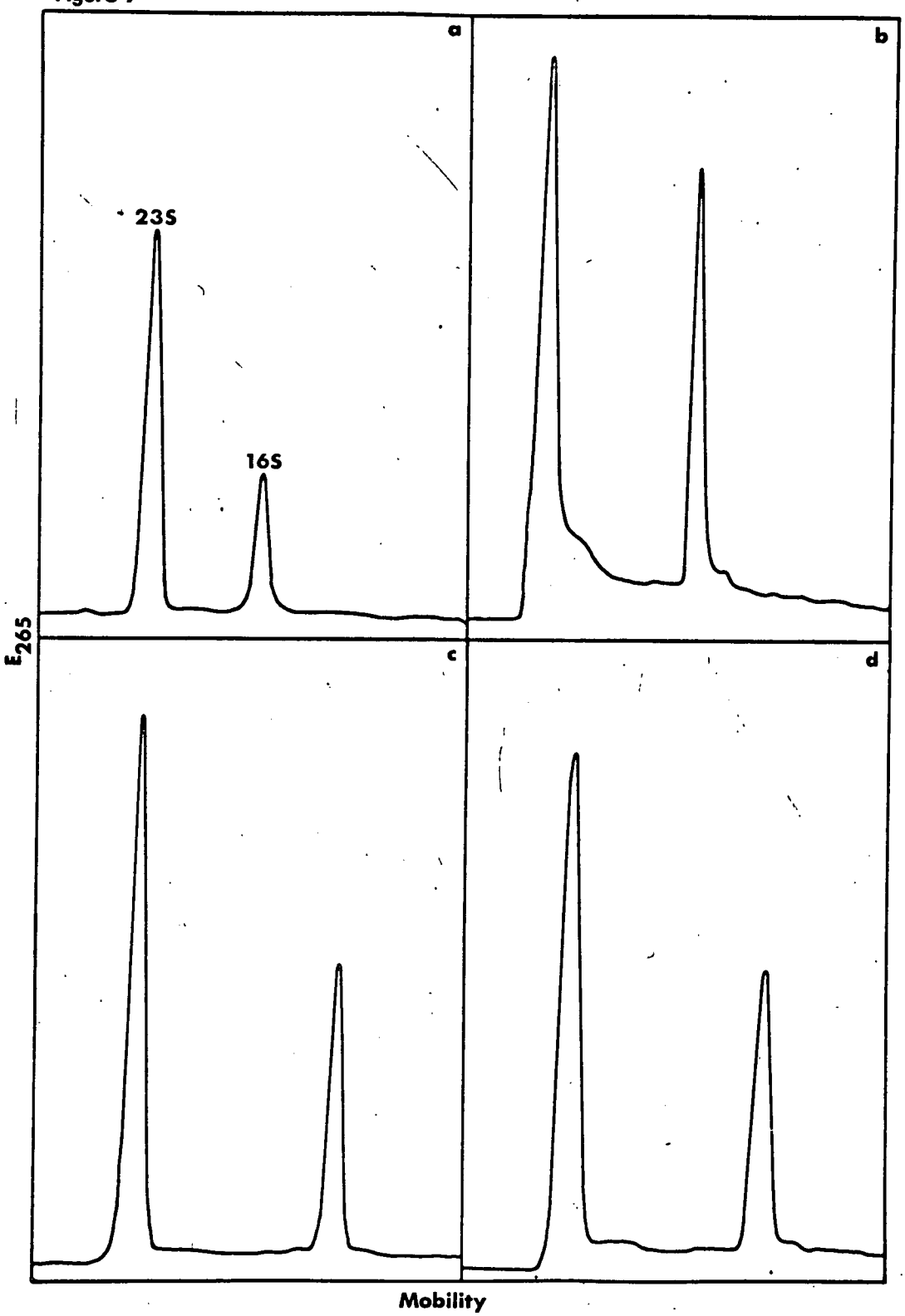
a) untreated sample in E buffer fractionated for 3 hr on a 2.4% acrylamide E buffer gel at room temperature.

b) denatured RNA (0.25 hr) in UE buffer fractionated for 3 hr on a 2.0% acrylamide UE buffer gel at 50°C.

c) denatured RNA (0.25 hr) in UE buffer fractionated for 3 hr on a 2.4% acrylamide E buffer gel at room temperature.

d) denatured RNA (3.25 hr) in UE buffer fractionated for 3 hr on a 2.4% acrylamide E buffer gel at room temperature.

Figure 9



Although continued denaturation during the 3 hr fractionation reduces the ratio even further to 2.00, this final result can be obtained for the normal E buffer fractionation system by increasing the pre-incubation of the sample to 3.25 hr (Figure 9d and Table 4). It is clear then that the characteristic profile obtained after denaturation during fractionation, with tails to the light side of the peaks (Figure 9b), is a result of the release of smaller components during actual fractionation. This can be prevented by short denaturation followed by fractionation under non-denaturing conditions.

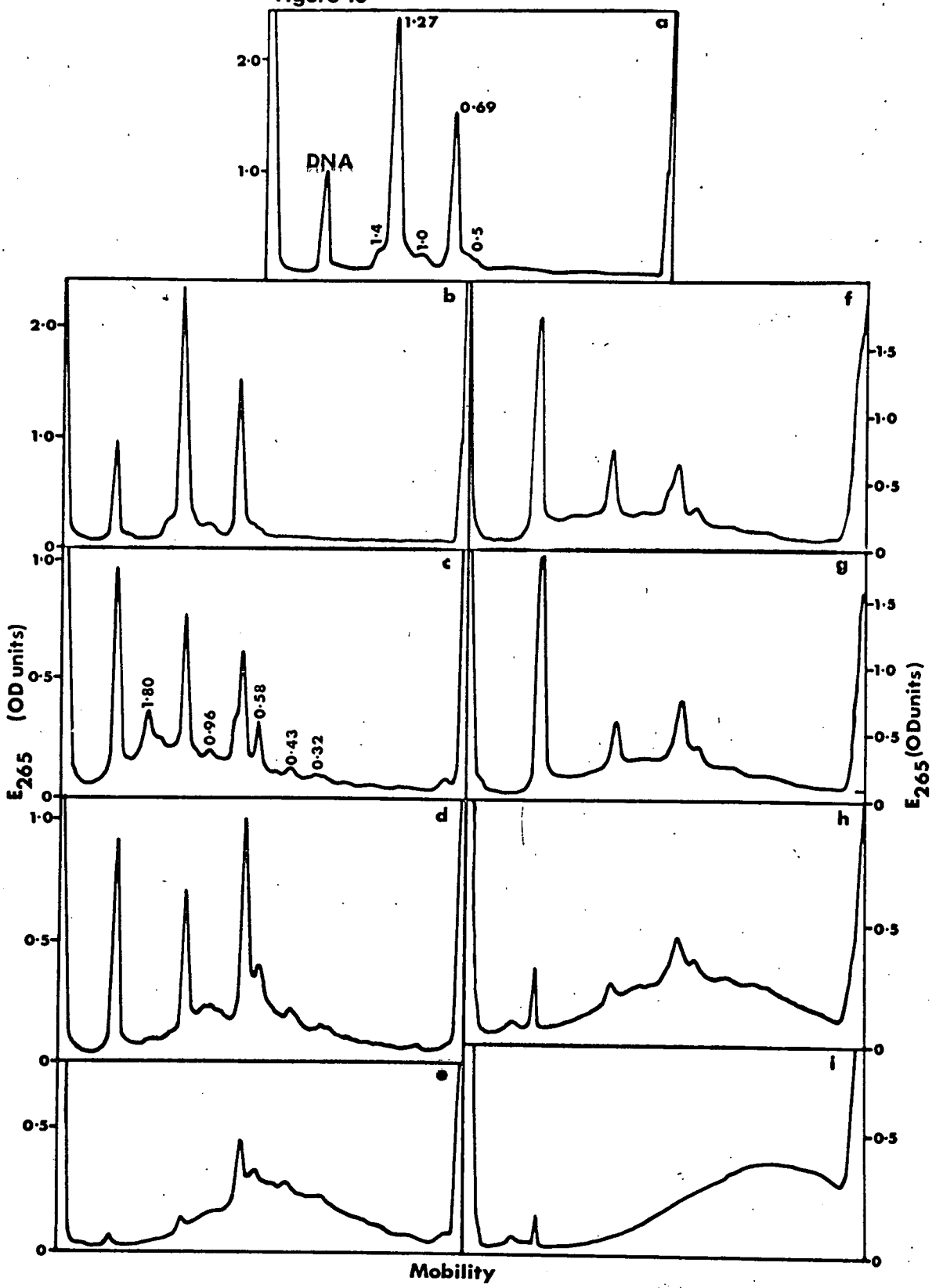
As well as releasing molecular fragments of random size, denaturation also releases, from some rRNA preparations, RNA fragments of particular size in sufficient quantity for them to appear as discrete peaks on gel fractionation. Figure 10 shows the gel fractionation profiles of pea epicotyl total nucleic acid after incubation at a range of temperatures in E or UE buffer. Fractionation of the untreated sample resolves the DNA, the two rRNAs with molecular weights of 1.27 and 0.69 x 10^6 daltons plus small peaks of the organelle RNAs at 1.0 and 0.5 x 10^6 and a trace of 0.69 x 10^6 aggregate at 1.4 x 10^6 daltons (Figure 10a). The fractionation profile is not altered by treatment of the preparation in E buffer at 30°C for 5 min (Figure 10b), but after 5 min at 50°C (Figure 10c) both the 1.27 and 0.69 x 10^6 dalton peaks have greatly reduced in amount (relative to the DNA) and the former is reduced in size to 1.22 x 10^6 due to the loss of the 7S component. Additional peaks are also present at 0.32, 0.43, 0.58, 0.96 and 1.80 x 10^6 daltons, the latter being an aggregate peak of 25S and 18S rRNA, and there is a background of heterogeneous molecules with a mean at about 1.3 x 10^6 daltons. Treatment at 70°C results in a similar profile of peaks except that the aggregate at 1.80 x 10^6 is virtually absent and the heterogeneous background is increased with the mean nearer to 0.7 x 10^6 daltons (Figure 10d). Exposure to 90°C denatures the DNA which then remains on the top of the gel, and

Figure 10.

The effect of temperature on the stability of pea rRNA.

Total nucleic acid was prepared from 3-5 day old dark grown pea epicotyls and fractionated on 2.4% acrylamide E buffer gels for 2.5 hr at room temperature (a). Samples were incubated for 5 minutes in either E buffer at 30°C (b), 50°C (c), 70°C (d) and 90°C (e) or UE buffer at 30°C (f), 50°C (g), 70°C (h) and 90°C (i), rapidly cooled and fractionated.

Figure 10


a
1·27
0·69
DNA
1·4
1·0
0·5
b
f
c
1·80
0·96
0·58
0·43
0·32
g
d
h
e
i
2·0
1·0
0·5
0
1·5
E265 (OD units)
E265 (OD units)
Mobility

results in the bulk of the RNA becoming heterogeneous, with a mean at 0.4 x 10^6 daltons (Figure 10e). Even after this treatment, traces of the 1.22 and 0.7 x 10^6 dalton rRNAs are present. Comparable treatment in the buffer supplemented with 7.0M urea results in a similar degree of degradation being achieved at a lower temperature. For example treatment at 30°C in the urea buffer (Figure 10f) gives a profile similar to that resulting from 50°C exposure in the electrophoresis buffer except that the aggregate peak is considerably less stable in urea. After treatment in the urea buffer at 70°C, there are still traces of the intact rRNA molecules on a high heterogeneous background with a mean about 0.5 x 10^6 daltons (Figure 10h) whereas exposure to 90°C removes all traces of discrete peaks, resulting in a heterogeneous distribution with an average size of 0.1 to 0.2 x 10^6 daltons (Figure 10i). Thus, if denaturation is measured in terms of the release of discrete fragments, the minimum temperatures required are 50°C in E buffer, and 30°C if this is supplemented with 7.0M urea. Slightly higher temperatures are in fact required in E buffer to destroy the aggregate peaks, but the aggregation can be prevented by reducing the concentration of the nucleic acid solution to below 1 mg ml^{-1}. The continued loss of the major RNA components as the temperature in UE buffer is increased above 60°C which, according to the hyperchromicity curves (Figure 5) produces complete denaturation, is a reflection of the increasing rate of thermal hydrolysis at increasing temperature. Although no new components were released between 50° and 70°C in E buffer, there could be quantitative changes not revealed by a visual examination of the gel profiles. Because of this, the effect of temperature on denaturation was further examined with 25S and 18S rRNA fractionated from pea epicotyl nucleic acids on a sucrose gradient. Fractions of the 25S and 18S RNAs were dissolved in E buffer to a concentration

of 500 $\mu g\ ml^{-1}$ and incubated at a range of temperatures for 5 min. Sucrose was added to a concentration of 12% prior to fractionation on 2.4% acrylamide E buffer gels for 2.5 hr at room temperature. The purification and low incubation concentration reduced aggregation to a negligible level. The amount of RNA recovered as 25S or 18S was determined from the E_{265} profile of the gels and corrected for slight variations in total gel loading. Figure 11 shows these values plotted against incubation temperature and confirms that the bulk of the loss of the two rRNA components takes place at 50°C under the incubation conditions used. Again, continued loss above this temperature is a reflection of the increasing rate of thermal hydrolysis. Thus in terms of the release of fragments as measured by loss of the 25S and 18S components, denaturation is virtually complete at slightly lower temperatures than those indicated by the hyperchromicity. Most of the loss of the rRNAs on denaturation takes place during the first five minutes and can amount to more than 60% for artichoke 25S RNA. Thereafter, there is a gradual exponential loss which is attributed to thermal hydrolysis of the polynucleotide chain during incubation and will be examined in detail later.

To ensure that there was no renaturation of fragments following denaturation, RNA preparations were incubated at 50°C in 7.0M UE buffer for 5 min (the time required to give complete denaturation with minimum thermal hydrolysis) and thereafter transferred to an incubation temperature of 25°C for periods up to 45 min prior to fractionation or normal E buffer gels. The gel profiles showed no recovery of the major component loss or change in pattern of any other type, indicating that there is no renaturation of RNA fragments.

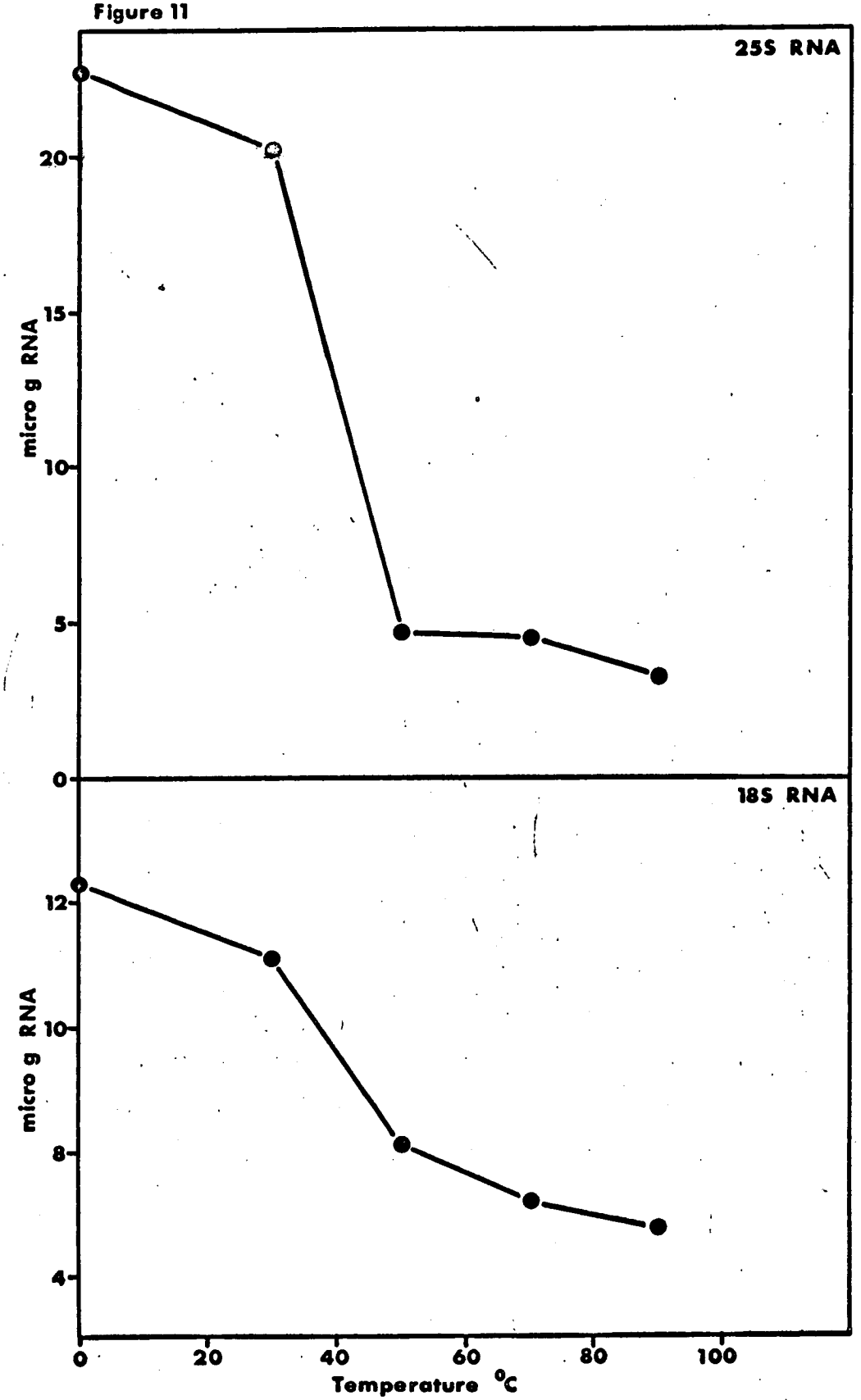
One further denaturation technique was studied, that of incubation at 60°C for 5 min in the presence of phenol as described by Higo, Higo and Tanifuji (1971). Total pea epicotyl nucleic acid was dissolved in 50mM

Figure 11.

The loss of pea 25 and 18S rRNA after denaturation in E buffer at various temperatures.

Pea 25 and 18S rRNA recovered from a sucrose density gradient fractionation of total pea nucleic acid were dissolved separately in E buffer and incubated at a range of temperatures for 5 min. Samples were fractionated on 2.4% acrylamide E buffer gels for 2.5 hr at room temperature and the amount of 25 and 18S rRNA left after denaturation determined by weighing tracings of the peaks from the gel profiles.

Figure 11


25S RNA
18S RNA
micro g RNA
micro g RNA
20
15
10
5
0
12
10
8
4
0
20
40
60
80
100
Temperature °C

NaCl, 10mM Tris-HCl (pH 8.0) at approximately 1 mg ml^{-1}, an equal volume of phenol mixture was added and the mixture shaken at 60°C for 5 min. The two phases were separated after cooling, by centrifugation and the aqueous phase removed and shaken with 2 volumes of ether to remove any dissolved phenol. The two phases were again separated by centrifugation and the aqueous phase removed and sucrose added to 8–12% prior to fractionation on 2.4% acrylamide E buffer gels at room temperature for 3 hr. Even after this denaturation, intact 25S (minus 7S) and 18S RNA are still present (Figure 12).

To determine the rate of thermal hydrolysis, samples of RNA were incubated in either E or UE buffer, both containing 12% sucrose, at constant temperature. Samples were removed at intervals and fractionated either on normal E buffer gels at room temperature or 2.0% acrylamide gels containing 7.0M urea at 50°C. The amount of each of the two major rRNA components remaining after incubation was determined by tracing and weighing the peaks from the E_{265} profile and correcting for differences in the total loaded as a result of evaporation during incubation. Where the incubation temperatures exceeded the temperature for complete denaturation, samples were loaded onto gels immediately. Where they were lower, samples were incubated for 5 min at 60°C prior to fractionation. Where E buffer alone was the incubation medium, the samples were made 7.0M with respect to urea and incubated for 5 min at 60°C prior to fractionation. Figure 13 shows the loss of mouse 18S RNA incubated in UE buffer containing 12% sucrose at 50°C plotted as $\log_{10}$ of concentration against time. The linear relationship between the two parameters indicates that the reaction involved has first order (or pseudo first order since both water and urea are in excess) reaction kinetics. The slope of this and similar plots enabled k, the rate constant for hydrolysis of the rRNA components to be calculated from the

Figure 12.

The effect of denaturation at 60°C in the presence of phenol on the fractionation profile of pea total nucleic acid.

Pea total nucleic acid was dissolved in 50 mM Na Cl, 10 mM Tris-HCl (pH 8.0) and an equal volume of phenol mixture added. The mixture was shaken at 60°C for 5 min and, after cooling, the phases were separated by centrifugation. The aqueous phase was shaken with 2 volumes of ether to remove any dissolved phenol and the two phases again separated by centrifugation. The aqueous phase was made 8-12% with respect to sucrose prior to fractionation.

a) untreated sample in E buffer fractionated on a 2.4% acrylamide E buffer gel for 2.5 hr at room temperature.

b) phenol treated sample fractionated on a 2.4% acrylamide E buffer gel for 3.0 hr at room temperature.

Figure 12

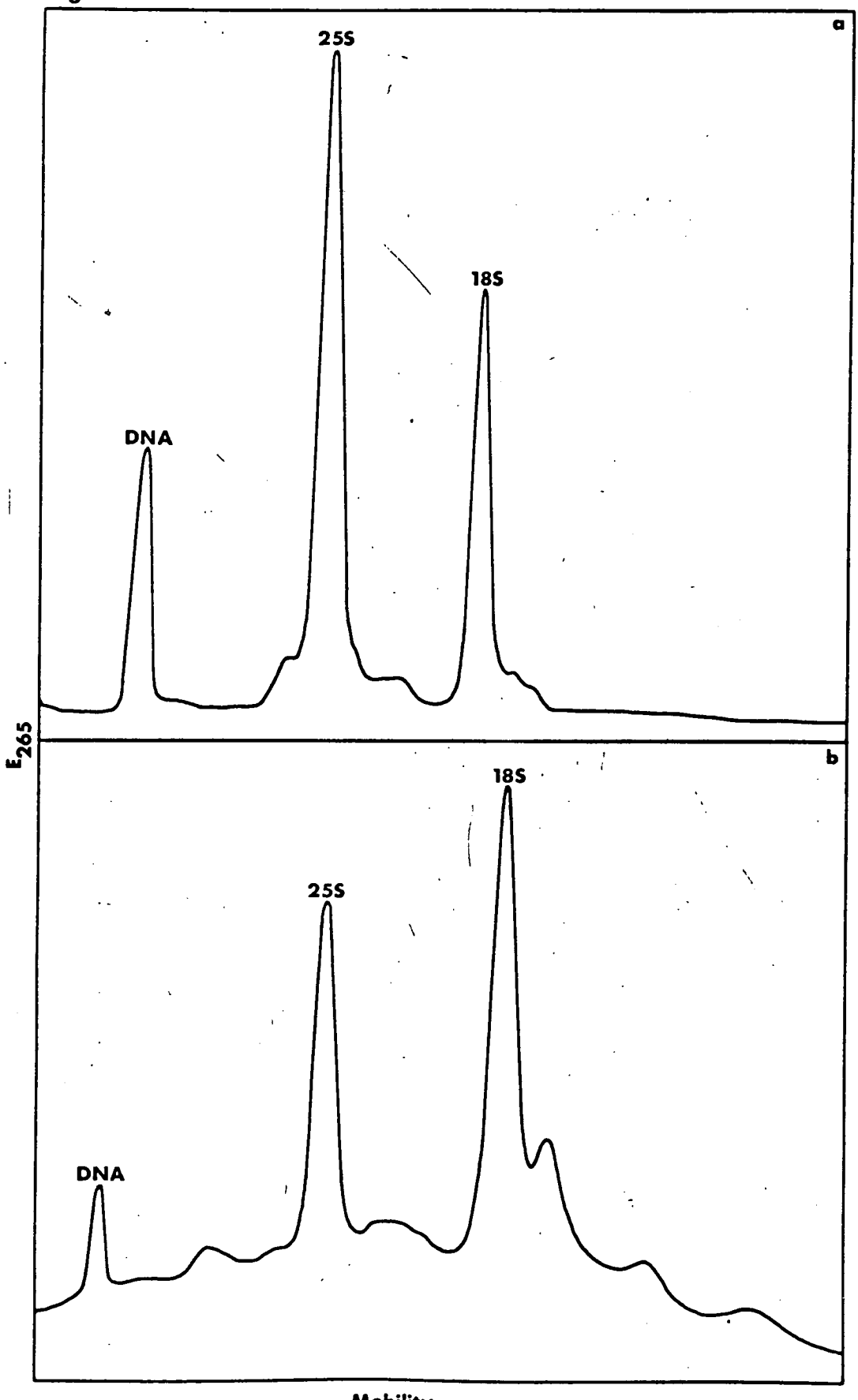
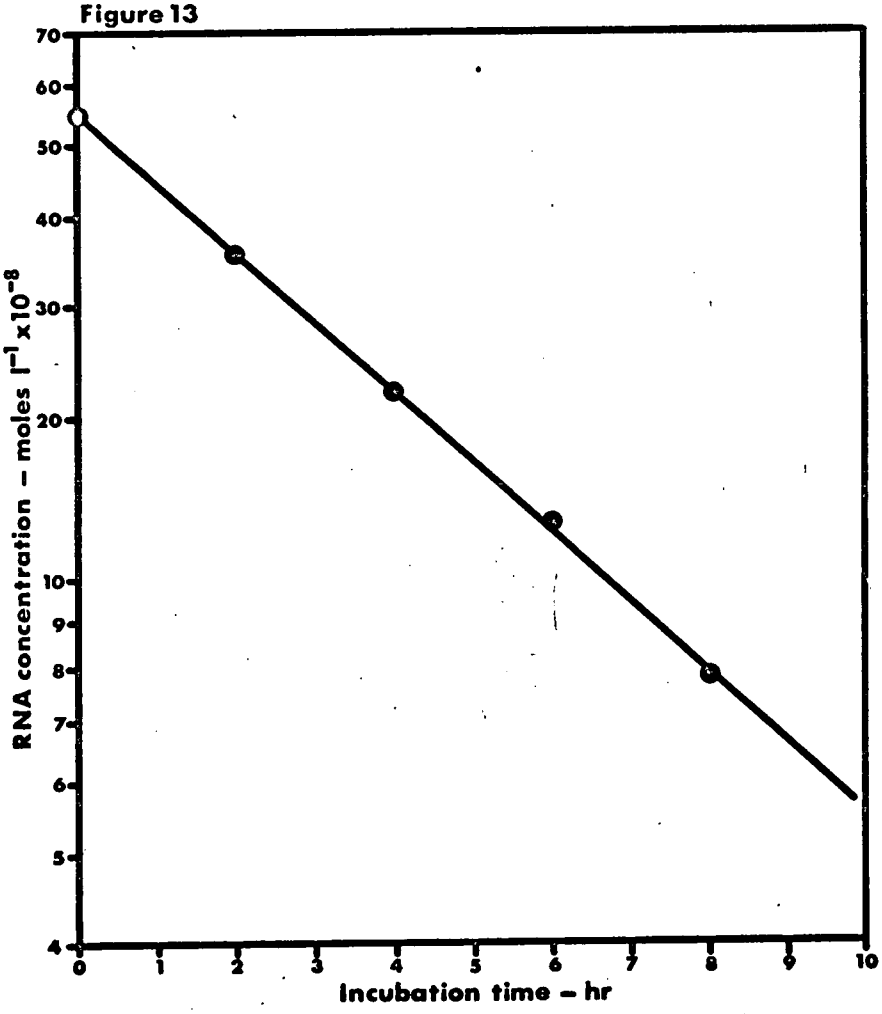


Figure 13.

The thermal hydrolysis of mouse 18S rRNA.

Mouse rRNA was dissolved in UE buffer and incubated at 50°C. Samples were removed at times and fractionated on 2.4% acrylamide E buffer gels at room temperature. The amount of 18S rRNA at each time was determined by weighing tracings of the peak from the gel profiles and correcting for variations in loading.

Figure 13


RNA concentration – moles $l^{-1} \times 10^{-8}$
70
60
50
40
30
20
10
9
8
7
6
5
4
0
1
2
3
4
5
6
7
8
9
10
Incubation time – hr

equation

$$k = \frac{\log \frac{Co}{C_t} \times 2.303}{t}$$

where Co and C_t are the concentrations in moles l^{-1} at time '0' and time 't' respectively. In practice, the values of Co and C_t used were expressed in terms of the primary measurements (mg paper per µl sample) since conversion of these to moles l^{-1} involved a constant which is eliminated by the calculation of $\frac{Co}{C_t}$. Since the hydrolysis of one phosphodiester bond in a molecule means that that molecule is lost from the major peak, k, when divided by the number of bonds per molecule gives a measure of k_b, the rate constant for phosphodiester bond hydrolysis (Table 5). In order to eliminate the possibility that nuclease contamination in the preparations could be responsible for the hydrolysis, the k_b at 60°C in UE buffer was determined for pea epicotyl nucleic acid prepared by a normal Kirby extraction procedure, and then for the same preparation further purified by procedures which should remove any traces of nuclease which had survived the phenol extraction. To 'electrophoretically' purify the rRNA, pea epicotyl nucleic acid was fractionated on 2.4% acrylamide E buffer gels at room temperature for 3.0 hr and the regions containing the 25 and 18S rRNA cut out as a number of 0.2 mm slices. The RNA was recovered from these by two consecutive elutions in 1.0 ml 1.0M lithium acetate, 0.5% SLS at 25°C, the first for 14 hr, the second for 5 hr, and collected by centrifugation at 100,000 g for 17 hr. To purify the RNA by precipitation from guanidinium chloride, an adaptation of the procedure of Cox (1968) was used. A sample of the original preparation in 50mM Na Cl, 10mM Tris-HCl (pH 7.4) was made 4.0M with respect to guanidinium chloride (BDH, Aristar grade) and, after the addition of 0.5 volumes ethanol, precipitated overnight at 0°C. The precipitate was collected by centrifugation (2,000g, 10 min), washed with

Table 5. The rate of phosphodiester bond hydrolysis (k_b) for rRNA under a range of incubation conditions.

Tissue	Preparation	Hydrolysis conditions	k_b (sec^{-1})	
			25/28S rRNA	16/18S rRNA
E. coli (1.1 and 0.56 x 10^6 daltons)	Standard Kirby (1)	50°C UE buffer	1.40×10^{-8}	1.80×10^{-8}
	Standard Kirby (2)	50°C UE buffer	4.04×10^{-8}	3.49×10^{-8}
Artichoke (1.3 and 0.7 x 10^6 daltons)	Standard Kirby	50°C UE buffer	1.40×10^{-8}	3.70×10^{-8}
Mouse (1.7 and 0.7 x 10^6 daltons	Standard Kirby	50°C UE buffer	2.40×10^{-8}	2.39×10^{-8}
		Mean	2.58×10^{-8}	
Mouse	Standard Kirby	50°C E buffer	6.09×10^{-9}	9.40×10^{-9}
		Mean	7.75×10^{-9}	
Pea (1.3 and 0.7 x 10^6 daltons)	Standard Kirby	60°C UE buffer	2.44×10^{-7}	2.70×10^{-7}
	electrophoretically purified	60°C UE buffer	2.47×10^{-7}	2.29×10^{-7}
	guanidinium chloride precipitated	60° UE buffer	2.50×10^{-7}	2.51×10^{-7}
		Mean	2.49×10^{-7}	

Table 5 legend.

Purified rRNA was incubated as shown and fractionated on either 2.4% acrylamide E buffer gels at room temperature or 2.0% acrylamide UE buffer gels at 50°C. The concentration of intact rRNA molecules was determined from the gel profiles and plotted against time and from the slope, the rate constant for hydrolysis of the RNA (k) was calculated. These were converted to the rate constant for phosphodiester bond hydrolysis (k_b) by dividing by the number of bonds per molecule.

Since 50°C in E buffer does not result in complete denaturation, samples incubated under these conditions were made 7.0M with respect to urea and incubated at 50°C for a further 5 min immediately prior to electrophoresis.

80% ethanol, 0.2% SLS and reprecipitated from 150mM sodium acetate, 0.5% SLS by the addition of two volumes of ethanol. The values of k_b determined using these preparations are identical with that obtained for the original preparation (Table 5) indicating that if the hydrolysis is due to ribonuclease, the enzyme is not removed by further purification as described above.

The relative integrity of old and newly synthesised ribosomal RNA

Artichoke explants cultured for 20 hr were labelled with ^{32}Porthophosphat (33 μCi ml^{-1}, 10 Ci mg^{-1}P, Amersham) for 4 hr and the nucleic acid was prepared by normal Kirby extraction. The sample was split into two, one of which was fractionated directly on 2.4% acrylamide E buffer gels while the other was taken up in UE buffer and incubated for 5 min at 60°C prior to similar fractionation (Figure 14). Since the bulk of the RNA in the preparations originates from ribosomes already present in the tuber, the E_{265} profile represents 'old' RNA while the radioactivity histogram represents 'new' RNA. The absence of discrete fragment peaks (Figure 14b) indicates that the

Table 6. Recoveries and specific activities of 'old' and 'new' artichoke rRNA.

	1.33 x 10^6 daltons	0.68 x 10^6 daltons
Percentage recovery of 'old' rRNA (E_{265})	38	63
Percentage recovery of 'new' rRNA (radioactivity)	50	71
Specific activity of native rRNA (cpm ug^{-1})	1,800	2,040
Specific activity of denatured rRNA (cpm ug^{-1})	2,280	2,340

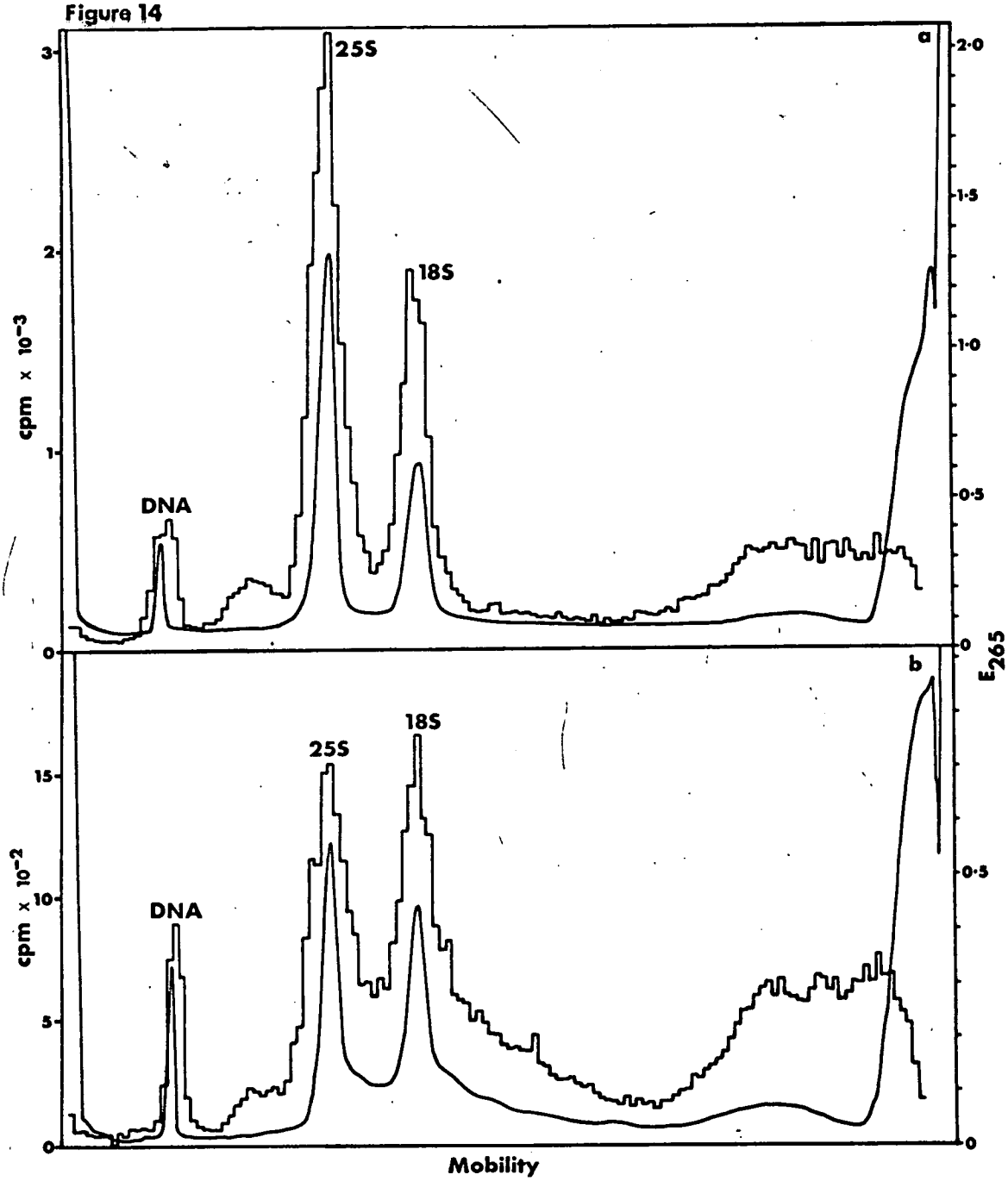
Recoveries were calculated from the profiles in Figure 14 as the amount of each component still present after denaturation. The values are corrected for the loss of the 7S RNA from the 1.33 x 10^6 dalton component and for random hydrolysis resulting from the denaturation treatment (5 min, 60°C in UE buffer, followed by fractionation on 2.4% acrylamide E buffer gels).

Figure 14.

Thermal stability of old and newly synthesised artichoke rRNA.

Nucleic acid was extracted from 20 hr artichoke explants labelled for 4 hr with ^{32}P orthophosphate (33 uCi ml^{-1}, 10 Ci $mg^{-1}P$, Amersham) and fractionated on 2.4% acrylamide E buffer gels at room temperature for 1 hr 50 min (a) native and (b) denatured for 5 min at 60°C in UE buffer. Continuous profile (E_{265} represents old RNA and histogram (radioactivity per 0.5 mm slice) represents newly synthesised RNA.

Figure 14


a
25S
18S
DNA
cpm x 10⁻³
3
2
1
0
2·0
1·5
1·0
0·5
0
b
25S
18S
DNA
cpm x 10⁻²
15
10
5
0
0·5
0
E265
Mobility

amount of specific cleavage of the polynucleotide chain is low. When the percentage recoveries of the 'old' and 'new' rRNA are calculated (Table 6), the loss on denaturation is shown to be greater for 'old' rRNA than for 'new'. This is also shown in the specific activity of the denatured rRNA being somewhat higher than that of the native rRNA (Table 6).

Kinetics and synthesis of 7S rRNA

As has been mentioned earlier, the 28S RNA of animals and the 25S RNA of plants lose one specific fragment on denaturation, the 7S RNA (Pene, Knight and Darnell, 1968; Payne and Dyer, 1972). In artichoke, the size of this component is 50,000 daltons, determined by co-electrophoresis with 5S (37,900 daltons) and 4S (25,000 daltons) as standards on 7.5% acrylamide E buffer gels (Figure 15b and d). The denaturation in this case was in E buffer at 60°C for 5 min. Concommittant with this release, the molecular weight of the 25S RNA decreases from 1.33×10^6 to 1.28×10^6 daltons when compared with E. coli rRNA standards (molecular weights 1.10×10^6 and 0.56×10^6 daltons) (Figure 15a and c). The exact timing of the cleavage of 7S in relation to processing of rRNA was studied in artichoke tissue. The radioactivity profile of nucleic acid from 3-day-old artichoke explants labelled for 1.5 hr with ^{32}Porthophosphate (33 μCi ml^{-1}, 5 Ci mg^{-1} P, Amersham) fractionated on 2.2% acrylamide E buffer gels for 3.5 hr shows that the 25S rRNA (1.33×10^6 daltons) and its immediate precursor (1.39×10^6 daltons) form a double peak (Figure 16a). After denaturation at 60°C for 5 min in E buffer, this peak splits into two (Figure 16b), the precursor maintaining its size of 1.39×10^6 daltons, while the labelled 25S peak decreases with the E_{265} peak to a molecular weight of 1.28×10^6 daltons as the 7S is released from it. The separation suggests that there is no apparent intact 1.33×10^6 dalton component after denaturation. To confirm this, 1 mm slices were cut from a frozen gel similar to that shown in Figure

Figure 15.

The release of 7S rRNA by thermal denaturation.

Artichoke nucleic acid was fractionated on 2.4% (a and c) or 7.5% (d and e) acrylamide gels for 3 hr either in a native state (a and b) or after incubation at 60°C for 5 min (c and d). The molecular weights of the 2.4% acrylamide gel components were determined by co-electrophoresis with E. coli rRNA (1.1×10^6 and 0.56×10^6 daltons) and the 7S rRNA by comparison with those of 4S and 5S RNAs (25,000 and 37,900 daltons). The molecular weights in 'a' and 'c' are in daltons x 10^{-6}.

Figure 15

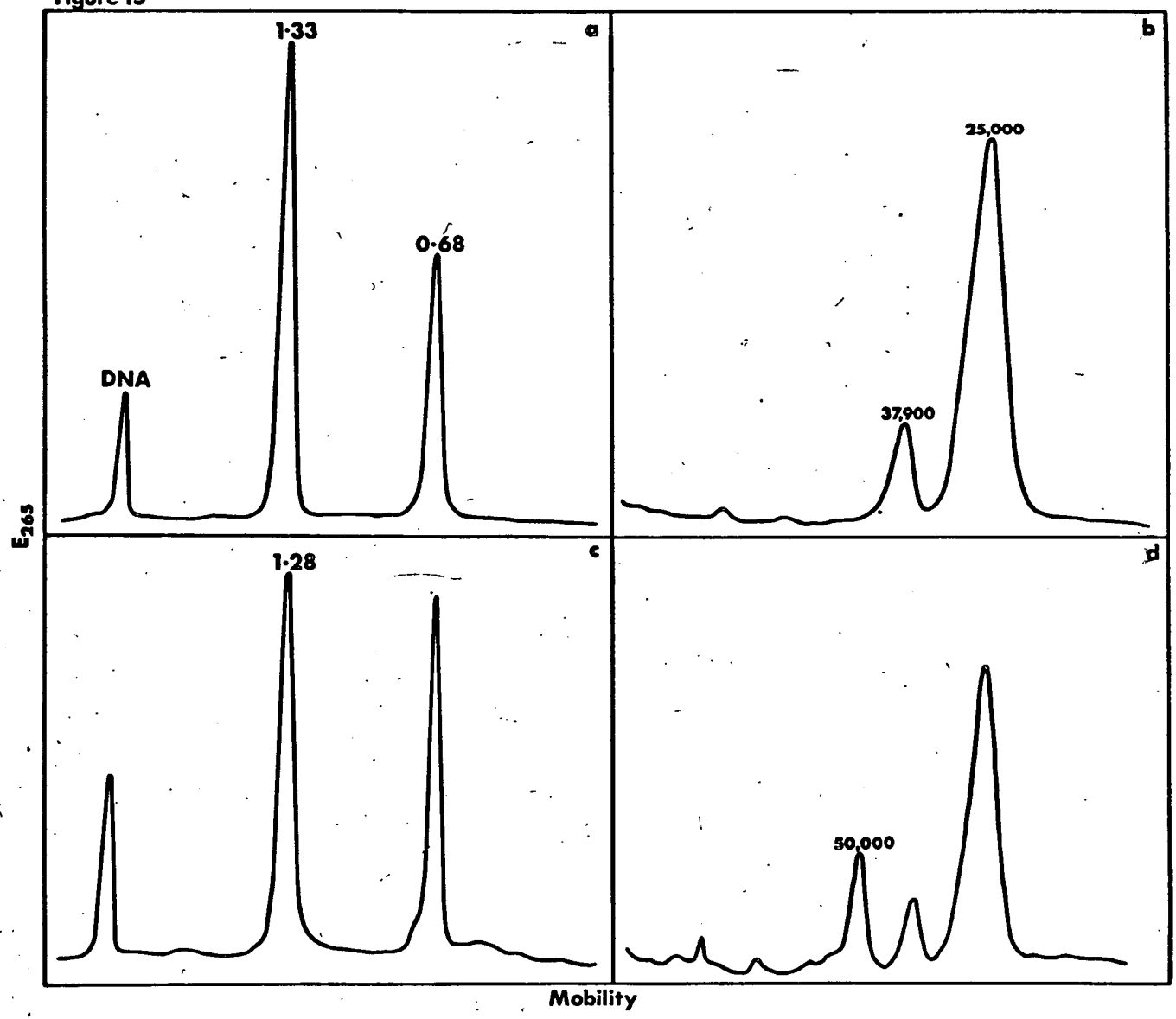
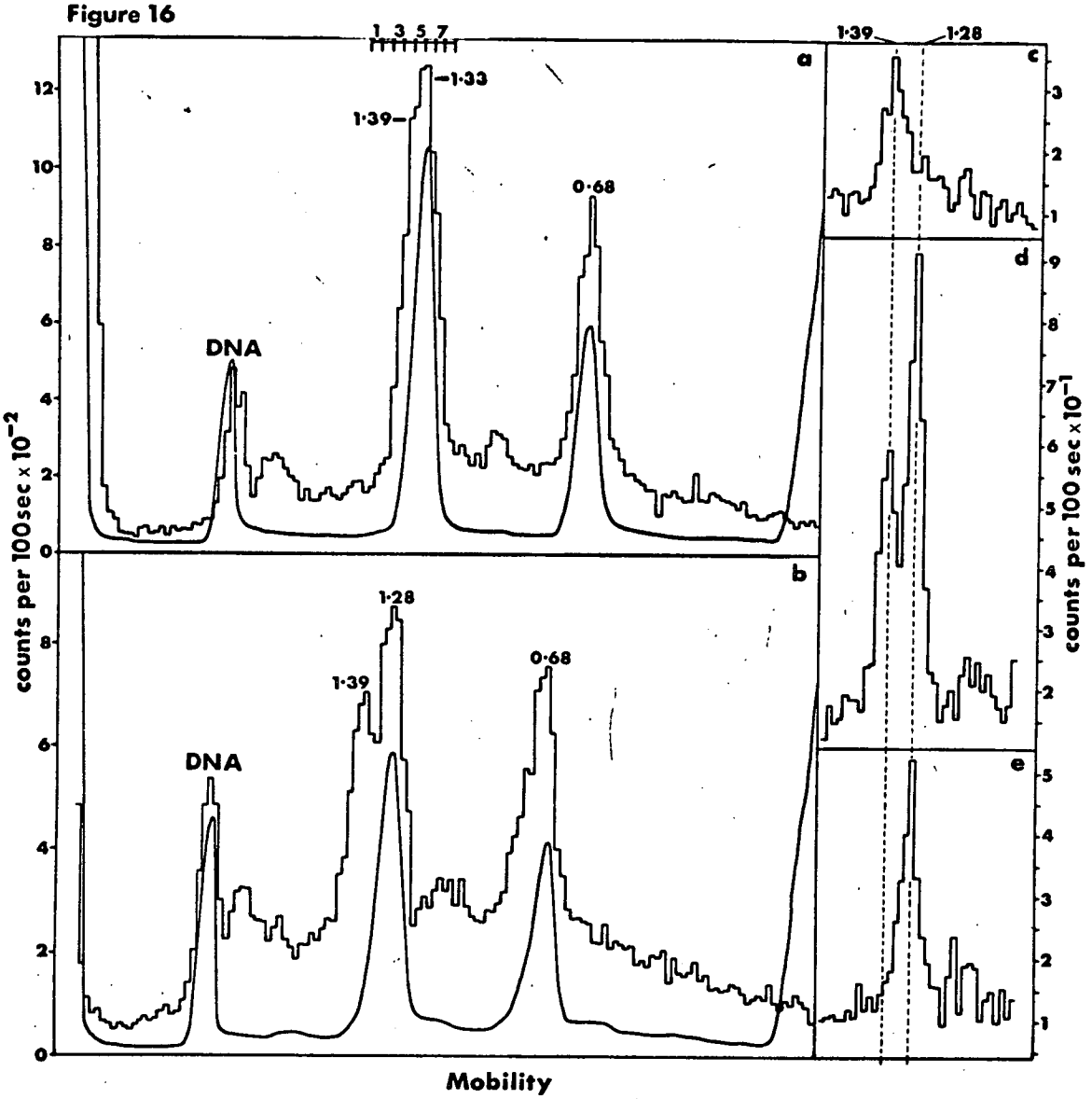


Figure 16.

The thermal denaturation profiles of ^{32}P orthophosphate labelled artichoke rRNA.

Artichoke tissue was labelled with ^{32}P orthophosphate (33 $\mu Ci\ ml^{-1}$, 5 Ci $mg^{-1}P$, Amersham) for 1.5 hr and the nucleic acid was fractionated by electrophoresis on 2.4% acrylamide E buffer gels at room temperature for 2.5 hr. a) native, b) after incubation at 60°C for 5 min. c), d) and e) show the radioactivity profiles of refractionated denatured (50°C, 5 min) RNA from slices through the combined 1.39×10^6 and 1.33×10^6 dalton radioactive peak as indicated in a). c) slice 3, d) slice 5 and e) slice 7. ~ E_{265}, ⎍ ^{32}P orthophosphate. The molecular weights are determined against E. coli rRNA as standards (1.1 and 0.56×10^6 daltons). Molecular weights are in daltons $\times 10^{-6}$.

Figure 16


1 3 5 7
a
1·33
1·39
0·68
DNA
b
1·28
1·39
0·68
DNA
c
1·39
1·28
d
e
counts per 100 sec x 10⁻²
counts per 100 sec x 10⁻¹
Mobility

16a, as indicated by the bar above the 25S/25S-precursor peak. These were placed, together with E. coli rRNA as standards, on top of 2.4% acrylamide E buffer gels at 50°C in an incubator. After 5 min at this temperature with 10V maintained across the gels to ensure that the RNA did not diffuse from the gel surface, the voltage was increased to 50V for a further 15 mins to electrophorese the sample and marker into the new 2.4% acrylamide gels. Thereafter, the electrophoresis tank was removed from the incubator and, after cooling down to room temperature, electrophoresis was continued for a further 3.25 hr. The heavy side of the double peak (slice 3, Figure 16c) shows only the 1.39×10^6 dalton precursor and the light side (slice 7, Figure 16e) only the 1.28×10^6 dalton mature peak while a fraction from the middle of the combined peak (slice 5, Figure 16d) shows both the 1.39×10^6 and 1.28×10^6 dalton peaks with no indication of a discrete component of intermediate size.

The position of the 7S within the 1.39×10^6 precursor was investigated by radioactive incorporation. Three-day-old artichoke explants were labelled with ^{32}P orthophosphate (33 μCl ml^{-1}, 5 Ci mg^{-1} P, Amersham) for varying times and total nucleic acid was extracted by the normal Kirby method. These samples, after denaturation in E buffer at 60°C for 5 min were fractionated on 2.4% and 7.5% acrylamide E buffer gels for 3 hr at room temperature. The specific activities of the 1.28×10^6 and the 7S RNAs were measured from summed counts in each component and the amount of RNA as determined from the peak area. For labelling periods of 40 min and less, the specific activities of the components was too low (< 50 cpm μg^{-1}) to give reliable results. The increase in specific activity and the ratio of the specific activities of the 7S and the 1.28×10^6 dalton component are shown in Table 7. Over the labelling period, the specific activity of the 7S was initially three times greater than that of the 1.28×10^6 dalton molecule; but by 6 hr the two

Table 7. Specific activities of 50,000 and 1.28 x 10^6 dalton rRNA species.

Duration of label	Ratio of specific activities		Approximate specific activities cpm μg^{-1}
	Sp. Act. 50,000 / Sp. Act. 1.28 x 10^6		
	Experiment 1	Experiment 2	
50 min	-	2.38	100
60 min	2.84	1.95	350
75 min	1.30	-	550
6 hr	-	1.06	4,390

^{32}P orthophosphate labelled artichoke nucleic acid was prepared and purified. Specific activities were determined from the E265 and ^{32}P orthophosphate profiles of nucleic acid samples incubated at 60°C for 5 min in E buffer on gels as in Figure 15c and d.

specific activities were similar. This initial high specific activity of the 7S indicates that the 7S is located at the 3' end of the 1.39 x 10^6 dalton molecule, since this end of the precursor would contain the first incorporation of nucleotides to appear in the mature molecules.

PART 1

DISCUSSION

In 1971 Higo, Higo and Tanifuji reported that pea 25S rRNA sedimented at 17-18S after incubation at 60°C in the presence of phenol, thus re-opening the controversy over the integrity of the RNA from the large sub-unit of eukaryote ribosomes. Similar claims have also been made for soybean (Yokoyama, Mori and Matsushita, 1973). The integrity of 28S rRNA was studied in many labs in the mid 60s. Bramwell and Harris (1967) using HeLa RNA and Petermann and Pavlovec (1963) using rat liver rRNA demonstrated either partial or complete conversion of 28S rRNA after denaturation to a 16S product. Riley (1969) developed a technique using exonuclease digestion, which compared the rate of breakdown of his sample (HeLa or E. coli) with that of a standard (usually the RNA from the small ribosomal subunit) and concluded that the formation of the 28S and 23S RNA species involved the association of smaller molecules in the 16-18S size range. Midgley (1965 a, b) and Midgley and McIlreavy (1967) used the technique of periodate oxidation followed by reaction with [carbonyl-^{14}C] isonicotinic hydrazide (isoniazid) to label the 3' end of E. coli rRNA and claimed that the number of labelled ends per unit nucleotide was the same for the 23 and 16S species although Midgley (1965a) claimed that this result only held for RNA prepared "under certain extraction conditions". More recently, however, Hunt (1970) using the same technique, has shown that the 28S rRNA from rabbit reticulocytes, after allowing for the extra end as a result of the 7S rRNA molecule (Pene, Knight and Darnell, 1968) has less than half the number of ends per unit nucleotide of the 18S. The early work of Granboulan and Scherrer (1969) in visualising the RNA directly under the electron microscope gave $\frac{23S}{16S}$ or $\frac{28S}{18S}$ length ratios for rRNA from E. coli, HeLa

and duck erythroblasts as 2, while more recently, Wellauer and Dawid (1973) showed by refinements of the same technique that the $\frac{28S}{18S}$ ratio for HeLa rRNA was 3. Therefore, although under normal fractionation the RNA from the large subunit of both prokaryote and eukaryote ribosomes is intact, breaks can be introduced and it is this that has caused confusion over the size and integrity of this rRNA species.

At the same time, it has been shown that the 26S rRNA of insects and some lower eukaryotes does break down on denaturation to give molecules in the 16 to 18S range. Bostock, Prescott and Lauth (1971) show that the 26S rRNA of Tetrahymena pyriformis is converted to an 18S product by 5 min denaturation at 50°C when the Na Cl concentration is less than 0.5M while co-incubated Chinese hamster ovary rRNA remains at the same size. Stevens and Pachler (1972) have demonstrated a similar result for Acanthamoeba castallani although they were able to show that the 26S rRNA breakdown products (0.80×10^6 daltons) are slightly larger than the 16S rRNA (0.60×10^6 daltons). Silk moth (Applebaum, Ebstein and Watt, 1966), Drosophila (Greenberg, 1969), Aedes aegypti cell culture (Dalgarno, Hosking and Shen, 1972), wax moth (Ishikawa and Newburgh, 1972) and honey bee (De Lucca, Giorgini and Calabrese, 1974) 26S rRNAs are all converted to an approximately 18S product under conditions which leave either an internal (ribosomal precursor RNA) or external (co-incubated mammalian or E. coli rRNA) standard intact. Granboulan et al (1969) also show that the length of the 26S rRNA from Bombyx mori as measured under the electron microscope was approximately halved after heating. More recently Shine and Dalgarno (1973) have examined a range of insects from the Hymenoptera, Lepidoptera, Diptera, Coleoptera Orthoptera, Isoptera, Blattoidea and Thysaura, all of which show the 26S to 18S rRNA conversion. Four examples of the Hemiptera were also examined, three from the suborder Homoptera and one from the suborder Heteroptera. Two of the Homoptera, both species of aphid, did not show the

breakdown and in addition, their rRNAs were both larger than those of the other insects examined. They also showed that the insect 26S rRNA contains the 7S RNA described by Pene et al (1968) in mammalian 28S rRNA. Shine et al (1973) also present results for the number of 3' unsubstituted termini using the periodate-isoniazid technique. They obtained values of 1 for E. coli 23S rRNA, 2 for cultured baby hamster kidney or rat liver cell 28S rRNA and 3 for cultured Aedes aegypti cell 26S rRNA. After heating, the mammalian 28S rRNA was virtually the same size but had only one 3' unsubstituted terminus while the 7S RNA which was released carried the other. Similarly, when the A. aegypti 26S rRNA was heated the 3' terminal label moved to the 18S and 7S positions on sucrose gradients. The conclusion from this data is that only the rRNA from the large ribosomal subunit of the majority of insects and some lower eukaryotes can be shown to be split into 2 parts whose size is in the 16-18S range. This break must occur late in processing since precursor molecules do not show this phenomenon. Since in many of the quoted cases, mammalian or E.coli rRNA was co-incubated to show that the resulting conversion of the insect 26S to 18S rRNA was not caused by residual nuclease activity, the conclusion must be that the former are covalently bonded chains and that reported conversions of these rRNAs to 16S to 18S fragments are artefacts of methodology. One further justification for claiming these latter conversions as artefactual can be shown by an examination of the results of Giorgini and De Lucca (1973) who claim to show just such a result for the 28S rRNA of the rattlesnake, Crotalus durissus terrificus. Their initial results using sucrose density gradients show that after heating, the RNA has a very broad sedimentation profile with a peak at approximately 16S. When, however, the RNA is fractionated by gel electrophoresis albeit poorly, both 28S and 18S rRNA are still present but at decreased amounts with a corresponding increase in the heterogeneous background. These results with Crotalus show the same

trend as described here for pea epicotyl rRNA (Figure 10). It is clear then that sucrose density gradient analysis as used in the earlier studies (eg. Bramwell and Harris, 1967) lacks the resolution necessary for showing details of rRNA breakdown. Hunt (1970) and Shine and Dalgarno (1973) discuss difficulties with the periodate-isoniazid reaction which could explain some of the early results with this technique. The data presented here show that when plant cytoplasmic rRNA is denatured, a percentage of the molecules break down to smaller fragments as has been shown for plant chloroplast rRNA but that there is always a residual percentage of molecules which remain as covalently bonded chains.

The current model for the structure of the two major rRNA components is that up to 70% of the polynucleotide chain takes part in intramolecular base pairing, comprising short hair-pin loops of 20-40 nucleotides in length (Fresco, Albert and Doty, 1960; Cox, 1966; Cox and Kanagalingam, 1967; Cox, Gould and Kanagalingam, 1968; Attardi and Amaldi, 1970) of the type described by Tinoco, Uhlenbeck and Levine (1971). Similar results have been shown for the 5S rRNA by Jordan (1971). The hair-pin loop sizes are average values however and may bear little relationship to the actual loop sizes in the molecule. It is envisaged that either *in vivo* or during extraction, those loops that are exposed at the surface of the ribosome are broken by nuclease attack (Hartman and Clayton, 1974) but that the two rRNA fragments which are thus produced are held together by the region of double helix which originally subtended the loop. On denaturation, therefore the fragments separate as has been shown in pea (Payne and Loening, 1970), *E. coli* (Hartman and Clayton, 1974) rat (Kokileva, Mladenova and Tsanev, 1971) and chloroplast (Ingle, 1968; Leaver and Ingle, 1971) rRNA. The temperature that is required for this is related to the length and G-C content of the double helical region. A comparison of Figures 11 and 1 show that the bulk of the loss of the rRNA

components occurs at temperatures very much less than those required to produce complete denaturation as measured by hyperchromicity. Tinoco, Borer, Dengler, Levine, Uhlenbeck, Crothers and Gralla (1973) have shown for one possible secondary structure of a 26 nucleotide fragment from R17 virus that the Δ G for the loop content is +9 and for the double helical region is -17.8. Their theory predicts that as the number of unbonded bases (m) in a loop increases the Δ G increases such that for any value of m greater than 30, Δ G is given by 3.5 + 2 log m. Where the loop has been broken, it can be considered as having a very large value of m, and hence the positive Δ G for the loop will approach or exceed the stabilising negative Δ G of the double helical region. A similar conclusion is predicted by the work of Scheffler, Elson and Baldwin (1970). Thus broken hair-pins should melt at a lower temperature than intact hair-pins since they will have a less negative net Δ G and be considerably destabilised.

From the data presented here, it is clear that whether they have been subjected to either a denaturation cycle (i.e. pre-incubation followed by fractionation on normal E buffer gels at room temperature) or fractionation under denaturing conditions (E buffer gels containing 7.0M urea at 50°C) a percentage of each of the two large rRNA components persists, although it must be borne in mind that the 25S-28S rRNA from eukaryotes has lost the 7S molecule. The effect of random hydrolysis during denaturation complicates the gel profiles. The tails on the light side of the peaks on hot urea gel profiles (eg Figure 9b) are a direct result of this thermal hydrolysis during fractionation and are caused by the fragments thus produced moving away from the parent molecule during the course of electrophoresis. These are not evident on the room temperature E buffer gels because the rate of hydrolysis is very much lower and many of the breaks are likely to be in regions of secondary structure where, at these temperatures, the ends will be held

together. The values quoted for the rate constant of phosphodiester bond breakage, k_b (Table 5) are taken as being those for thermal rather than nuclease hydrolysis since SLS, a potent nuclease inhibitor, was present in all incubating media and because neither electrophoretic purification nor precipitation from guanidinium chloride which Cox (1968) claims removes all traces of ribonuclease, resulted in any change in the value of k_b. Similar values for k have been found for dimethyl phosphate at 100°C (1×10^{-8} sec^{-1}; Burton, Mhala, Oldham and Vernon, 1960) and dibenzyl phosphate at 75°C (4×10^{-7} sec^{-1}; Kumamoto and Westheimer, 1955). Eigner, Boedtker and Michaels (1961) using TMV-RNA as a substrate and the initial slope of $\frac{M_o}{M_t}$ against t (where M_o is the molecular weight at time zero and M_t the molecular weight at time 't') to determine k, quote the value for k in 10mM phosphate buffer (pH 7.1) at 57°C as 1.4×10^{-8} sec^{-1} which compares favourably with the value of k_b determined here for E buffer at 50°C of 7.75×10^{-9} sec^{-1} (Table 5). Ginoza (1958) showed that for TMV-RNA, the half life during incubation at 55°C in 0.1M phosphate buffer (pH 7.4) was 39 min as determined by retained infectivity of the RNA, and concluded that inactivation was caused by breaking one phosphodiester bond. A value for k_{TMV} at 50°C in E buffer can be calculated by multiplying the k_b for these conditions by the number of bonds (5,900), giving 4.6×10^{-5} sec^{-1}. By putting this value into the equation for the half life ($t_{\frac{1}{2}} = \frac{0.6932}{k}$), the $t_{\frac{1}{2}}$ of TMV at 50°C in E buffer is calculated to be 25 min. This compares well with Ginoza's value allowing for the difference in incubation conditions and the fact that it is possible that not all phosphodiester bond breaks would lead to a loss of infectivity of the molecule.

Using the moderately severe denaturation conditions of 50°C in UE buffer, mouse 28S rRNA (1.8×10^6 daltons) has a k of 1.4×10^{-4} sec^{-1}. The $t_{\frac{1}{2}}$ under these conditions is 82.5 min. Increasing the incubation temperature

to 60°C, reduces the $t_{\frac{1}{2}}$ to 8 min. The percentage of intact molecules in mouse liver 28S rRNA preparations estimated after 5 min denaturation at 50°C in UE buffer was routinely 20% and so, combining this with the very short half life of this species under denaturing conditions and the lack of resolution of sucrose density gradients, it is easy to see why it was concluded that mammalian 28S rRNA was produced as a result of aggregation and did not exist as a covalently bonded molecule.

Reijnders, Sloof, Sival and Borst (1973) have recently questioned the validity of the determination of RNA molecular weights by electrophoresis of the native molecules with standards, usually the 23S and 16S of E. coli. Their earlier studies showed that the electrophoretic mobility of yeast mitochondrial rRNAs varied markedly with temperature (Grivell, Reijnders and Borst, 1971) and from this they concluded that the very low GC content (26% GC compared with 46% GC for yeast cytoplasmic rRNA) meant that the RNA was denaturing over the range 10-50°C. The implication was that electrophoretic mobility was more dependent on secondary structure than had been previously found (Loening, 1969). They went on to demonstrate (Reijnders et al, 1973) that using 8.0M urea gels at 60°C, the molecular weight of mouse 28S rRNA as determined relative to E. coli 23 and 16S rRNA is 1.48×10^6 daltons (corrected to include the 7S molecule) concluding that this is its true molecular weight and that the molecular weight determined using non denaturing conditions (1.80×10^6 daltons) is incorrect. They also show that the non aqueous gel conditions described by Staynov, Pinder and Gratzer (1972) give incorrect estimates of molecular weight because of pH gradients set up in the gels during electrophoresis, but that adding water to 2% and buffering the system at pH 6.0 gives the same results as those obtained by their own hot urea gel method. One final technique which has been used to remove secondary structure from rRNA is reaction with formaldehyde at 63°C for 15

mins (Boedtker, 1971). However, this is not a valid denaturation agent since formaldehyde reacts with the amine groups of the bases, modifying their structure which effectively increases their molecular weight (Hall and Doty, 1959; Feldman, 1973), and prevents the hydrogen bonds reforming and the secondary structure recovering on cooling. Virtually identical results as those described by Reijnders et al (1973) have been shown here using 7.0M urea gels at 50°C, but different conclusions have been drawn. Table 2 and Figure 8 show that the apparent molecular weight of mouse 28S rRNA decreases from 1.80 to 1.41 x 10^6 daltons (although part of this is due to the loss of the 7S molecule) when it is electrophoresed under continuous denaturing conditions and similar though smaller changes are shown for other rRNA species. The fact that the mouse 28S rRNA regains its 'native-state' mobility allowing for the loss of the 7S moiety on re-electrophoresis (see Results part 1) indicates that it has recovered almost all of its native shape, the conclusion being that while no renaturation of separated fragments with complementary sequences could be shown, complementary sequences within intact molecules, since they are spacially linked and are hence in a very high effective concentration, renature almost immediately on cooling.

The decision whether a linear relationship between $\log_{10}$ molecular weight and mobility exists for native or denatured molecules therefore rests on whether the true molecular weight of mouse 28S-7S rRNA is 1.75 x 10^6 or 1.41 x 10^6 daltons. The determination of molecular weight either by sedimentation velocity or sedimentation equilibrium relies on factors which are difficult to estimate; β and $\bar{v}$ for the former and $\bar{v}$ alone for the latter, where β is the shape-volume coefficient and $\bar{v}$ the partial specific volume. From sedimentation velocity studies, the molecular weight is

normally calculated using the Scheraga-Mandelkern equation (Scheraga and Mandelkern, 1953) which assumes that the molecule can be considered as an effective hydrodynamic ellipsoid for the purposes of the calculation. The values of β determined by Kurland (1960) indicate that the molecule must take the form of a prolate ellipsoid with a/b lying between 4 and 8 (where a is the semi-axis of revolution and b the equatorial radius). Kurland (1960) uses values for the molecular weights of E. coli 23 and 16S RNA in the Scheraga-Mandelkern equation to get β and it is this value that is used by Petermann and Pavlovec (1966). Where others use different values of β (e.g. Stanley and Bock, 1965) no derivation is given. Thus a constant shape for all rRNA molecules is assumed. $\bar{v}$ has been calculated by Stanley and Bock (1965) and Petermann and Pavlovec (1966) as 0.53 ml g^{-1} and by Reijnders et al (1973) as 0.51 ml g^{-1}, however molecular weights calculated from sedimentation equilibrium data using these two values differ by only 0.06 x 10^6 daltons (Reijnders et al, 1973). The range of molecular weights of mammalian 28S rRNA determined from sedimentation studies is from 1.90 x 10^6 daltons (HeLa: McConkey and Hopkins, 1969) through 1.65 x 10^6 daltons (Jensen sarcoma: Hamilton, 1967) to 1.57 x 10^6 daltons (Rat: Reijnders et al, 1973) and yet, according to Loening (1968) HeLa and rat 28S RNA co-electrophorese on normal E buffer gels. This range of values gives little indication as to which of the two methods of electrophoresis gives the true linear relationship. Hunt's (1970) determination of the weight of rabbit reticulocyte 28-7S rRNA as being 1.53 x 10^6 daltons, using the periodate-isoniazid reaction, would appear to favour the lower value for the molecular weight. However, Shine and Dalgarno (1973) using the same technique to determine the number of ends in rRNA preparations assumed a molecular weight of 1.7 x 10^6 daltons for BHK 28S rRNA before and after loss of the

7S to show that the native molecule contained 1.99 3' ends while the denatured molecule contained 0.94 3' ends, thus indirectly confirming the assumed molecular weight of 1.7×10^6 daltons. Two attempts have been made to measure the lengths of cytoplasmic rRNA directly using the electron microscope. Granboulan and Scherrer (1969) spread denatured rRNA from a number of species and measured the contour lengths. They calculated the molecular weight of duck erythroblast and HeLa 28S rRNA both to be 1.61×10^6 daltons. Wellauer and Dawid (1973) measured the length of the partially denatured HeLa 28S and of a more fully denatured sample after reaction with formaldehyde, in each case with E. coli rRNA as an internal standard and calculated the molecular weight of the 28S rRNA after loss of the 7S to be 1.75×10^6 daltons. Thus both direct measurements favour the higher molecular weight. Since the most recent techniques give high values for the molecular weight of mammalian 28S rRNA, it was concluded that the true molecular weight of mammalian 28S RNA is 1.75×10^6 daltons and that native but not denatured molecules show linear relationships with electrophoretic mobility. This does, however, assume that there is a fair degree of homology between the molecular weights of 28S rRNA from mammalian sources.

The data presented and reviewed here postulates a model for rRNA which assumes a large amount of secondary structure in the form of hair-pin loops. Some of these loops, as a result of their orientation within the 'molecular shape' are more liable to nuclease attack than others. However, although breaks may be introduced into the polynucleotide chain, presumably at the tips of these loops, the broken ends are held together by the residual base pairing. If the molecule is denatured, fragments are released. On cooling, these fragments never renature together although the secondary structure

within the intact chains and fragments does recover to a considerable extent. The molecular shape must be present for the molecule to show the linear relationship between electrophoretic mobility and $\log_{10}$ molecular weight.

Kokileva et al (1971) showed for rRNA extracted from rat livers labelled for 2 hr or a number of days that the older rRNA contained relatively fewer intact polynucleotide chains than the newer, indicating that at least some of the breaks are introduced in vivo during the ageing of the rRNA. The results on artichoke rRNA shown in Table 6 indicate a similar trend although the differences are very much smaller. It is important to note, however, that the tissues are physiologically very different. The liver cells are continuously functioning and ageing until death while the artichoke tuber tissue, although old in finite terms is physiologically dormant. If the in vivo breaks are only inserted during the active life of the molecule, this would explain the smaller differences in integrity between old and new rRNA obtained for artichoke than for rat liver.

As has been mentioned earlier in connection with the integrity and molecular weight of rRNA, denaturation of the rRNA from the large subunit of 80S ribosomes but not 70S ribosomes, releases a small molecule, variously described as 7S, 28sA, 5.8S and 1 RNA, which in the native state is hydrogen bonded to the 28S rRNA molecule (Pene, Knight and Darnell, 1968; Eliceiri and Green, 1969; Plagemann, 1970; Sy and McCarty, 1970; Payne and Dyer, 1972; Shine and Dalgarno, 1973). Hunt (1970) has demonstrated that native 28S rRNA from rabbit reticulocytes contains 2 distinct 3' end groups (-pGpU and -pGpCpU) while denatured 28S rRNA contains only one (-pGpU), the other appearing with a separate molecule designated 6S. Its molecular weight varies from 45,000 to 46,000 daltons in HeLa and rat (Pene et al, 1968; Sy and McCarty, 1970) to 50,000 to 51,000 daltons in sea urchin

and a range of plants (Sy and McCarty, 1970; Payne and Dyer, 1972; Figure 15).

Pene et al (1968) showed that the labelling kinetics of the 7S are virtually identical to those of the 28S in HeLa rRNA unlike the 5S which labels very much more slowly, indicating that the 7S is part of the precursor molecule and is cleaved off at one of the final processing stages. Weinberg and Penman (1970) come to the same conclusion but do not present the evidence. If the 7S is split from the precursor chain at the final processing step, the 25-28S rRNA molecule should never exist as a covalently bonded unit. The data presented in Figure 16 confirms this. The final processing involves the conversion of one molecule of 1.39×10^6 daltons to one molecule of 1.28×10^6 daltons with one molecule of 50,000 daltons hydrogen bonded to it. The loss of 60,000 daltons at this stage could occur by several methods, either by an endonucleolytic double break in the polynucleotide chain or two consecutive single strand breaks (Figure 17, model 1) or a single endonucleolytic break followed by exonuclease digestion (Figure 17, model 2 or 3). The 60,000 dalton fragment predicted by model 1 was never observed, mitigating against this scheme, unless the fragment were very rapidly degraded. Model 2 would predict the existence of intact polynucleotide chains ranging in size from 1.34×10^6 to 1.28×10^6 daltons, and model 3, from 110,000 to 50,000. Neither of these situations was convincingly observed from gel fractionations, however it has been suggested that such exonuclease digestions must happen very rapidly since other internal intermediates could not be found at earlier stages of processing in other organisms (Weinberg and Penman, 1970; Wellauer and Dawid, 1973). It is also, of course, possible that the discarded 60,000 dalton fragment is at the other end of the molecule but as it would require to be cleaved

Figure 17.

Alternative models for the cleavage of 7S rRNA in artichoke.

Figure 18.

Proposed processing scheme for artichoke rRNA.

Molecular weights are in daltons x 10^{-6}.

Figure 17

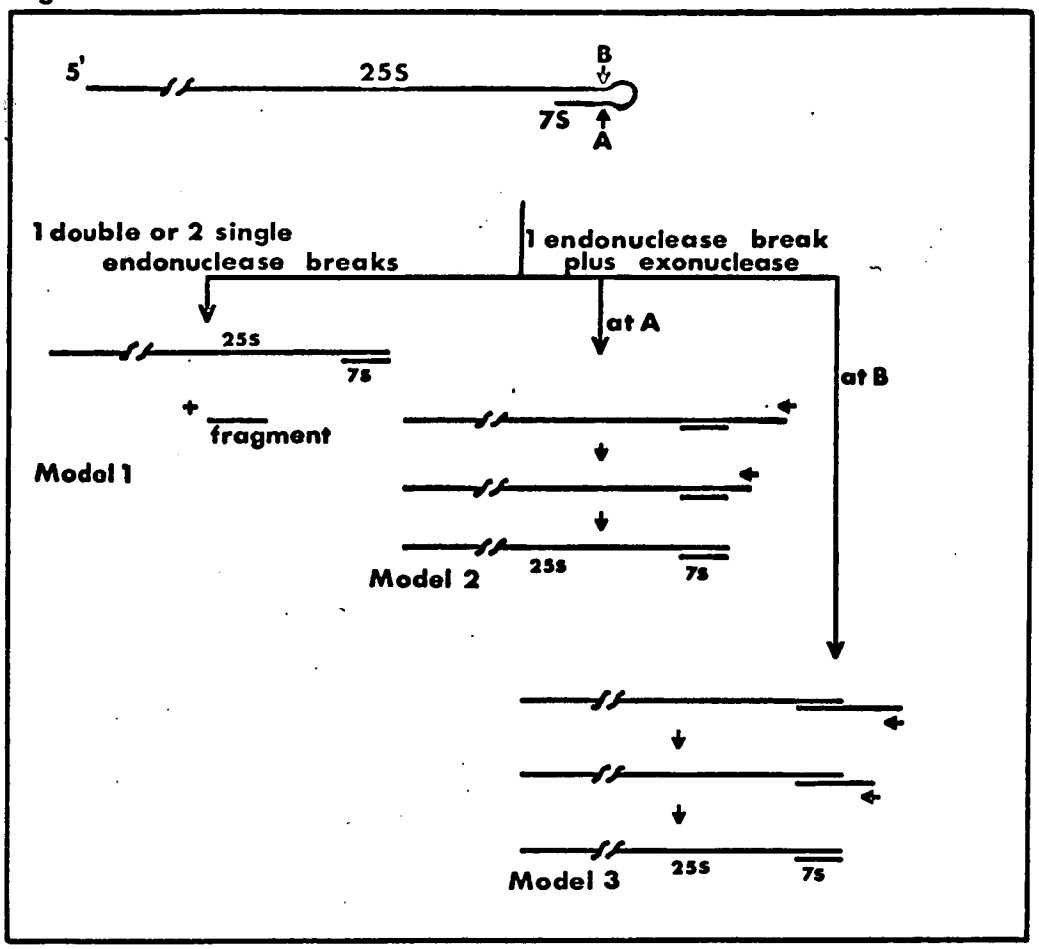
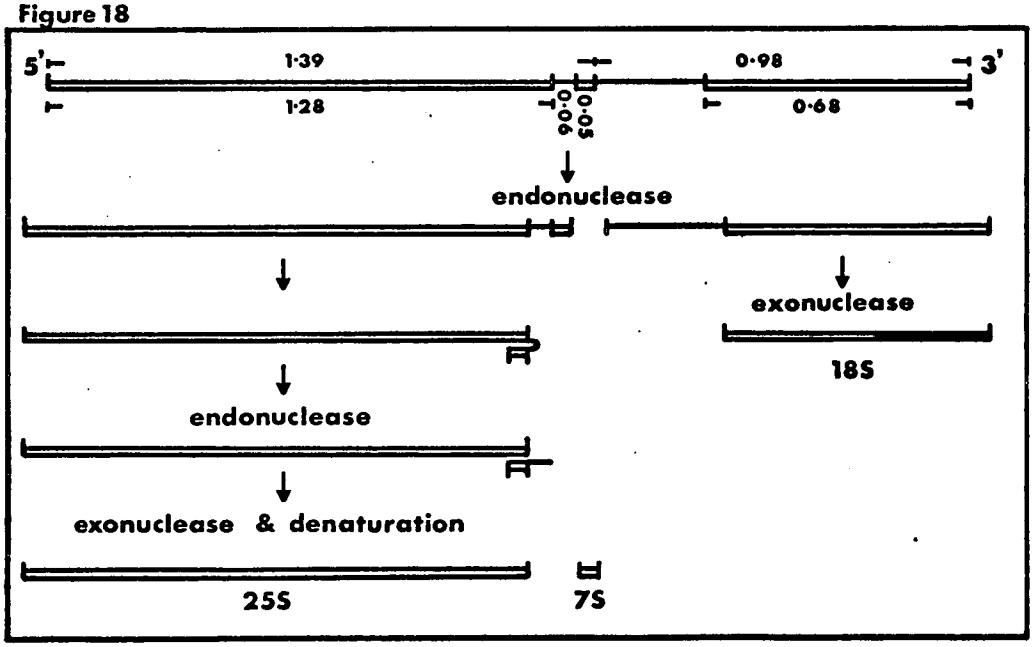


Figure 18



at exactly the same time as the 7S to fit the observed molecular sizes, this model is considered unlikely.

Since nucleotides are added to the 3' end of the precursor chains during synthesis, the 3' end of the 1.39×10^6 dalton precursor should have a higher specific activity than the 5' end. Thus, by comparing the specific activities of the 50,000 and the 1.28×10^6 dalton molecules, it should be possible to determine the location of the 7S within the 1.39×10^6 dalton precursor. Table 7 shows the results of an experiment designed to demonstrate this. In theory, the ratio at the shortest times should be very high, but the situation is complicated by the rising specific activity of the precursor pool and the gradient of uptake and incorporation through the explant tissue. Nonetheless, it is clear that the 7S specific activity is initially higher than that of the 1.28×10^6 dalton molecule and rapidly drops towards the steady state value of 1 (determined as 1.06 after 6 hr labelling, Table 7). The labelling times used in these experiments are very much shorter than those of Pene et al (1968) when they showed that the 7S and 28S had similar labelling kinetics, data used to argue that they originate from the same polynucleotide chain. The results presented here do not therefore invalidate their original conclusions.

The positioning of the 7S at the 3' end of the 25-28S species in the initial transcription product agrees with the work of Choi and Busch (1970) which suggested that it could not be at the 5' end in rat since in hot phenol extracted RNA, the 45S, 32S and 28-7S molecules share a common 5' terminus. Speirs and Birnstiel (1974) have shown by hybridization of sheared ribosomal cistrons in Xenopus laevis that the 7S gene lies between the 28 and 18S genes, and from separations between the three, that it must lie in the transcribed region between the two main genes. Combining this conclusion with the

data presented here in artichoke indicates that the 25-28S rRNA is at the 5' end of the initial transcription product as concluded by Wellauer and Dawid (1973) from their secondary structure maps of HeLa rRNA precursors. This disagrees with the original work of Siev, Weinberg and Penman (1969) who, using cordycepin (3' deoxyadenosine) to prematurely terminate chains, claimed that 18S rRNA but not 28S could be made from prematurely terminated 45S precursor. This suggested that 18S rRNA was at the 5' end of the precursor. Reeder and Brown (1969) showed that E. coli DNA dependent RNA polymerase initiated specifically at the 5' end of the 18S gene in Xenopus laevis DNA and proposed that the same initiation site would be used by the X. laevis polymerase, results which again suggest that 18S rRNA is at the 5' end of the precursor. Both of these results, however, can be challenged, the former on the basis of possible secondary effects of cordycepin on processing and the latter on the possibility of differing specificities of the two polymerases.

By considering all the experimental evidence and making the assumption that the same system holds for animals and plants, the probable order and spacing within the original transcription product and the processing steps for artichoke rRNA can be deduced (Figure 18). The molecular weights were determined initially by co-electrophoresis with E. coli rRNA (1.10 and 0.56 x 10^6 daltons) and thereafter by comparison with the 1.33 and 0.68 x 10^6 dalton mature rRNA components. The precursor sizes have a larger error as a result of the reduced resolution obtained from slicing the gels for counting. The model requires the existence of two endonucleases with different specificities or 1 endonuclease with two different specificity 'factors' and a 5' to 3' exonuclease. This scheme does not agree with the 'preferred' model of Perry and Kelley(1972) which suggests that the

non-conserved fragments are at the 3' ends of the intermediate precursors in mouse L cell rRNA as deduced from 3' to 5' exonuclease digestion of these precursors *in vitro*. However, Wellauer and Dawid (1973) show that the 24S non-conserved portion at the 3' end of the 45S precursor which would be an obvious target for such an enzyme is in fact removed *in vivo* by an endonuclease, to be broken down separately which suggests that unless the two species studied are markedly different in their method of processing the rRNA precursor molecules, the postulates of Perry and Kelley (1972) are unlikely to be true.

PART 2

THE CHARACTERISATION AND POLY (A) CONTENT OF ARTICHOKE RNA

Extraction conditions for artichoke nucleic acid.

Brawerman, Mendecki and Lee (1972) reported that a large proportion of the non ribosomal polysomal RNA paritioned with the phenol layer when polysomes in neutral 0.1 M tris buffer in the absence of detergents were shaken with an equal volume of water saturated phenol. When, however, this phenol phase was then shaken with 0.1 M tris buffer at pH 9.0, the RNA moved back into the aqueous phase. They concluded that the effect was due to the ionic strength of the solution since the neutral tris was largely in ionic form and its reduced ionic strength at pH 9.0 could be replaced by an increase in Na Cl concentration. Total nucleic acid was therefore prepared from artichoke explants labelled for varying times after two days growth with ^{32}P orthophosphate (33 μCi ml^{-1}, 20 mCi mg^{-1} P, Amersham). Tris buffer at pH 7.4 was used with and without adjusting the aqueous phase after the first phenol extraction to 0.5 M Na Cl. Tris buffer at pH 9.0 was used without the Na Cl adjustment. In addition, explants labelled with ^{32}P orthophosphate over the whole two day growth period were extracted at pH 7.4 with the Na Cl concentration adjustment of the first aqueous phase, and explants labelled for 2 hr were homogenised in the absence of any detergent in 50 mM Na Cl, 10 mM Tris-HCl pH 7.4 prior to deproteinisation with phenol mixture at pH 7.4 without the Na Cl concentration adjustment. As a final comparison, 1.33×10^6 dalton rRNA was eluted for 2 hr at 50°C in 0.9 M Na Cl, 0.09 M sodium citrate from a 2.4% polyacrylamide gel fractionation of nucleic acid extracted from explants labelled for 3 days. The base composition of these samples was determined. Figure 19 shows typical nucleotide

Figure 19.

High voltage electrophoretograms of ^{32}P orthophosphate labelled artichoke RNA hydrolysates.

Artichoke RNA precipitated from and washed with 5% TCA was hydrolysed with 10% piperidine, 10 mM EDTA - sodium salt for 48 hr and electrophoresed on Whatman No. 1 chromatography paper in 7.5% glacial acetic acid, 0.75% pyridine (pH 3.5-4.0) for approximately 2 hr. After drying in air, the papers were cut into 0.5 mm strips and counted in 10 ml toluene - butyl PBD scintillator (4 gl^{-1}) in an SL 31 liquid scintillation spectrometer.

a) electrophoretogram from total RNA labelled for 1 hr with ^{32}P orthophosphate (33 $\mu Ci\ ml^{-1}$, 20 $mCi\ mg^{-1}P$, Amersham).

b) electrophoretogram from 3 day labelled 1.33×10^6 dalton rRNA eluted from a gel

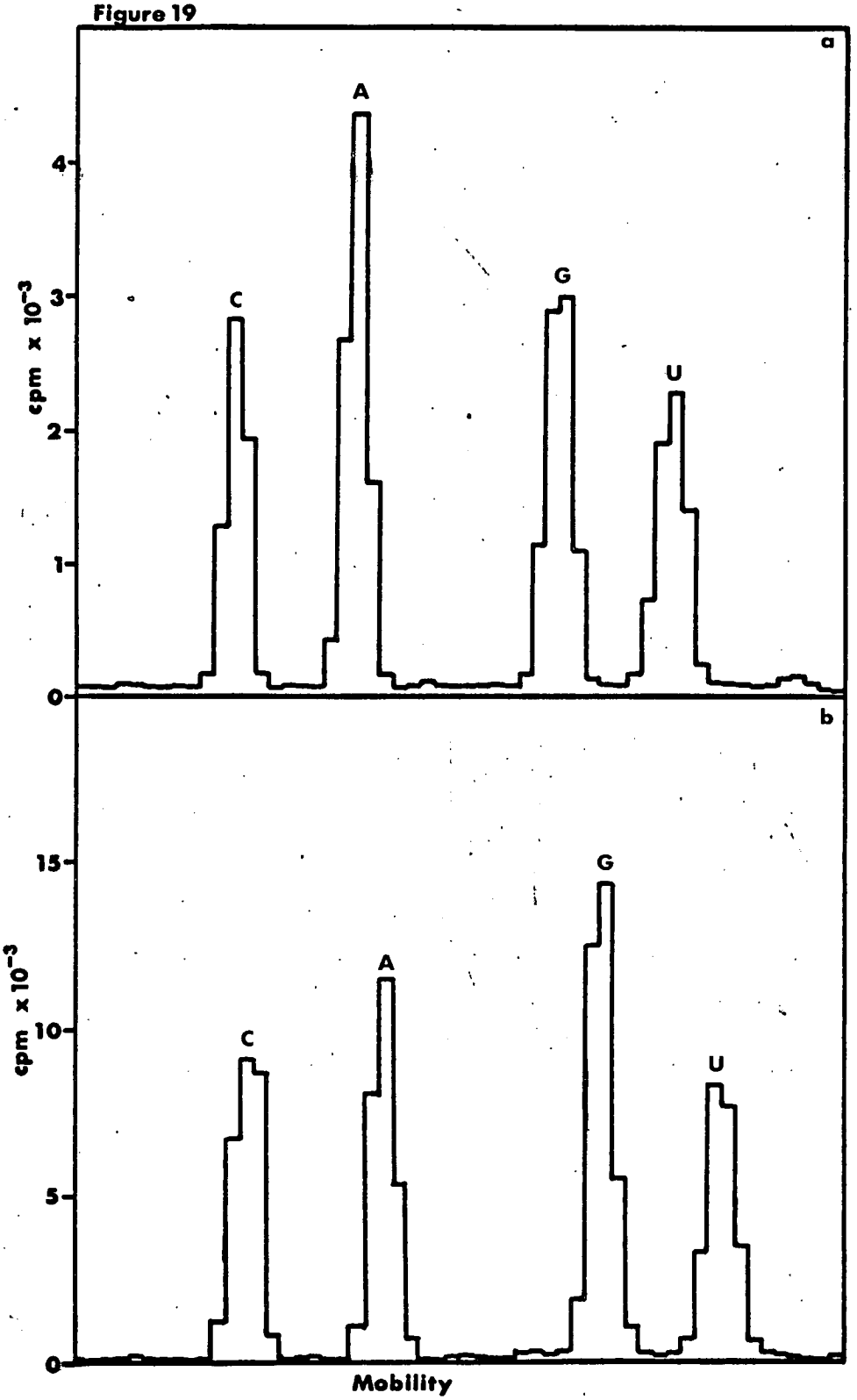
C: cytidylic acid

A: adenylic acid

G: guanylic acid

U: uridylic acid

Figure 19



profiles of piperidine - EDTA hydrolysates of a total nucleic acid sample (Figure 19 a) and of purified 1.33 x 10^6 dalton rRNA (Figure 19 b) after 2 hr high voltage electrophoresis. The base composition of the RNA was determined by summing the total counts in each nucleotide peak, subtracting the background and expressing each as a percentage of the total radioactivity (Table 8). Neither the pH nor the adjustment

Table 8.

Label duration hr	Extraction conditions	Percentage base composition			
		CMP	AMP	GMP	UMP
0.5	pH 9.0	20.7	31.1	23.7	24.5
1.0		19.9	31.8	23.7	24.6
1.5		20.7	31.2	24.3	23.8
0.5	pH 7.4	20.4	32.1	22.9	24.8
1.0		20.7	30.7	24.0	25.6
1.5		21.6	31.5	23.1	23.9
2.0		20.2	30.8	24.4	24.6
0.5	pH 7.4 with 0.5 Na Cl	20.8	32.1	22.8	24.3
1.0		19.5	30.0	25.3	25.3
1.5		20.5	30.7	24.1	24.7
2.0	No detergent	22.0	23.5	27.2	27.2
2 day total RNA	pH 7.4 with 0.5M Na Cl	24.0	25.3	27.4	23.3
2 day 1.3 x 10^6 dalton RNA	Eluted from gel	23.5	23.7	31.6	21.1

Base composition analysis of ^{32}P orthophosphate labelled RNA prepared by a range of extraction conditions.

The percentage base composition of the ^{32}P orthophosphate labelled RNA samples was determined from electrophoretograms similar to those shown in Figure 19, by expressing the counts in each nucleotide peak as a percentage of the total nucleotide counts after background subtraction. The extraction conditions indicate variations from the Kirby technique as described in the text.

of the first aqueous phase to 0.5 M Na Cl made any marked difference to the base composition. Omission of the detergents from the homogenisation, or labelling for two days resulted in the purified RNA being predominately ribosomal, with the adenylic acid content reduced from approximately 31% to 24% and the guanylic acid content increased from approximately 24% to 27%, the latter tending towards the value for 1.33×10^6 dalton rRNA of 32%. The results indicate that if detergents are omitted from the initial homogenisation, there is considerable loss of adenylic acid rich RNA, the non-ribosomal polydisperse RNA which accounts for more than 50% of newly synthesised RNA (Ingle, Key and Holm, 1965). When, however, detergents are present during homogenisation, the ionic strength dependent partition of RNA observed by Brawerman et al (1972) does not appear to occur. Since, however, ribonuclease activity is reduced at higher pHs, (maximum activities are usually in acid or neutral pHs) it was decided to use pH 9.0 Tris-HCl without the addition of any Na Cl to the first aqueous phase for the extraction of all the artichoke nucleic acid samples used in this section (hereafter referred to as the modified Kirby method).

Growth and labelling of artichoke explants.

Artichoke explants were labelled for 2 hr periods with ^{32}P orthophosphate (33 μCi ml^{-1}, 20 Ci mg^{-1} P, Amersham) during the first 85 hr of growth. The growth of the explants was monitored by following the change in cell number (Figure 20) and shows an exponential increase over the duration of the experiment. The uptake of ^{32}P orthophosphate by the explants was estimated by counting duplicate 10 μl samples from the final aqueous phases of phenol extracts, dried onto 21 mm GF/C filters (Figure 21) and shows that although the cell number is increasing

Figure 20.

Cell number increase in artichoke explants during growth.

Five explants cultured for varying times were digested in 3 ml of 5% chromium trioxide for one to three days at 0°C and the cell number in the suspension determined using a Fuchs Rosenthall haemocytometer slide. 6 grids were counted to give statistical accuracy and the cell number corrected to that for one explant.

Figure 21.

Uptake of ^{32}P orthophosphate by artichoke explans during growth.

Artichoke explants were labelled for 2.0 hr periods with ^{32}P orthophosphate (33 μCi ml^{-1}, 20 Ci mg^{-1} P, Amersham) during growth. The uptake of ^{32}P orthophosphate was determined by counting duplicate 10 μl samples from the final aqueous phase of a modified Kirby extraction dried onto Whatman GF/C filters. The values obtained were corrected to the uptake per explant.

Figure 22.

Incorporation of ^{32}P orthophosphate into RNA by artichoke explants during growth.

Explants were cultured and labelled as for Figure 21. The counts incorporated were determined from TCA prcipitates of purified nucleic acid preparations collected by suction filtration onto GF/C filters and corrected to the incorporation by one explant. The counts incorporated into DNA which are included in this value were 5 to 7% of the total counts as determined from gel fractionation profiles.

N.B. The bars on the time axis represent days.

Figure 20

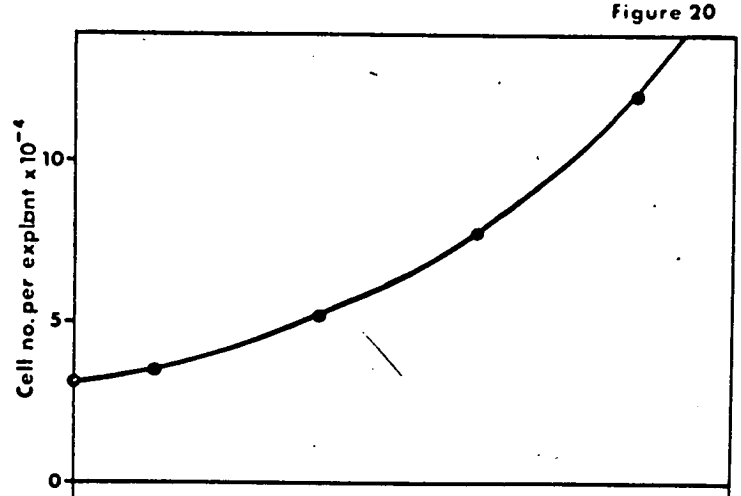


Figure 21

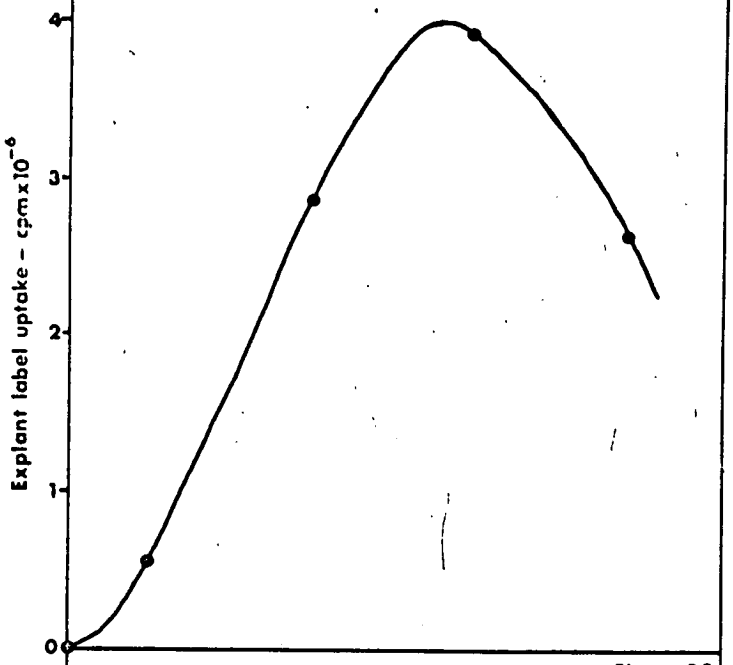
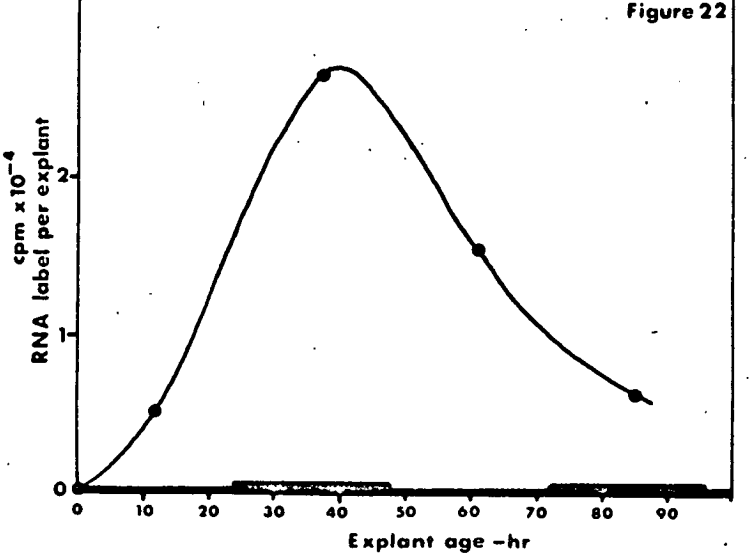


Figure 22



over the 85 hr period, the uptake of label reaches a maximum during the third day and thereafter decreases. This is reflected in the incorporation of ^{32}P orthophosphate into nucleic acid as determined by TCA precipitation of purified nucleic acid preparations (Figure 22) and the specific activity of the RNA (Figure 23) although both these parameters peak earlier (35-40 hr) than does the uptake (50-60 hr). The reason for the presence of this maximum of uptake and incorporation is shown in Figure 24. Initially there is a rapid increase in RNA which on an explant basis levels off after approximately 45 hr and remains more or less constant up to 85 hr. When expressed on a cell basis, the RNA peaks after approximately 40 hr. Then as the cells continue to divide, the RNA per cell decreases towards the zero time value. When the newly synthesised RNA is fractionated into polydisperse RNA and rRNA (mature and precursor molecules) (Figure 25), it can be seen that the percentage contribution of the polydisperse fraction increases considerably during the growth period. Both fractions show maxima, but they are separated by approximately 15 hr (Figure 26).

Cellular fractionation of cultured artichoke explants.

Explants were cultured for 69 hr and labelled for 1 hr with ^{32}P orthophosphate (100 μCi ml^{-1}, 10 Ci mg^{-1} P, Amersham). Nuclear and polysome fractions were prepared and the total nucleic acid extracted from each stage of the preparation. Figure 27 shows the E_{265} and radioactivity profiles of these samples fractionated on 2.4% acrylamide E buffer gels. The nucleic acid from the initial homogenisation (Figure 27 a) shows a typical 'total' profile. The E_{265} shows peaks of DNA and the two mature rRNA components at 25S (1.33×10^6 daltons) and 18S (0.68×10^6 daltons). The radioactivity profile from 1 mm slices again shows a peak of DNA and peaks corresponding to the 25S and 18S molecules

Figure 23.

Incorporation of ^{32}P orthophosphate into RNA by artichoke explants during growth expressed as specific activity.

Explants were cultured and labelled as for Figure 21. The incorporation of ^{32}P orthophosphate per unit RNA was determined from 2.4% acrylamide E buffer gel profiles of samples of total nucleic acid from explants of different ages, fractionated for 1 hr 50 min at room temperature.

Figure 24.

Synthesis of rRNA during the growth of artichoke explant.

The amount of rRNA (25S and 18S) in explants of different ages was determined from the E_{265} profiles of the gels used for determining the incorporations quoted in Figure 23 and corrected to amounts of rRNA per explant. Further correction to amount of rRNA per cell was made using the data quoted for the increase in cell number per explant shown in Figure 20.

●——●: rRNA per explant

o----o: rRNA per cell

N.B. The bars on the time axis represent days.

Figure 23

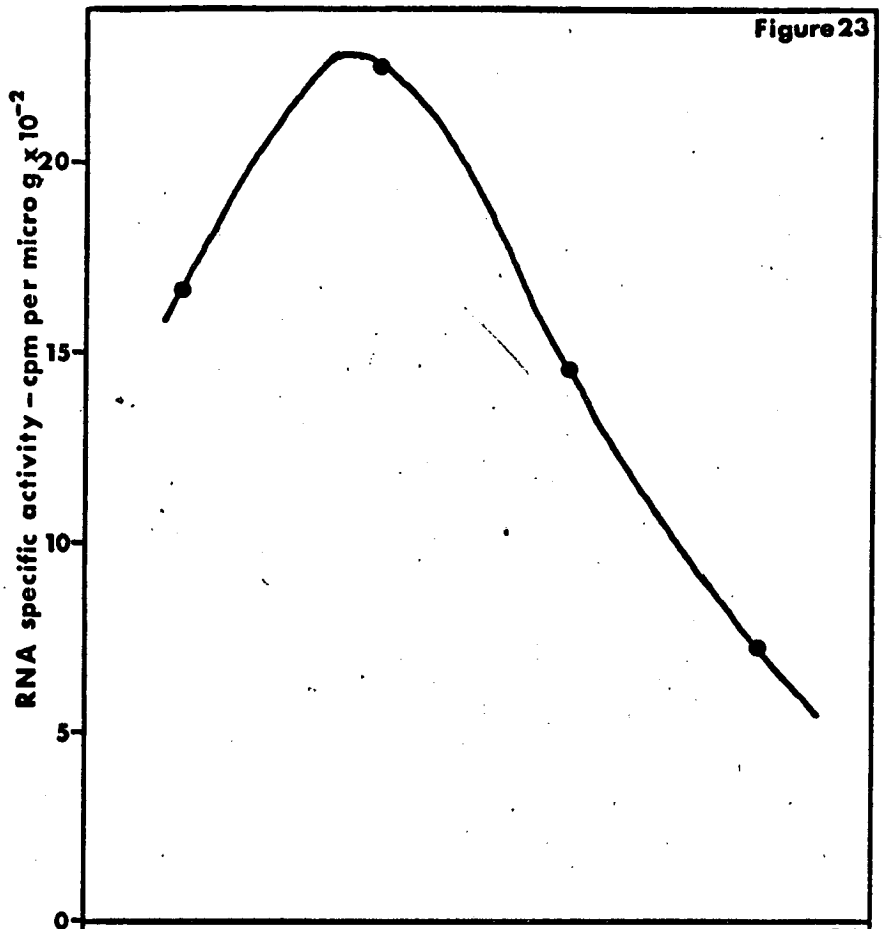


Figure 24

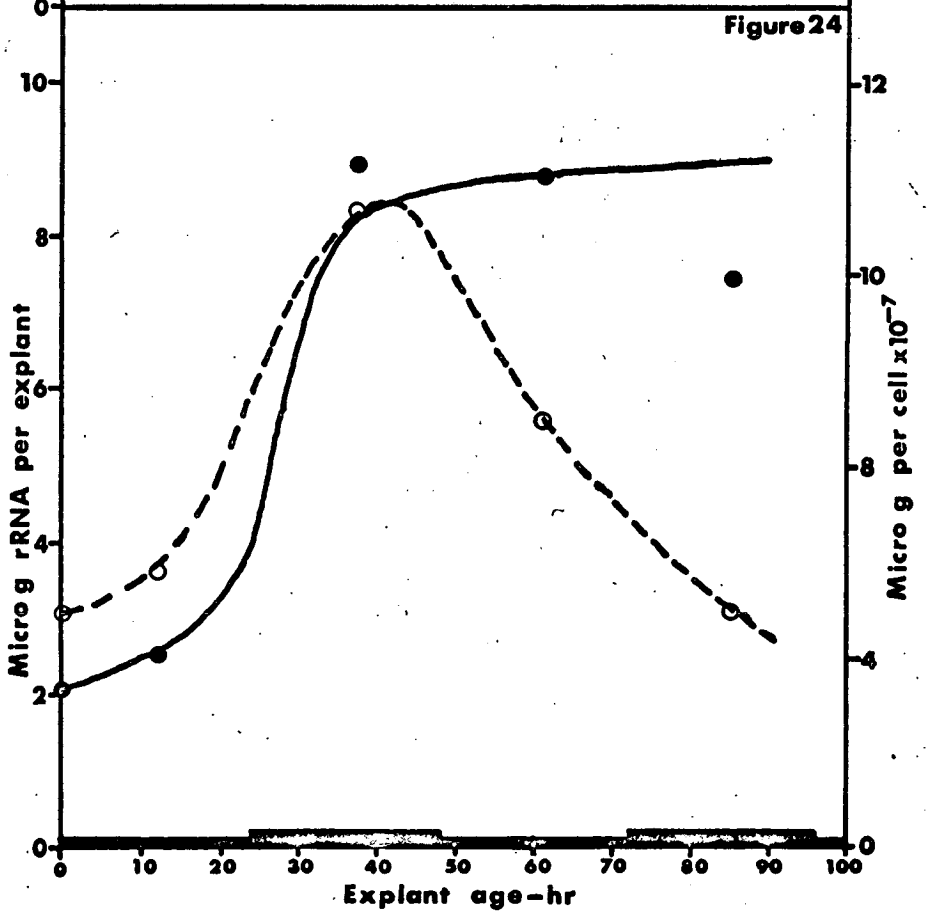


Figure 25.

The percentage incorporation of ^{32}P orthophosphate into various classes of newly synthesised RNA during artichoke explant growth.

The incorporation of ^{32}P orthophosphate into newly synthesised RNA in explants of varying ages was determined from the gel profiles described in Figure 23. This incorporation was split between rRNA including rRNA precursors (the peaks on the profile) and polydisperse RNA (the background on the profile) and expressed as a percentage at each time.

□——■: rRNA incorporation

○——○: polydisperse RNA incorporation

Figure 26.

The incorporation of ^{32}P orthophosphate into various classes of newly synthesised RNA during artichoke explant growth.

The data plotted shows the incorporation in finite terms of ^{32}P orthophosphate into the two classes of RNA during explant growth described in Figure 25.

□——■: rRNA incorporation

○——●: polydisperse RNA incorporation

N.B. The bars on the time axis represent days.

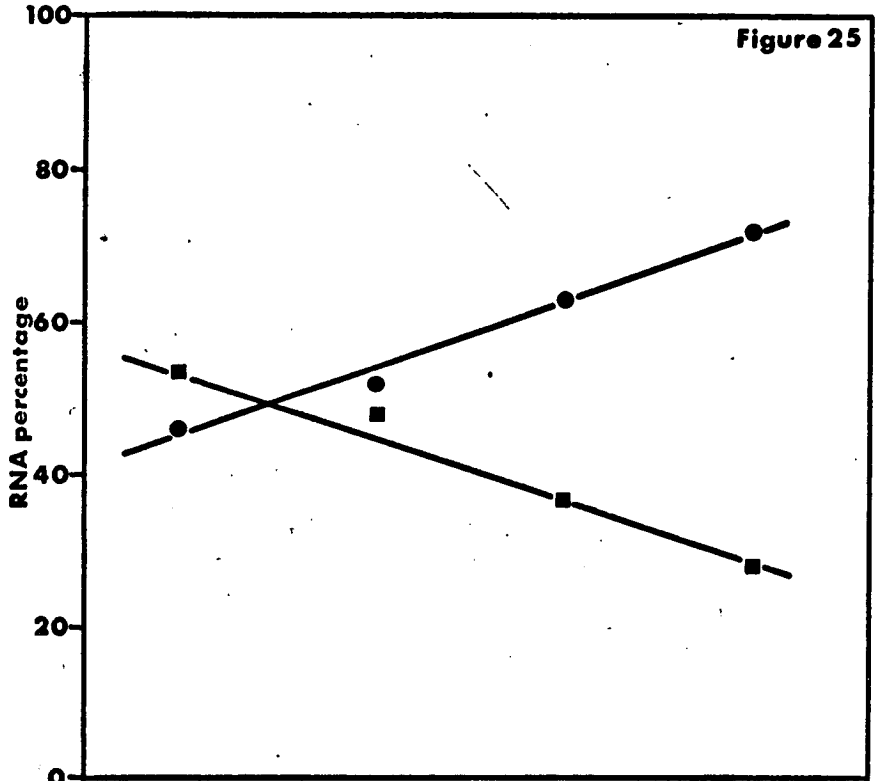


Figure 25
RNA percentage
100
80
60
40
20
0

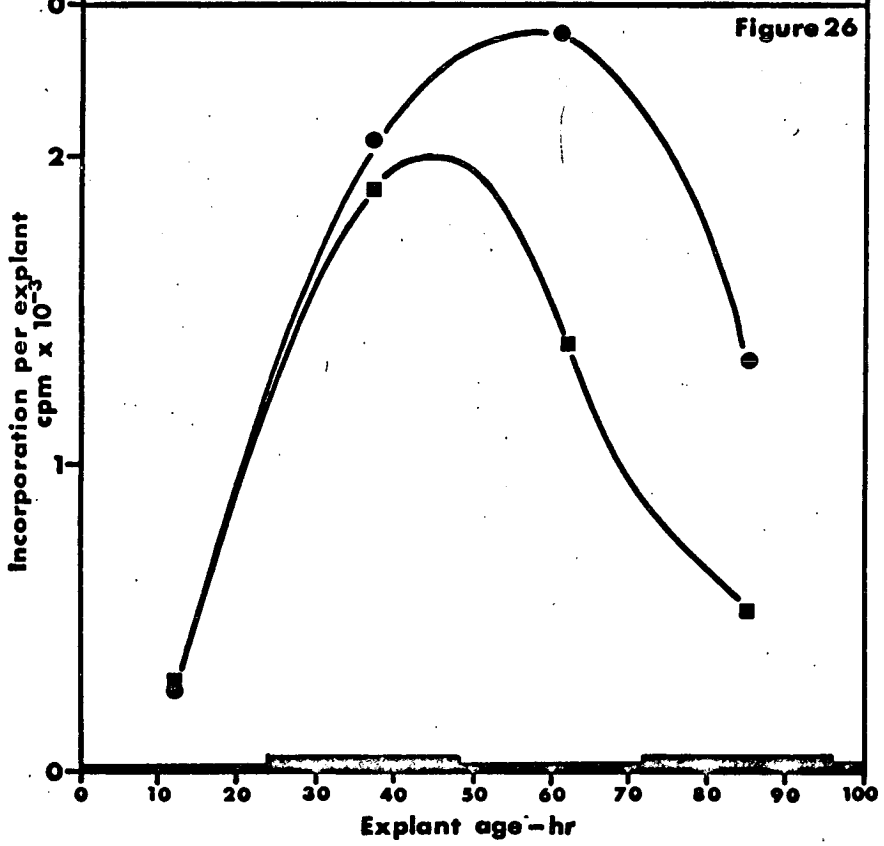


Figure 26
Incorporation per explant
cpm x 10⁻³
2
1
0
0
10
20
30
40
50
60
70
80
90
100
Explant age—hr

together with two new peaks, one at 2.4 x 10^6 daltons which is the primary transcript and one at 0.98 x 10^6 daltons, the precursor to the 18S. By comparison with the mobility of its E_{265} counterpart, the radioactivity peak associated with the 25S can be seen to have a midpoint slightly larger than that of the 25S indicating that it is the double peak of mature 25S and its immediate precursor (1.39 x 10^6 daltons) which are not resolved under the fractionation conditions used here. The radioactivity peaks are superimposed on a polydisperse background containing 53% of the total radioactivity. Routine denaturation as described in Part 1 indicated that none of these components was an artefact of aggregation.

After centrifugation at 10,000 g for 10 min all the DNA had pelleted, as had the ribosomal precursors (Figure 27 c). Once again the radioactivity peak associated with the 25S is rather larger than the E_{265} marker indicating that it contains the 1.39 x 10^6 dalton precursor in large amounts. It is not possible to determine how much of the mature 25S radioactivity has pelleted as denaturation of samples labelled at the high level used here results in too much RNA breakdown for the 1.39 x 10^6 and 1.28 x 10^6 dalton peaks to be as clearly separated as in Figure 16 where a lower concentration of ^{32}P orthophosphate was used to label the tissue. Only 8% of the label in mature 18S rRNA appears in the 10,000 g pellet. The E_{265} profile shows that approximately 5% of the mature 25S and 18S rRNAs pellet at this stage and hence the radioactivity peak associated with the 25S rRNA must contain at least 5% of the 1.33 x 10^6 dalton component. The bulk of the polydisperse RNA (70%) remains in the supernatant after centrifugation at 10,000 g (Figure 27 b) as does 95% of the mature 25S and 18S rRNA. The radioactivity peak associated with the 25S rRNA fractionates exactly with its E_{265}

Figure 27.

Nucleic acid content of stages during cell fractionation of artichoke explants.

69 hr old explants were labelled for 1 hr with ^{32}P orthophosphate (100 μCi ml^{-1}, 10 Ci mg^{-1} P, Amersham) and the tissue used for a polysome extraction. Nucleic acid was prepared from each stage by the modified Kirby method and fractionated on 2.4% acrylamide E buffer gels for 3.5 hr at room temperature. a) initial homogenate, b) 10,000 g supernatant, c) 10,000 g pellet, d) 50,000 g pellet and e) 50,000 g supernatant.

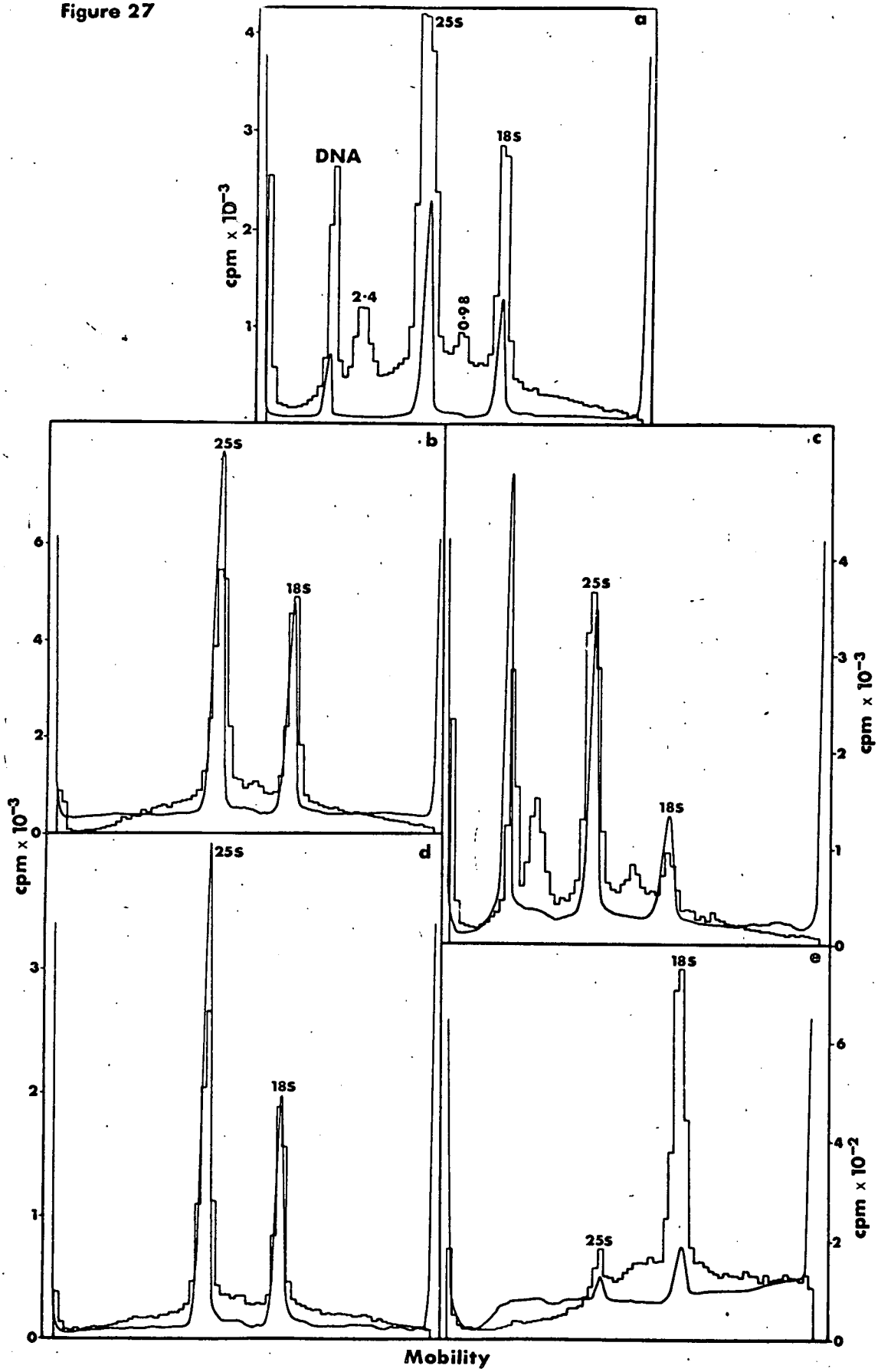

: E_{265}


: ^{32}P orthophosphate determined from 1 mm gel slices.

Molecular weights are in daltons x 10^{-6}.

Loadings
b: 20% of total sample
c: 50% of total sample
d: 17% of total sample
e: 50% of total sample

Figure 27


a
25s
18s
DNA
2·4
0·98
cpm x 10⁻³
b
25s
18s
c
25s
18s
cpm x 10⁻³
d
25s
18s
cpm x 10⁻³
e
18s
25s
cpm x 10⁻²
Mobility

counterpart, indicating that there is little, if any of the precursor present. This primary centrifugation pellets DNA and many RNA molecules associated with the nucleus or nucleolus (rRNA precursors) and only 30% of the polydisperse RNA. The pellet at this stage contains cell wall fragments, cytoplasm, nuclei and nucleoli (Figure 28). It is probable that the 5% of total mature 25S and 18S molecules which pellet represent clumps of cytoplasm. When the 10,000 g supernatant is further centrifuged at 50,000 g over a 1.0 M sucrose cushion, approximately 90% of the labelled RNA sediments (Figure 27 d). The supernatant, including the cushion contains 17% of the polydisperse RNA and 15% of the 18S but only 1% of the 25S RNA label. This centrifugation appears to leave small quantities of the 40S and traces of the 60S ribosomal subunits and possibly informosome like particles containing the polydisperse RNA (Silverstein, 1973) in the supernatant. From the relative amounts of the ribosomal subunits, however, it is clear that virtually all the 80S ribosomes and polysomes have sedimented. Figure 29 summarises the fate of the labelled RNA during the course of cell fractionation. Approximately 60% of the total polydisperse RNA is associated with the polysome fraction while 30% is retained in the nucleus. The remaining 10% of the polydisperse RNA which remains in the supernatant after centrifugation at 50,000 g is of uncertain origin and may be associated with informofers released from danaged nuclei (Samarina, Lukanidin and Georgiev, 1973), true cytoplasmic informosomes or mRNA ribonucleoprotein particles released from polysomes (Silverstein, 1973).

Analysis of poly (A) in total nucleic acid preparations.

Ten percent of the counts in total nucleic acid prepared from tissue labelled for 1 hr with ^{32}P orthophosphate was stable to RNAase A

Figure 28.

Artichoke explant polysome homogenate - 10,000 g pellet.

Portions of the pellet from a 10,000 g centrifugation of the initial homogenate used for a polysome extraction from artichoke explants were mixed on a slide with pyronin-methyl green and photomicrographs taken.

The micrographs show cell wall debris (cw), cytoplasm (cy), nuclei (n) and nucleoli (no). Magnification: x 900.

Figure 28.

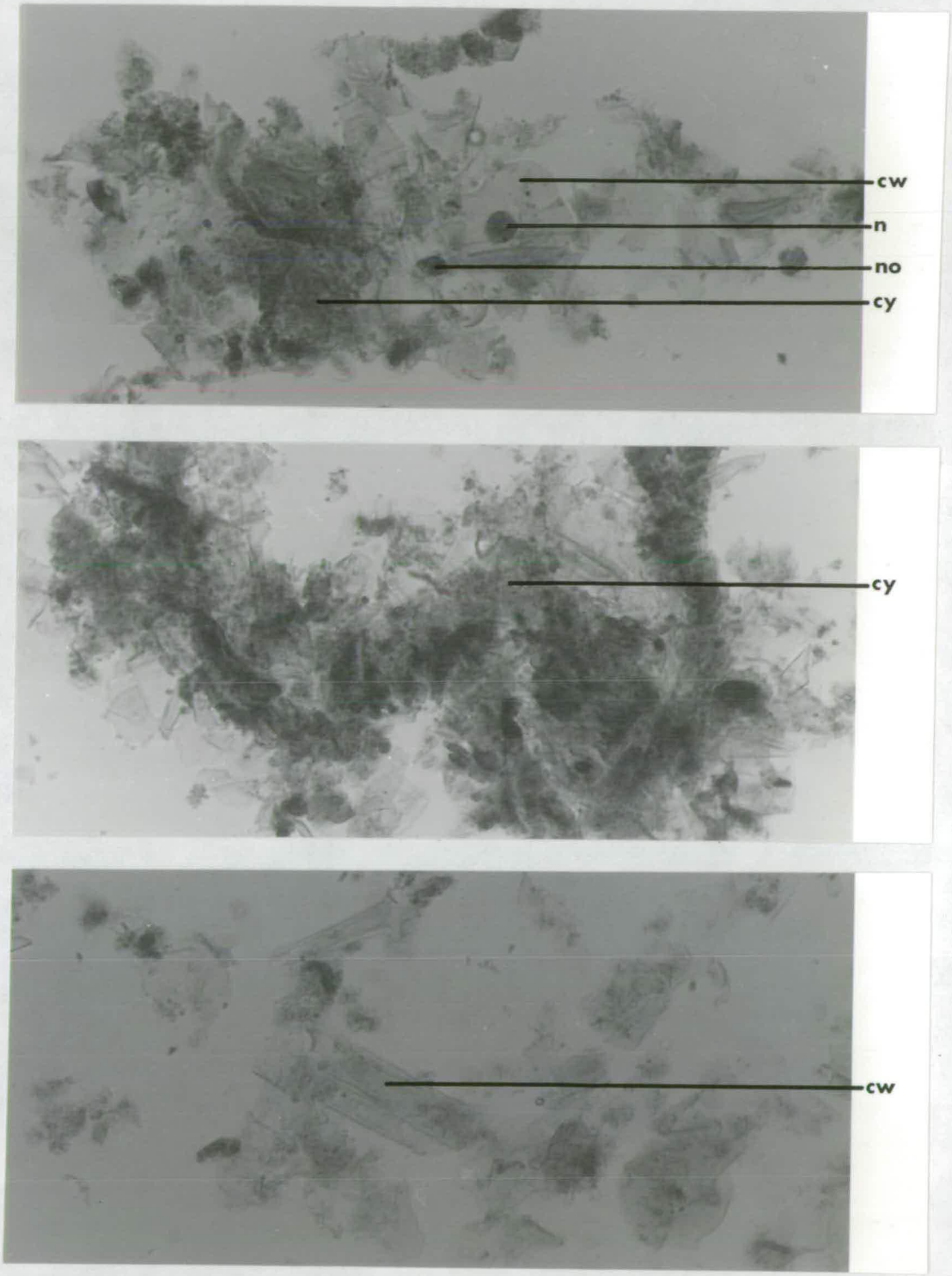


Figure 29.

Homogenisation

10,000 g centrifugation

Supernatant	Pellet
65% 25S and 25S precursor rRNA	100% DNA
92% 18S rRNA	100% 2.4×10^6 dalton rRNA precursor
70% polydisperse RNA	100% 0.98×10^6 dalton rRNA precursor
	35% 25S and 25S precursor rRNA
	8% 18S rRNA
	30% polydisperse RNA

10,000 g

Supernatant

50,000 g centrifugation

Supernatant	Pellet
1% 25S rRNA	99% 25S rRNA
15% 18S rRNA	85% 18S rRNA
17% polydisperse RNA	83% polydisperse RNA

Distribution of newly synthesised RNA during cell fractionation
The component percentages are derived from gel profiles similar to those shown in Figure 27. Each value is the mean of replicate extractions. The percentages in the supernatant and pellet are expressed as percentages of the sample prior to centrifugation.

and RNAase T1. Routinely half of these counts were also stable to RNAase T2, but base composition analysis of this radioactivity showed that the residual label was not in ribonucleotides. Part of it could be attributed to either incompletely digested or totally undigested DNA. Digestion of labelled artichoke rRNA purified by electrophoresis showed that only 0.5% of the total counts precipitated as poly (A) in the assay used. This figure remained constant with a 10-fold difference in rRNA input. The poly (A) content of total RNA labelled for 1 hr with ^{32}P orthophosphate was therefore 5%. Base composition analysis of a total nucleic acid sample digested for 0.5 hr with RNAase A and RNAase T1 showed that the residual poly (A) was only 64% adenylic acid. Over the course of a 2.0 hr nuclease digestion, the percentage of residual poly (A) decreased from 5.6% to 5.0%, a result which is only barely significant (routinely, percentage stabilities to ribonuclease were found to be $\pm$ 0.5%). Figure 30 a shows the base composition of residual poly (A) at 0.5 hr intervals during the 2.0 hr nuclease digestion. Guanylic acid is the largest contaminant, 18.6% after 0.5 hr digestion, but is reduced to 8.9% after 2.0 hr. The total counts used in each base composition analysis were of the order of 850 cpm and hence, with a machine background of 45 cpm, the values for the cytidylic acid and uridylic acid contamination after 1.5 hr digestion are approaching the practical limit of the technique, since 2.0% of the total counts as one nucleotide is equivalent to a total of 43 cpm which, spread over 3 or 4 fractions, is only 10-12 cpm above background. Thus, after 0.5 hr ribonuclease digestion, 64% of the residual counts (approximately 5% of the total) are in adenylic acid. Increasing the digestion to 2.0 hr increases the purity of the poly (A) to 83% (Figure 30 b).

Figure 30.

Base composition of artichoke poly (A)

Artichoke nucleic acid prepared from explants which had been labelled for 1 hr with ^{32}P orthophosphate (100 μCi ml^{-1}, 10 Ci mg^{-1} P, Amersham) was digested with RNAase A and RNAase T1 for increasing times. The base composition of the residual counts was determined by high voltage electrophoresis of the piperidine - EDTA hydrolysates. a) shows the time course of the residual poly (A) with increasing time of nuclease digestion. The zero time value is that of a total sample. The 0.5 hr and subsequent values are for residual poly (A) which comprised approximately 5% of the total counts at time zero.

: adenylic acid, A

: guanylic acid, G

: cytidylic acid, C

: uridylic acid, U

b) shows the electrophoretogram used to obtain the 2.0 hr percentages. The machine background is 45 cpm. The bars on the base line indicate the fractions used in determining the counts in each nucleotide.

Figure 30a

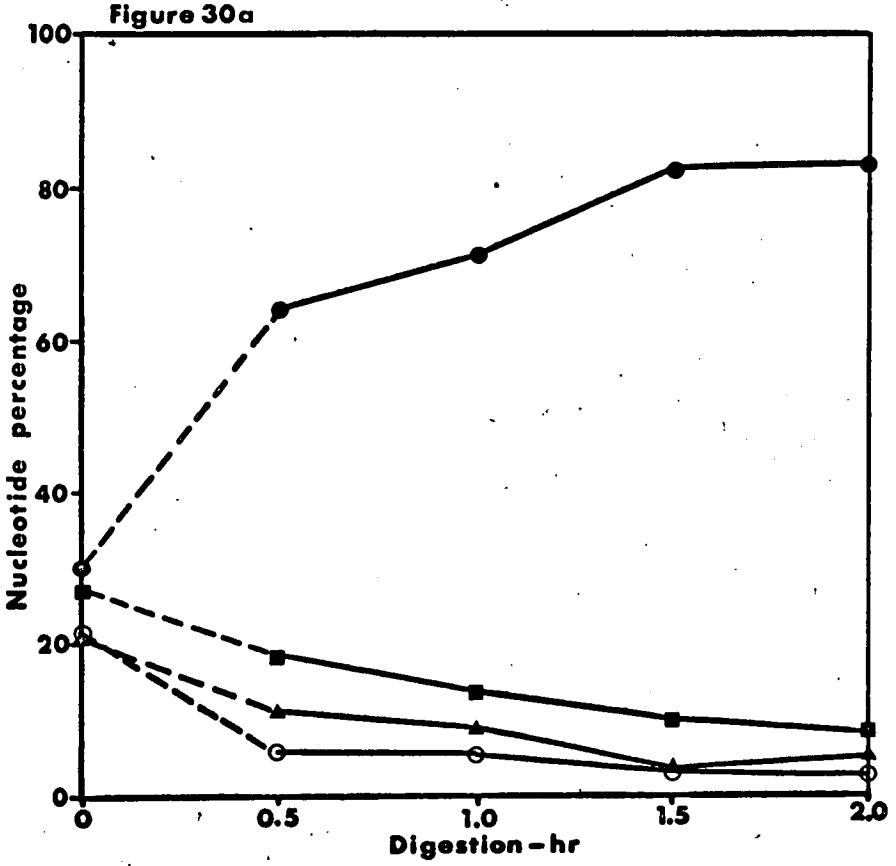

100
80
60
40
20
0
Nucleotide percentage
0
0.5
1.0
1.5
2.0
Digestion – hr

Figure 30b

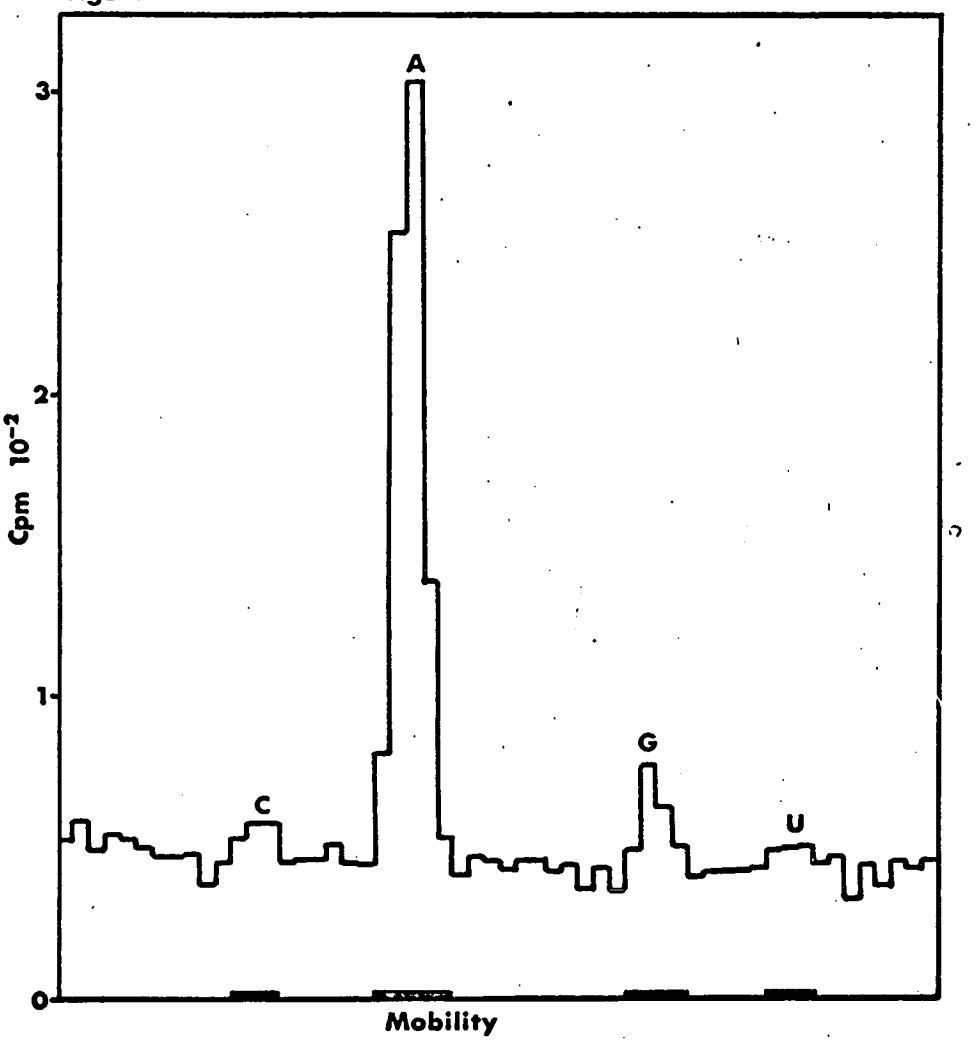


Figure 31 shows the radioactivity profile from a 7.5% acrylamide E buffer gel of an RNAase A and RNAase T1 digest of a total nucleic acid preparation from 67 hr explants labelled for 1 hr with ^{32}P orthophosphate. Assuming a comparable mobility of poly (A) with 4S and 5S RNA (see Discussion), the $\log_{10}$ linear relationship between molecular weight and mobility indicates that the broad range of poly (A) size has a maximum of 250 nucleotides with a major peak at 160 nucleotides and minor peaks at 70 and 40 nucleotides respectively. To prepare this sample for electrophoresis approximately 400 μg total nucleic acid was digested with 67 $\mu g\ ml^{-1}$ RNAase A and 333 units ml^{-1} RNAase T1 in a total volume of 3 ml for 0.25 or 1.25 hr. The two digestions showed identical gel profiles whether fractionated with or without unlabelled carrier RNA. Both samples after base composition analysis were approximately 80% pure adenylic acid.

The poly (A) content was determined for nucleic acid fractions eluted from a 2.4% polyacrylamide E buffer gel of total nucleic acid extracted from 72 hr explants labelled for 1 hr with ^{32}P orthophosphate (Figure 32). The radioactivity profile was determined from the Cerenkov radiation of wet gel slices prior to elution. Table 9 shows the percentage of poly (A) from the various fractions and the poly (A) content corrected for the percentage of non-polydisperse RNA in each fraction. The corrected poly (A) content decreases as the size of the polydisperse RNA increases as would be expected if the size of the poly (A) was within a limited range as has been already shown. If it is assumed that all polydisperse RNA molecules contain poly (A) then, from the corrected percentage poly (A) and the mean molecular weight, the average size of the poly (A) sequence from each fraction can be calculated (Table 9). The size range of poly (A) approximately falls into that determined

Figure 31.

The size of artichoke poly (A)

Explants were labelled with 1 hr ^{32}P orthophosphate (100 $\mu Ci\ ml^{-1}$, 10 Ci mg^{-1} P, Amersham) and the nucleic acid prepared by the modified Kirby method. The poly (A) was prepared from total nucleic acid by ribonuclease digestion and collected by ethanol precipitation in the presence of unlabelled poly (A) (Boehringer mannheim). The purified poly (A) obtained by 0.25 hr digestion was fractionated on a 7.5% acrylamide E buffer gel for 3.0 hr at room temperature and the profile determined by counting 1.0 mm slices. The arrows indicate the distances migrated by 4S and 5S RNA on a comparable gel.

Figure 31

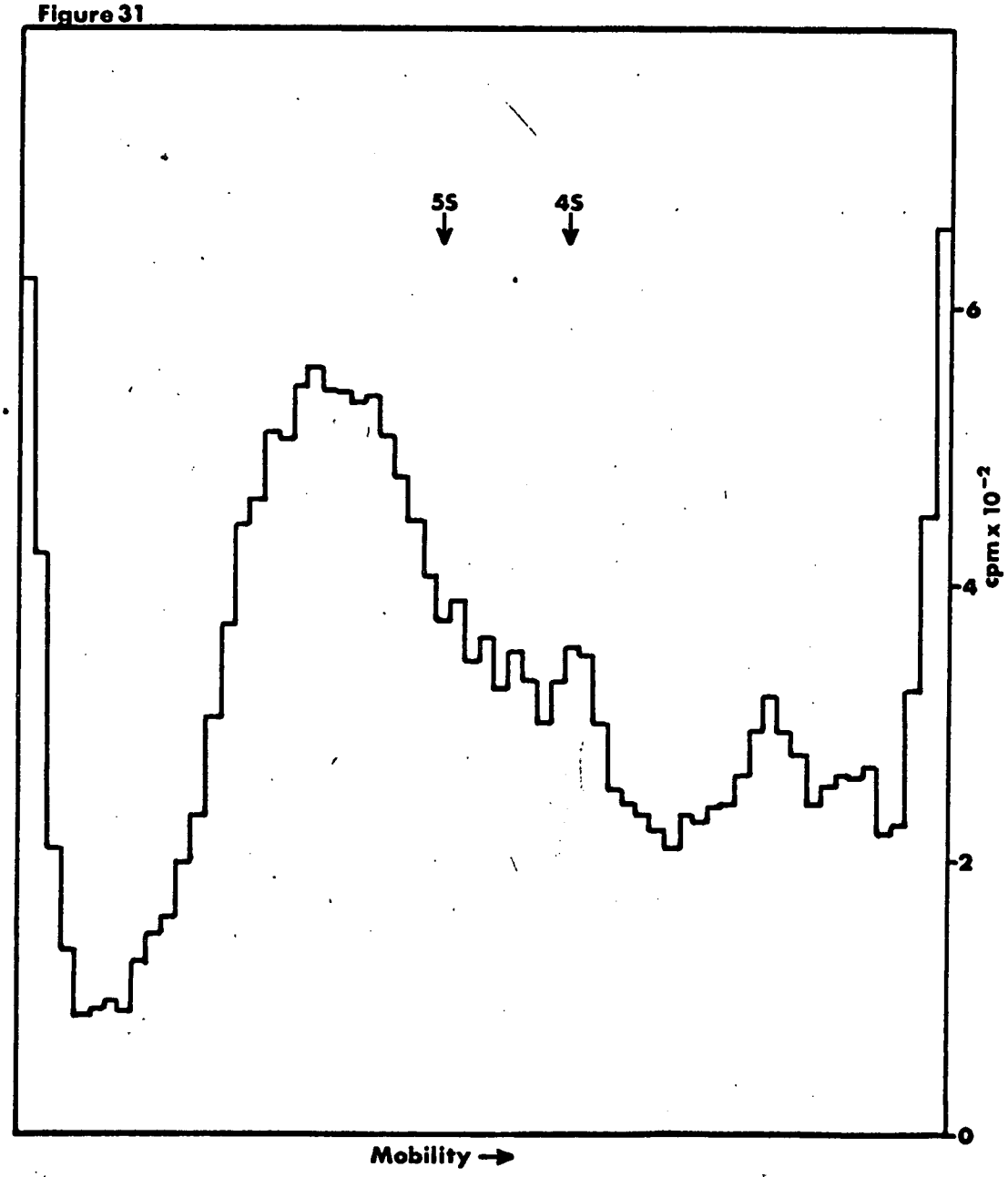


Figure 32.

Polyacrylamide gel fractionation of rapidly labelled artichoke nucleic acid for recovery of purified fractions.

Explants were labelled for 1 hr with ^{32}P orthophosphate (100 μCi ml^{-1}, 10 Ci mg^{-1} P, Amersham) and nucleic acid prepared from them by the modified Kirby method. 100 μg was fractionated on a 2.4% polyacrylamide E buffer gel for 2.5 hr at room temperature and the radioactivity profile determined by counting the Cerenkov radiation from paired wet 0.5 mm gel slices. The nucleic acid was eluted from the gel slices comprising the fractions marked on the figure and the poly (A) content of these determined (Table 9).

The DNA peak contains the 2.4×10^6 dalton rRNA precursor peak which is not resolved under these fractionation conditions.

Figure 32

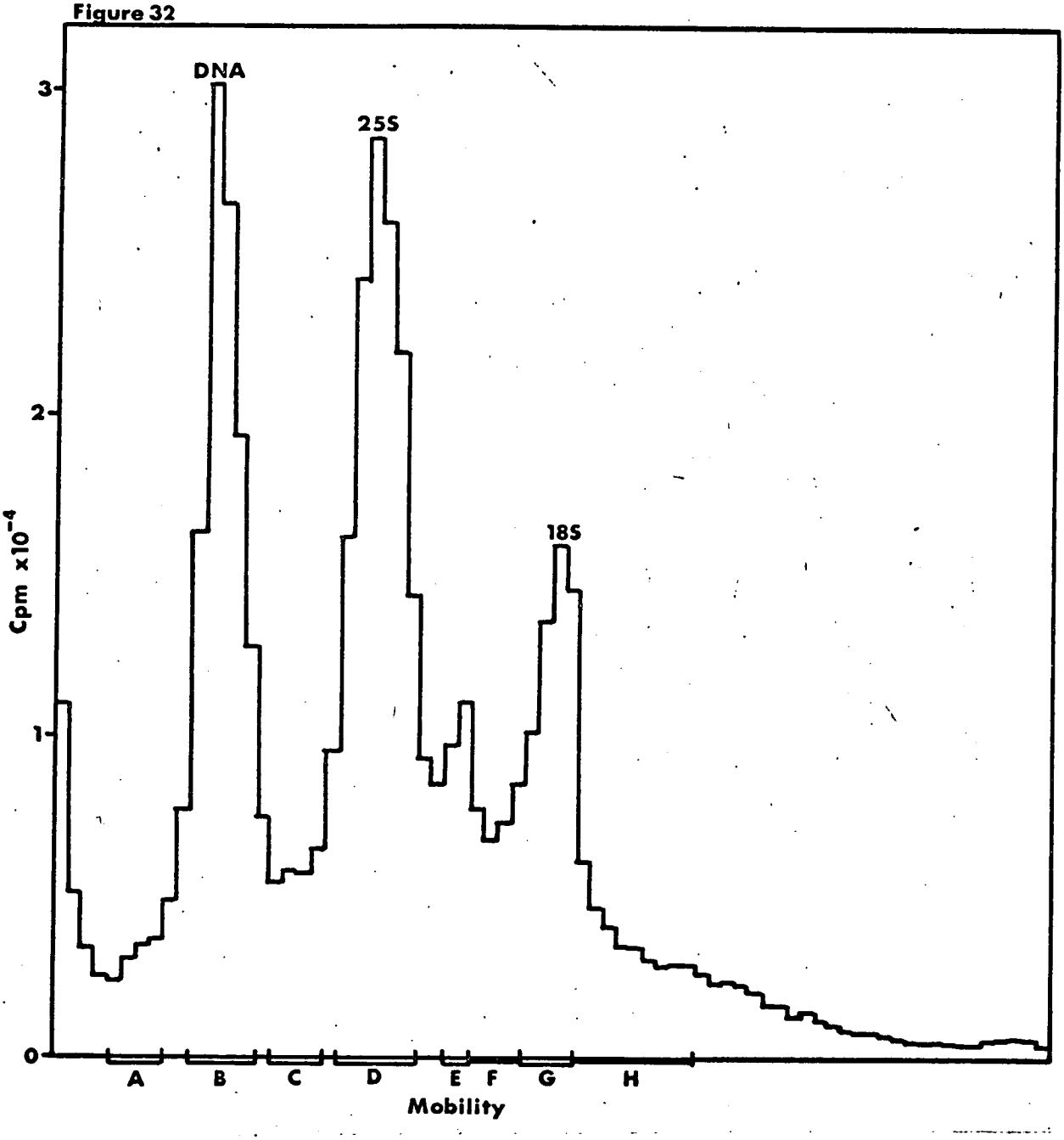


Table 9.

Fraction	% poly (A)	% polydisperse	corrected % poly (A)	mean polydisperse molecular weight (x 10^{-6}) daltons	mean size of poly (A) (nucleotides)
A	0.7	100	0.7	3.2	65
B	1.1	23	4.7	2.3	310
C	4.0	100	4.0	1.9	230
D	2.1	37	5.7	1.4	235
E	5.1	77	6.6	0.98	190
F	7.7	100	7.7	0.85	192
G	3.4	45	7.6	0.70	155
H	8.0	100	8.0	0.52	122

Poly (A) content and size in RNA of fractions eluted from a polyacrylamide gel of ^{32}P orthophosphate labelled total nucleic acid.
The letters refer to fractions eluted from the polyacrylamide gel shown in Figure 32. The poly (A) content is determined from the percentage of total counts in each fraction which are stable to ribonuclease, and are corrected to the percentage of poly (A) in polydisperse RNA by dividing by the percentage of polydisperse RNA in each fraction determined from the gel profile. The mean size of the poly (A) in nucleotides is determined by dividing the average molecular weight of each fraction, as determined from the gel profile, by the percentage poly (A) in polydisperse RNA, and again by the average molecular weight of a nucleotide.

by gel fractionation of poly (A). This suggests that the bulk of polydisperse RNA molecules contain poly (A) sequences.

To examine this further nucleic acid samples extracted from 72 hr explants labelled for 1 hr with ^{32}P orthophosphate were loaded in 1 ml 0.5 M Na Cl, 10 mM Tris-HCl, pH 7.4 (loading buffer) onto 100 mg oligo (dT) cellulose (type T-2, Collaborative Research, Inc.) in a jacketed column at 25°C. The oligo (dT) cellulose had previously been washed with 50 ml loading buffer at 25°C and 50 to 100 ml 10 mM EDTA-sodium salt, 10 mM Tris-HCl, pH 7.4 (elution buffer) at 40°C. The sample (approximately 100 µg) was passed through the column at 46 ml hr^{-1} and the column was then washed with 4 ml loading buffer at 25°C. The temperature was increased to 40°C and 5 ml elution buffer was pumped through. The loading plus washing volume (5 ml) and the total elution volume (5 ml) were collected, made up to 50 µg nucleic acid ml^{-1} by the addition of purified mouse rRNA and the nucleic acid recovered by overnight precipitation with 2 volumes of ethanol at 0 to 4°C.

Using these loading conditions, the 100 mg column had a capacity of approximately 100 µg poly (A) (Boehringer, Batch 7213205). In a normal sample there will be less than 10 µg poly (A). Under these conditions, the column retains in excess of 90% of the poly (A) loaded.

Figure 33a shows the electrophoretic profile of the total nucleic acid sample which contained 6.1% poly (A) or, when corrected, 9.3% of the polydisperse RNA (65% of total counts). After being passed through the oligo (dT) cellulose column, the polydisperse background is reduced to 54% (Figure 33b) and the poly (A) content reduced to 2% of total RNA label, i.e. 3.7% of polydisperse RNA. The column eluate (Figure 33c) shows only polydisperse RNA with a mean molecular weight of 0.9×10^6 daltons containing 11% poly A which would indicate an average poly (A)

Figure 33.

Oligo (dT) cellulose affinity chromatography of total artichoke rapidly labelled nucleic acid.

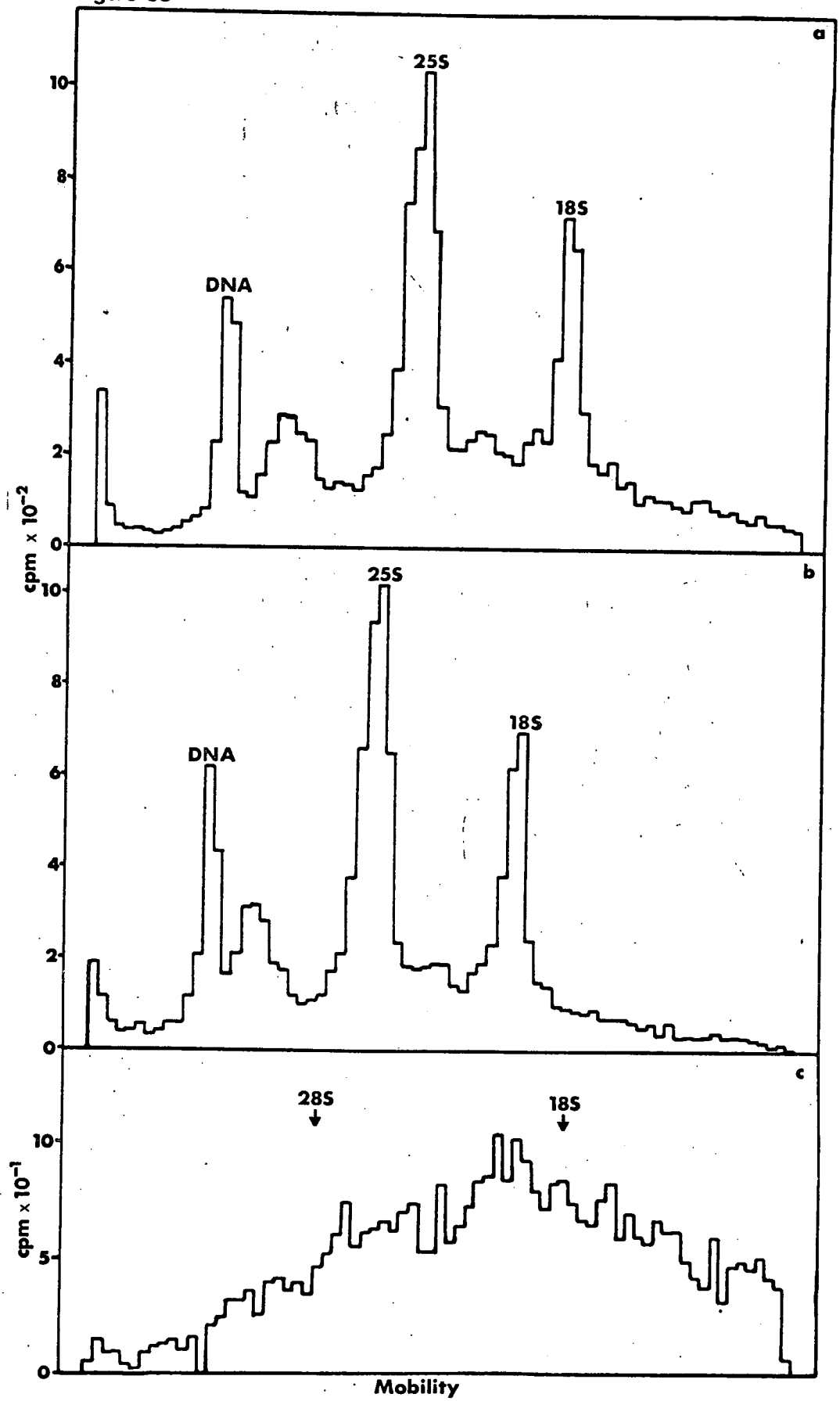
Total nucleic acid was prepared from explants labelled for 1 hr with ^{32}P orthophosphate (100 μCi ml^{-1}, 10 Ci mg^{-1} P, Amersham) dissolved in 0.5M NaCl, 10 mM Tris-HCl, pH 7.4 (loading buffer) and loaded in 1 ml onto 100 mg oligo (dT) cellulose at 25°C. The column was washed with 4 ml loading buffer and, after increasing the temperature to 40°C, eluted with 5 ml 10 mM EDTA-sodium salt, 1- mM Tris-HCl, pH 7.4. The washing and elution fractions were made up to 50 μg ml^{-1} with purified mouse rRNA and the nucleic acid recovered by ethanol precipitation. The samples were fractionated on 2.4% acrylamide gels for 3 hr at room temperature.

a) total nucleic acid

b) column wash

c) column eluate

Figure 33



size of 280 nucleotides. The column retained only approximately 15% of the total label, equivalent to 23% of the total polydisperse RNA. It was not possible to obtain complete binding of poly (A) containing RNA with this preparation, possibly because of residual non-protein contamination interfering with the binding.

Poly (A) content of cell fractions.

Figure 34 shows the E_{254} profile of polysomes extracted from 74 hr explants labelled for 1 hr with ^{32}P orthophosphate and fractionated on a 10-60% linear sucrose gradient. The low percentage of polysomes is typical of explants of this age. The radioactive profile (from TCA precipitates of 0.5 ml fractions) indicates that there has been considerable incorporation into the polysome region of the gradient. The major fractions marked were collected from an identical gradient and the polysomes precipitated. Nucleic acid was prepared from these by the modified Kirby method described earlier and the poly (A) content of the fractions determined. In addition, all the fractions were analysed on 2.4% acrylamide E buffer gels (Figure 35). Fraction 1 comprising ribosomal subunits and ribosomes has only 0.6% poly (A), a value comparable with that of pure rRNA. Figure 35a shows that this fraction contains 31% polydisperse RNA. This represents 39% of the total polydisperse RNA loaded onto the gradient, indicating that at least 39% of the polydisperse RNA which sediments with the polysomes does not contain poly (A). Fraction 2, the main part of the polysome region, is approximately 60% polydisperse RNA (Figure 35b) accounting for 43% of the total polydisperse RNA loaded onto the gradient. The poly (A) content of Fraction 2 was 4.9% which, after correction, indicates that the polydisperse RNA is 8.0% poly (A). Fraction 3, from the heavy polysome region of the gradient

Figure 34.

Sucrose density gradient fractionation of cultured artichoke explant polysomes.

Explants were labelled for 1 hr with ^{32}P orthophosphate (100 μCi ml^{-1}, 10 Ci mg^{-1} P, Amersham) and the polysomes extracted and fractionated on 20 ml 10 to 60% linear sucrose density gradients for 3 hr at 70,000 g (MSE 50, 3 x 20 swing-out rotor). The gradients were pumped out from the bottom at 46 ml hr^{-1}, the E_{254} profile recorded (continuous line) and 0.5 ml fractions collected. The RNA was collected from these fractions by cold 5% TCA precipitation followed by suction filtration and the ^{32}P in each fraction determined (broken line).

Figure 34

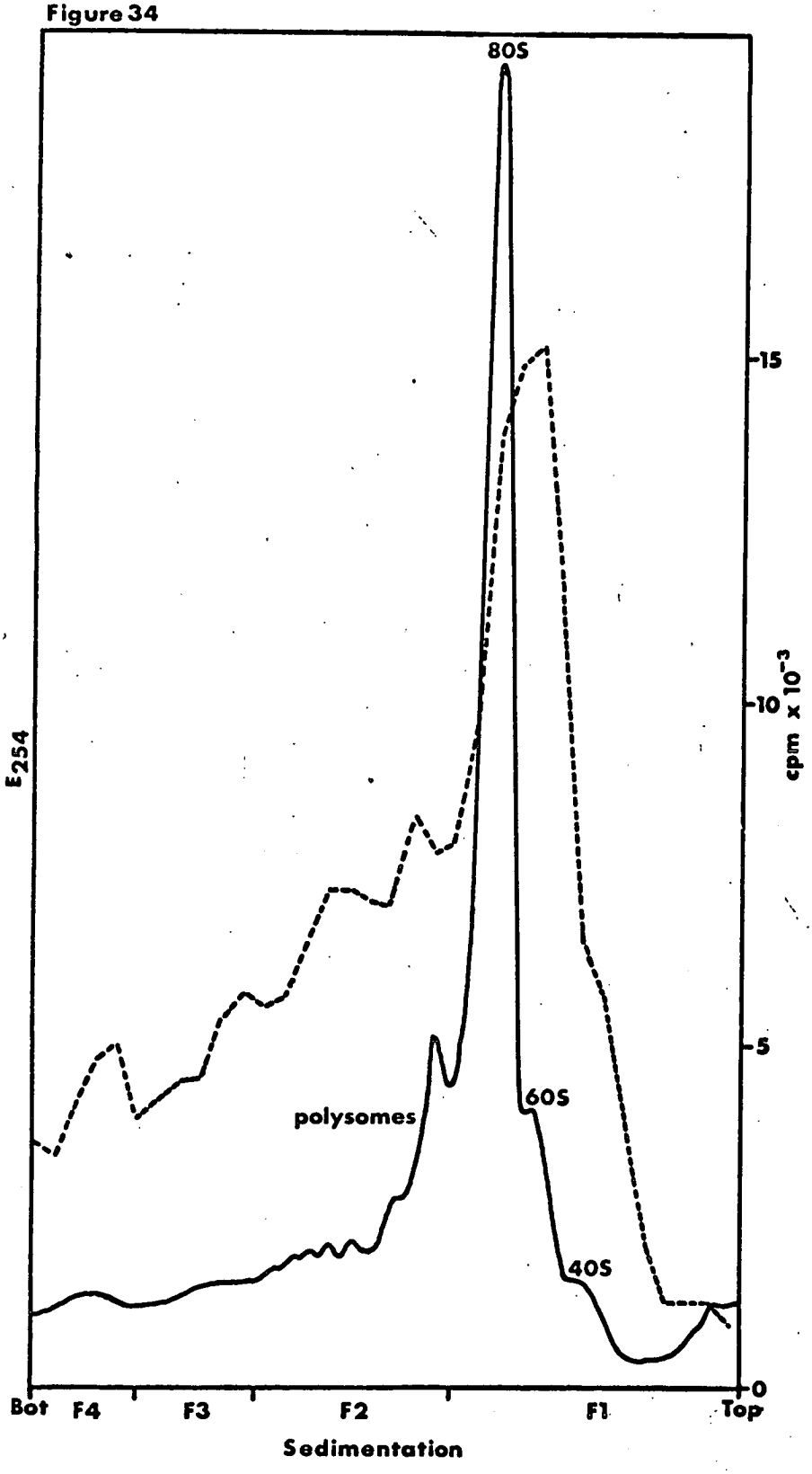
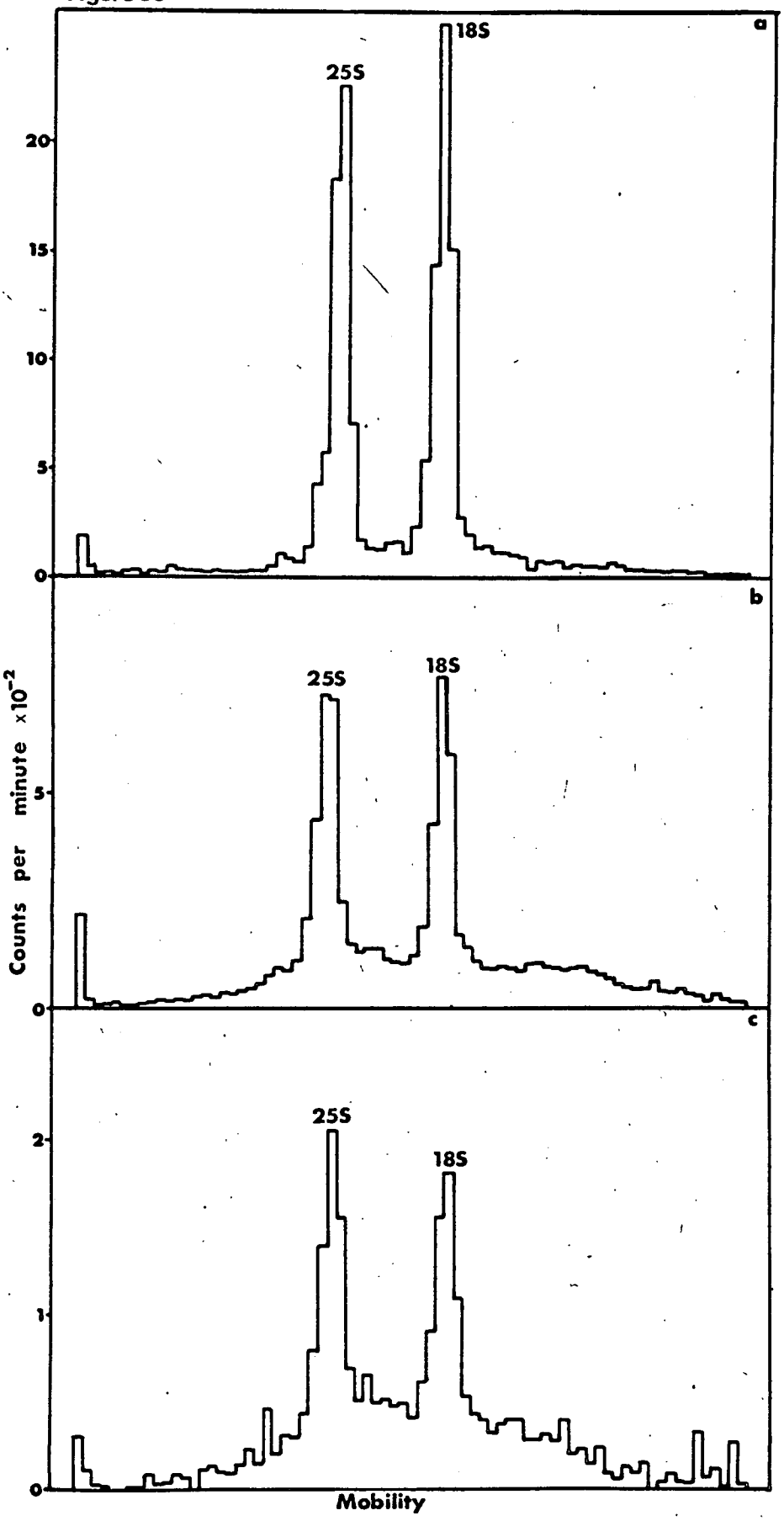

80S
15
10
5
0
E_{254}
cpm x 10^{-3}
polysomes
60S
40S
Bot
F4
F3
F2
F1
Top
Sedimentation

Figure 35.

The rapidly labelled RNA profiles of RNA from ^{32}P orthophosphate labelled polysome fractions.

The ribosomes and polysomes were collected from fractions of a sucrose density gradient as indicated in Figure 34 (F1 to 4) by ethanol-Mg Cl_2 precipitation and nucleic acid prepared from these by the modified Kirby method. The nucleic acid was fractionated on 2.4% acrylamide E buffer gels for 3.5 hr at room temperature. Fraction 4 (Figure 34) did not yield enough radioactive RNA to give significant results.

Figure 35


a
18S
25S
20
15
10
5
0
b
25S
18S
Counts per minute x10⁻²
5
0
c
25S
18S
2
1
0
Mobility

comprises 70% polydisperse RNA and has a total poly (A) content of 6.5% (Figure 35c). This, after correction, indicates that the poly (A) content of the polydisperse RNA from Fraction 3 is 9.3%. Fraction 4 yielded very low levels of label and did not give significant results.

The data from Fraction 1 indicates that at least 39% of the polysomal RNA does not contain any poly (A). This was confirmed by oligo (dT) cellulose affinity chromatography of newly synthesised RNA prepared from a 10,000 g supernatant fraction, the bulk of which has been shown to be polysomal RNA; a mixture of polydisperse RNA and mature rRNA (Figure 27b). The content of poly (A) containing molecules was determined by its binding to an oligo (dT) cellulose column as described earlier for oligo (dT) cellulose affinity chromatography of a total RNA preparation. The preparation had a total poly (A) content of 4.3% which, when corrected for the percentage of polydisperse RNA (70% of total counts) indicated that the polydisperse RNA contained 6.1% poly (A) (Figure 36a). The poly (A) content of the column wash is completely reduced to the background level (0-0.5%) although 33% of the total polydisperse RNA is present. The column binds 47% of the total RNA label, all of which is polydisperse with a mean molecular weight of 0.8×10^6 daltons. The poly (A) content of the bound RNA is 7.5%, which combined with the mean molecular weight of the polydisperse RNA in this fraction, indicates a mean size for the poly (A) of 170 nucleotides. The value for the amount of polydisperse RNA in a 10,000 g supernatant fraction which does not bind to oligo (dT) cellulose and does not contain poly (A) of 33%, compares favourably with the amount of polydisperse RNA from a sucrose gradient which does not contain poly (A) (39%). From these two estimations, it appears that 30-40% of cytoplasmic polydisperse

Figure 36.

Oligo (dT) cellulose affinity chromatography of rapidly labelled artichoke nucleic acid extracted from a 10,000 g supernatant.

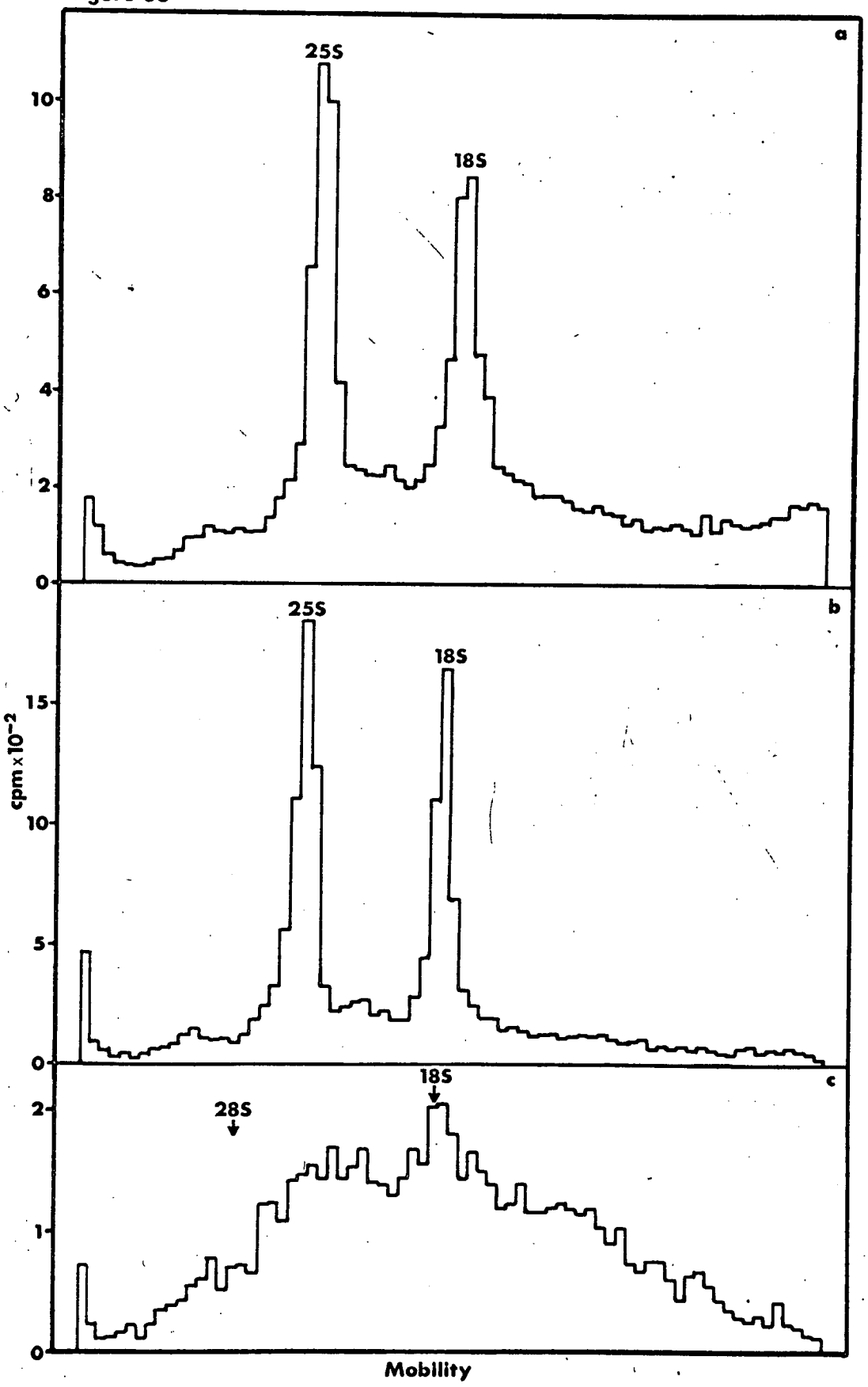
Explants were labelled for 1 hr with ^{32}P orthophosphate (100 $\mu Ci\ ml^{-1}$, 10 Ci mg^{-1} P, Amersham) and homogenised as for a polysome preparation. Nucleic acid was prepared from the supernatant of a 10,000 g centrifugation of the initial homogenisation, dissolved in 0.5M NaCl, 10mM Tris-HCl, pH 7.4 (loading buffer) and loaded in 1 ml onto 100 mg oligo(dT) cellulose at 25°C. The column was washed with 4 ml loading buffer and, after increasing the temperature to 40°C, eluted with 5 ml 10mM MEDTA-sodium salt, 10mM Tris-HCl, pH 7.4. The washing and elution fractions were made up to 50 $\mu g\ ml^{-1}$ with purified mouse rRNA and the nucleic acid recovered by ethanol precipitation. The samples were fractionated on 2.4% acrylamide gels for 3 hr at room temperature.

a) original sample

b) column wash

c) column eluate

Figure 36


a
25S
18S
10
8
6
4
2
0
b
25S
18S
15
10
5
0
cpm x 10⁻²
c
18S
28S
2
1
0
Mobility

RNA has no poly (A) sequences associated with it.

The 10,000 g pellet used for oligo (dT) cellulose affinity chromatography contains rather less contamination from mature rRNA than previously shown (compare Figure 37a with Figure 27c) but shows the same distribution of rRNA precursors, DNA and polydisperse RNA (Figure 37a). The poly (A) content was 8.1% which, when corrected for the polydisperse RNA content (66% of the total label) indicates that the poly (A) content of the polydisperse RNA fraction is 12.3%. Of the RNA which washed through the column, 54% is polydisperse. The total poly (A) content of this fraction (7.8%) when corrected for this polydisperse RNA indicates that 14.4% of the unbound polydisperse RNA is poly (A). Only 10% of the total counts bound to the oligo (dT) cellulose. This fraction has a very broad spectrum of molecular weights with a small amount of contamination from rRNA precursors and DNA (Figure 37c). The poly (A) content of this, largely polydisperse RNA fraction is 10.4%. Since binding of poly (A) containing molecules was incomplete, it was not possible to determine the percentage of polydisperse RNA molecules which contain poly (A) sequences.

Figure 37.

Oligo (dT) cellulose affinity chromatography of rapidly labelled artichoke nucleic acid extracted from a 10,000 g pellet.

Nucleic acid was prepared from the 10,000 g pellet obtained by the centrifugation described in Figure 36 and fractionated on oligo (dT) cellulose. The nucleic acid in the washing and elution fractions were recovered as described for Figure 36 and fractionated on acrylamide gels.

a) original sample fractionated for 3 hr on a 2.3% acrylamide E buffer gel at room temperature.

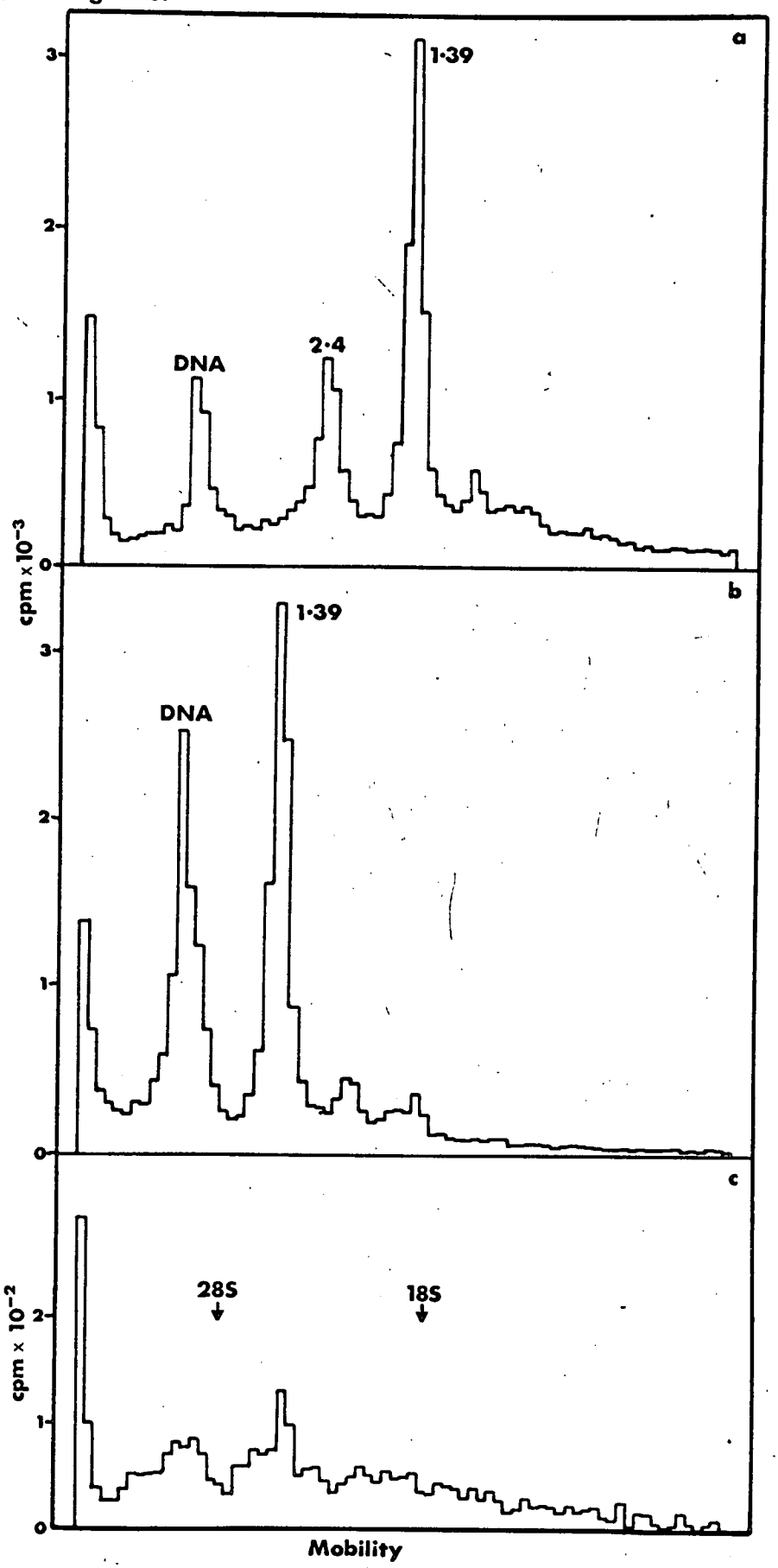
b) column wash fractionated for 3 hr on a 2.4% acrylamide E buffer gel at room temperature.

c) column eluate fractionated as in b).

Molecular weights are in daltons x 10^{-6}.

The DNA peak in b) contains the 2.4 x 10^{6} dalton precursor which is not resolved under the fractionation conditions used.

Figure 37



PART 2

DISCUSSION

Since Jacob and Monod (1961) first postulated the existence of a short-lived mRNA which functioned as the translation intermediate between the gene and its corresponding polypeptide product, mRNA has been searched for in the rapidly labelled polydisperse RNA fraction present in the nucleus and cytoplasm. In animal cells, cell fractionation has shown that a significant part, between one and two thirds and in one case up to 80%, of the nuclear rapidly labelled RNA is also rapidly destroyed, probably without leaving the nucleus (Scherrer, Latham and Darnell, 1963; Gvozdev and Tikhonov, 1964; Shearer and McCarthy, 1967). While the average size of the polydisperse RNA fraction dissociated from polysomes is 16 to 18S (Di Girolamo, Henshaw and Hiatt, 1974; Penman, Rosbash and Penman, 1970), that from the nucleus is very much larger at 50 to 70S (Attardi, Parnas, Hwang and Attardi, 1966; Scherrer, Marcaud, Zajdela, London and Gros, 1966). In plants, however, this very large molecular weight polydisperse fraction is not observed. For example, Ingle, Key and Holm (1965) from sucrose gradient analyses, describe the rapidly labelled fraction of total nucleic acid extracted from soybean hypocotyls as sedimenting in the 18 to 20S region of the gradients. In addition gel analysis of the DNA-like RNA (D-RNA) fraction from a MAK column separation of total soybean hypocotyl nucleic acid shows that this D-RNA is less than 32S in size (Ingle and Key, 1968).

The problems associated with the cellular fractionation of plant tissue are great. Because of the tough cell wall, the treatments required to break the cells damage a great many nuclei. By reducing the severity of this homogenisation, many of the cells are left intact.

For one or other of these reasons, then, the yield of intact nuclei from plant material is usually low. It is clear, however, that a 10,000 g centrifugation gives a reasonably good separation of the tissue homogenate into a nuclear (10,000 g pellet) and cytoplasmic (10,000 g supernatant) fraction. The bulk of the mature rRNA remains in the cytoplasmic fraction while the nuclear fraction contains all the DNA, all the rRNA precursors and only trace amounts of the mature rRNA molecules. Of the polydisperse RNA, 30% pellets with the nuclear fraction while the remaining 70% is in the cytoplasmic fraction. The 60% of total polydisperse RNA which sediments with polysomes after a 50,000 g centrifugation is a very much higher value than that previously reported by Lin, Key and Bracker (1966) where only 10 to 20% of the D-RNA was shown to be associated with polysomes in soybean root tips. This, however, may be a function of the tissue since in the artichoke explant system, as has been shown, the newly synthesised RNA is biased towards polydisperse RNA synthesis at the time studied (Figure 25), and the tissue is actively growing. Microscopic observation of this 10,000 g pellet (Figure 28) showed a great many apparently intact nuclei but also free nucleoli, indicating some nuclear damage. It is therefore possible that some of the polydisperse RNA in the 10,000 g supernatant may be of nuclear origin. The resulting distribution of such nuclear polydisperse RNA would depend on the type of particle released. Informofer complexes could contaminate the polysome preparation while, if they are in the unit form, they could remain in the 50,000 g supernatant fraction (Samarina, Lukaindin and Georgiev, 1973). The 17% of the 10,000 g supernatant polydisperse RNA which remains in the 50,000 g supernatant could therefore be either from these unit informofers or from truly cytoplasmic informosomes (Silverstein, 1973), however without a detailed examination of the nuclear RNP particles,

it is impossible to decide which. There is, of course, a degree of cytoplasmic contamination of the nuclear fraction. Since 5 to 10% of the mature rRNA appears in this fraction it is probable that this contamination will amount to no more than 5 to 10% of the total label. The degree of cytoplasmic contamination of the nuclear pellet can be reduced by low speed centrifugation (300 g) of the initial homogenate followed by resuspending and washing in homogenisation buffer (K. Chapman, personal communication).

There is considerable radioactivity in the monomer and subunit region of the polysome profile (Figure 33), 31% of which is in polydisperse RNA (Figure 34a) and may be associated with informosome particles rather than true ribosomes. Caesium chloride gradient centrifugation of the RNP particles prior to nucleic acid extraction would have to be used to determine whether this component represented RNA linked to informosomes or ribosomes since the former particles have a lower density (Ajtkhozhin and Akhanov, 1974). This technique could also be used to examine the nuclear particles and those which remain in the supernant after a 50,000 g centrifugation.

Although the very heavy nuclear polydisperse RNA of animal cells is not observed in the artichoke system, it is clear that the nuclear polydisperse fraction has a somewhat larger molecular weight range than that from the cytoplasmic fraction as shown in Figure 27 b and c. The mean molecular weight of the cytoplasmic polydisperse RNA is of the order of 1.1×10^6 daltons and although the bulk of the nuclear polydisperse RNA is very similar, there is a distinct shoulder on the heavy side which extends up to a molecular weight of approximately 3.0×10^6 daltons in agreement with the maximum size of D-RNA described by Ingle and Key (1968).

Georgiev has postulated a structure for the operon of higher animals (Georgiev, 1969, 1970; Georgiev et al, 1972) involving successive processing of a giant nuclear RNA transcript to a smaller unit which is then transported to the cytoplasm where it is translated. His model suggests that the parts of the giant nuclear RNA chains which are removed correspond to the promotor and activator regions of the gene. As further evidence for this model, nucleases capable of cleaving RNA have been shown to be associated with nuclear ribonucleoprotein particles (Niessing and Sekeris, 1970).

Plants therefore differ from animals in that their nuclear RNA is smaller while their cytoplasmic mRNA is larger, i.e. 1.1×10^6 daltons (from Figure 27 b) rather than 0.5×10^6 daltons (Di Girolamo et al, 1974; Penman et al, 1970). During maturation, therefore, plant mRNA is much more conserved than animal mRNA. This is analagous to the maturation of rRNA which is only 50% conserved in animals but 80% conserved in plants (Weinberg and Penman, 1970; Leaver and Key, 1970). Extrapolation of the Georgiev model may suggest that in plants, the operon size is smaller than in animals.

It is clear that there is a definite poly (A) component in artichoke rapidly labelled RNA. This is reflected in the adenylic acid content of artichoke rapidly labelled RNA of 31% (Table 8). Similar adenylic acid rich RNA has been shown in the D- and TB-RNA fractions of soybean rapidly labelled RNA from MAK columns (Key, Leaver, Cowles and Anderson, 1971; Vanderhoef, Schmid and Siegel, 1974). The percentage of adenylic acid in this poly (A) increases with time of digestion to a value of 83% after 2 hr. This compares with percentage purities of poly (A) from rice of 97.8% (Manahan et al, 1973) and from Vicia of 92.2% (Sagher et al, 1974). The base composition analysis technique

as described here cannot reliably distinguish a non adenylate nucleotide contaminant of less than 2% and hence the percentage purity of poly (A) cannot accurately be given as more than 94% adenylic acid. In all these cases of plant poly (A) the nucleotide which contributes most to the contamination is guanylic acid, being 8.9% in artichoke, 1.6% in rice (Manahan et al, 1973) and 5.8% in Vicia (Sagher et al, 1974). From nuclease digestion of total RNA, the poly (A) content is 5% which, when corrected for the percentage polydisperse RNA indicates that this fraction must contain 7% poly(A). Assuming an average size of polydisperse RNA of 1.1×10^6 daltons and that all polydisperse RNA molecules contain poly (A) its average size will be approximately 220 nucleotides. The size of poly (A) as determined from gel analysis of the RNA stable to RNAase A and RNAase T1 digestion showed a spread up to 250 nucleotides with a mean of 160 nucleotides. This is very similar to that reported for Vicia (Sagher et al, 1974). It is important to note, however, that the determination of exact molecular weights from polyacrylamide gel electrophoresis can only give accurate results if poly (A)s of known molecular weights are used as standards (Pinder and Gratzer, 1974; Kaufman and Gross, 1974), since the secondary structure of poly (A) is restricted to a simple coil and this sequence does not show the same relative mobility as 4S and 5S. Using the R_F values for poly (A) relative to tRNA of Pinder and Gratzer (1974) for 10% gels and the comparable R_F value obtained from Figure 31 for the 160 nucleotide peak on a 7.5% gel, the size of this peak can be estimated as being 140 nucleotides. However, the lower gel concentration used here means that this 140 nucleotide size is an underestimate and hence the actual sizes quoted for poly (A) in artichoke rapidly labelled RNA, although not exact, are close to the true value. Sagher et al (1974) confirmed their estimate of poly (A)

size from Vicia derived from electrophoretic mobility by sedimentation analysis using the relationship between the S-value and the molecular weight of poly (A) derived by Fresco and Doty (1957). Van de Walle (1973) and Higgins et al (1973) both use low concentration polyacrylamide gel electrophoresis to determine the size of their poly (A) samples. Pinder and Gratzer show that while a one hundred nucleotide poly (A) molecule has an R_F relative to tRNA of 0.6 in a 10% gel, in a 2.4% gel, its R_F relative to tRNA is 0.87. Thus the sizesof poly (A) estimated by Van de Walle (1973) and Higgins et al (1973) are underestimates. The true average size of mung bean poly (A) is therefore very similar to that reported here for artichoke poly (A) (Higgins et al, 1973). On the other hand, even allowing for the underestimate in size, the poly (A) from Zea appears to be rather smaller (Van de Walle, 1973). The data presented for the size of poly (A) in rice by Manahan et al (1973) is inconclusive, showing no clear peak on a gel fractionation profile. The average size of poly (A) from soybean TB-RNA, determined by electrophoretic mobility, as 160 nucleotides although in agreement with the average sizes quoted here, does not agree with the size determined from the adenosine to adenylate ratio of 72 nucleotides (Vanderhoef et al, 1974).

The large size of poly (A) in artichoke RNA agrees well with the data obtained by electrophoretic mobility for the size of poly (A) in animal cells of 200 nucleotides (Sheiness and Darnell, 1973) and confirmed by Mendecki et al (1972) by determination of the adenosine to adenylate ratio for purified poly (A). The homogeneity of animal poly (A) only holds for newly synthesised poly (A). As the age of the poly (A) increases, the modal size decreases and the size distribution becomes very much more heterogeneous (Sheiness and Darnell, 1973;

Brawerman, 1973) until it is very similar to the size distribution shown here for artichoke poly (A). Sheiness and Darnell (1973) and Brawerman (1973) also show that the average size of the nuclear fraction of poly (A) is larger than that of the polysomal fraction of poly (A). From the percentage poly (A) and average size of poly (A) containing RNA as bound by an oligo (dT) cellulose column, it has been shown that while the average size of poly (A) in RNA from a total sample is 280 nucleotides, that from the cytoplasmic fraction is only 170 nucleotides, indicating a similar trend. In addition, it has been shown from MAK column fractions that the size of poly (A) in soybean D-RNA (of nuclear origin) is larger than that in TB-RNA (of cytoplasmic origin) (Vanderhoef, et al, 1974). The spread of size of poly (A) observed in artichoke may be inherent to particular mRNA molecules. On the other hand, it may be a result of excessive nuclease digestion of large poly (A) sequences containing a percentage of non adenylate nucleotides which have been added to the poly (A) during synthesis as a result of mistakes by the polymerase. The fact that after a 2.0 hr nuclease digestion, artichoke poly (A) is still only 83% adenylate indicates that such mistakes do occur. It is also possible, however, that the poly (A) in artichoke may originate from mRNA molecules of varying ages. In this case, the size range may demonstrate the reduction in size of poly (A) with age mentioned earlier.

The modified Kirby method for extracting nucleic acid used here gives a preparation which contains DNA and many RNA molecules which do not contain poly (A). It is possible to enrich the preparation for polydisperse RNA and hence poly (A) containing molecules, but the separation of polydisperse RNA and ribosomal RNA is not complete (Ingle, Key and Holm, 1965). In addition, the use of inhibitors (e.g. low concentrations of actinomycin D) to preferentially inhibit rRNA

synthesis does not work well with plant tissue (Key and Ingle, 1969). The use of gel fractionation of total nucleic acid preparations, however, enables the relative amounts of rRNA and polydisperse RNA to be calculated and the poly (A) content corrected to the percentage polydisperse RNA as described earlier.

In order to study whether or not all the polydisperse molecules contain poly (A), two methods were used to purify the poly (A) containing molecules by specific binding to either poly (U) attached to Whatman glass fibre filters (GF/C) or oligo (dT) covalently linked to cellulose. The poly (U) filters, made as described by Sheldon et al (1972) were difficult to handle and the binding of RNA to them was very variable, presumably due to the lack of equilibrium between the poly (A) and poly (U). Oligo (dT) cellulose affinity chromatography was a much more effective method. As the material was supplied in a powder form, it could be used in a jacketed column, ensuring constant temperature during loading and elution and a controllable flow rate.

The oligo (dT) cellulose bound all the poly (A) containing RNA from the artichoke cytoplasmic fraction. The unbound RNA contained 0-0.5% poly (A), the level determined for rRNA in the assay used, but 33% of the total polydisperse RNA (Figure 36 b), indicating that 33% of the putative cytoplasmic mRNA does not contain poly (A). It is interesting to note that the polydisperse RNA from the ribosome and subunit region of a polysome gradient, amounting to 39% of the total polysomal polydisperse RNA (Figure 34 a) also contained no poly (A). Since the non poly (A) containing polydisperse RNA from the polysome gradient came from RNP particles less than 80S in size, it is possible that these molecules may be of nuclear origin (informofer units, Samarina, Lukaidin and Georgiev, 1973) or true cytoplasmic informosomes (Silverstein,

1973). Similarly, there is incomplete binding of polysomal mRNA from fibroblasts to oligo (dT) cellulose. The unbound fraction, which does not contain poly (A), has been shown by competitive hybridization to contain different sequences to those present in the bound, poly (A) containing mRNA (Johnson, Abelson, Green and Penman, 1974). This rules out the possibility that the absence of poly (A) from part of the mRNA fraction in this case is the result of nuclease activity. Although competitive hybridisation studies have not yet been made on the two fractions of artichoke polydisperse RNA, the fact that they are not significantly different in their molecular weight range (Figure 36 b and c) suggests that the absence of poly (A) and lack of binding is again not a result of nuclease activity unless the poly (A) length is more exposed than the rest of the molecule in a ribonucleoprotein particle such that a particle-associated nuclease could cleave the poly (A) segment from the molecule to produce a negligible change in molecular weight (Rosenfeld, Abrass and Perkins, 1972). The results with artichoke polydisperse RNA confirm the evidence of Adesnik et al (1972) with histone mRNA and Johnson et al (1974) with total fibroblast mRNA that there are two groups of mRNAs, one of which contains poly (A) sequences and one of which does not. This model, however, is somewhat confused by the evidence of Houdebine, Gaye and Favre (1974) who show that only 40% of the mRNA for α_s casein contains a poly (A) sequence.

Oligo (dT) cellulose did not bind all the poly (A) containing molecules from either the total preparation (Figure 35) or the nuclear fraction (Figure 37). However the molecules that were bound from the nuclear fraction showed a larger spread of molecular weight than those bound from the cytoplasmic fraction (compare Figure 37 c with Figure 36 c). This result is consistent with the precursor to mRNA in plants

being larger than the mature mRNA. Since these large molecules from the nuclear fraction contain poly (A), the poly (A) must be added prior to processing and transport.

There are several possible reasons why poly (A) containing molecules from the total and crude nuclear preparations do not bind to oligo (dT) cellulose. With very large RNA molecules, it is feasible that the long tail of non poly (A) RNA could destabilise the poly (A)-oligo (dT) hybrid and reduce the efficiency of binding. However, the size of the unbound polydisperse RNA (Figure 35 b and Figure 37 b) from gel analysis does not appear to be larger than the bound polydisperse RNA (Figure 35 c and Figure 37 c) which mitigates against this. A second possibility is that a proportion of total and nuclear polydisperse RNA molecules contain only short poly (A) sequences, insufficient to form stable hybrids. However, calculation of the average size of the poly (A) sequences in the unbound polydisperse RNA from the total and nuclear fractions from the percentage of poly (A) in the polydisperse RNA and the average size of the polydisperse RNA, assuming that each molecule contains poly (A) shows that the average poly (A) size is at least 100 nucleotides which does not greatly support this model. The final possibility, mentioned earlier, is that a contaminant of the nucleic acid preparation could be masking a proportion of the poly (A) sequences. Poly (A) will specifically bind to cellulose, probably to a lignin contaminant (De Larco and Guroff, 1973). It is feasible that such a contaminant could persist in the nucleic acid preparations from the total and nuclear fractions (which did initially contain cell wall contamination) and, under the high salt conditions used to bind the poly (A) to the oligo (dT) cellulose, mask some of the poly (A) sequences. To date it has not been possible to distinguish between these two latter alternatives.

The results presented here demonstrate the existence of poly (A) in a large proportion of polysomal mRNA molecules in artichoke. It is probable that these molecules are cleaved from the slightly larger nuclear poly (A) containing RNA molecules prior to transport to the cytoplasm. It is not yet clear whether the observed heterogeneity in the size of poly (A) from total polydisperse RNA is equally represented in the polydisperse RNA from the various cellular fractions.

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