Heterogeneous nuclear RNA in duck erythrocytes.

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CONTENTS

SUMMARY.

The pattern of RNA synthesis has been studied in immature erythrocytes present in the blood of anaemic ducks maintained *in vitro*. The majority of the RNA synthesised *in vitro* by these cells remains associated with the nuclear fraction and in non-denaturing sucrose gradients appeared to consist of heterogeneous RNA species of high sedimentation rate. This RNA has been called heterogeneous nuclear RNA (hnRNA) and under the conditions used in this work most of this hnRNA was not precursor rRNA.

Various RNA species were treated in sucrose gradients with formaldehyde, formamide and urea in amounts producing the denaturation of RNA secondary structure. In such denaturing gradients the hnRNA molecules appeared to be much smaller in size although they still sedimented heterogeneously, whilst cytoplasmic RNA species were affected to a much smaller extent. Reconstruction experiments in non-denaturing sucrose gradients using denaturated hnRNA produced large sedimenting species. Such results support the conclusion that hnRNAs are a heterogeneous mixture of molecules whose apparent large sizes are an artifact caused by aggregation.

Hybridisation experiments across non-denaturing and denaturing sucrose gradients containing hnRNA and cytoplasmic RNA were compared in order to study the relationships between hnRNAs and cytoplasmic mRNAs. Poly U hybridisation to detect poly A sequences was used to find mRNA - like sequences in both cytoplasmic RNA and hnRNA. Similarly, cDNA (DNA complementary to haemoglobin mRNA) hybridisation was used to identify haemoglobin mRNA sequences in cytoplasmic RNA and hnRNA. The effectiveness of these hybridisation techniques is discussed.

Using these hybridisation techniques, a 14 S hnRNA species containing both poly A and haemoglobin mRNA sequences and about three times longer than cytoplasmic haemoglobin mRNA has been isolated in denaturing sucrose gradients. The possible relationship between this 14 S hnRNA and cytoplasmic haemoglobin mRNA is discussed at length. It is concluded that the 14 S hnRNA species may be the nuclear precursor of the cytoplasmic haemoglobin mRNA and various schemes for its structure and processing are discussed.

ABBREVIATIONS

A,C,G,T,U	Adenine, cytosine, guanine, thymine, uracil.
cDNA	DNA complementary to haemoglobin messenger RNA.
cpm	Isotopic counts per minute.
DMSO	Dimethylsulphoxide.
DNA	Deoxyribonucleic acid.
DNase	Deoxyribonuclease.
EDTA	Ethylene diaminetetracetic acid.
hnRNA	Heterogeneous nuclear RNA.
MEMS	Minimum essential medium for suspension cultures.
mRNA	Messenger RNA.
poly A, poly U	Polyadenylic acid, polyuridylic acid.
RNA	Ribonucleic acid.
RNase	Ribonuclease.
rpm	Revolutions per minute.
rRNA	Ribosomal RNA.
SLS	Sodium lauryl sulphate.
SSC	Standard saline citrate.
TCA	Trichloroacetic acid.
Tris	Tris (hydroxymethyl) aminomethane.
tRNA	Transfer RNA.

INTRODUCTION

a) General

The work described here is concerned with the size of hnRNA and its relationships with cytoplasmic mRNAs. HnRNA is that portion of nuclear RNA having a wide range of size and heterogeneity, a DNA-like base composition and a very high turnover rate. In fact, most of the hnRNA turns over in the nucleus and never enters the cytoplasm (Houssais and Attardi, 1966; Warner et al, 1966). Superficially hnRNA has certain properties similar to mRNAs and it has been suggested that at least some of the hnRNA is the nuclear precursor of the cytoplasmic mRNAs.

One of the major problems involved in studying the relationship of the hnRNA to other RNA species is in the determination of the actual size and structure of hnRNAs. Large differences in the sedimentation coefficients of hnRNAs have been obtained in sucrose gradients by different workers and there is considerable evidence (see below), suggesting that these large differences in sedimentation coefficient are caused by the aggregation of hnRNA molecules. However, by using denaturing conditions that remove all aggregation and secondary structure, the actual sizes and molecular weights of denatured hnRNA molecules can be obtained. Denaturing conditions can not, of course, distinguish between the 'normal' secondary structure of hnRNA found in vivo and non-physiological aggregation between hnRNA molecules that may occur during the extraction and analysis of these molecules.

b) Secondary structure and aggregation of RNAs.

Generally, RNAs do not exhibit the same asymmetric, helical configurations found in DNA, but instead behave as irregularly coiled,

relatively compact, simple polynucleotide chains. Although the hydrogen bond forming potential of RNA is as great as that of DNA, its gross configuration in solutions does not reveal any such secondary structure. Yet most RNAs have a very compact shape (Doty et al, 1959) which suggests that there are significant numbers of intrachain contacts of hydrogen bonds which are localised and organised to produce a secondary structure. In some RNAs, notably rRNAs, as much as 40% of the residues in a simple polynucleotide chain can be brought into helical structures. The generally accepted model of RNA is of helical regions consisting of many hairpin-like sections of the chain with a small, but variable, number of bases unmatched in the loop.

Even the most stable helical configurations exhibit relatively great flexibility of the polynucleotide chains due to intense internal Brownian motion, and thus the polynucleotide chains exhibit lateral motion with respect to one another, (Doty et al, 1959). Thus it is likely that in helical structures there are unmatched bases or defects. Opposing pyrimidines can easily be accommodated with no steric hindrance and certain purine - pyrimidine pairs (G-U and A-C) might be unmatched and involve some distortion but would probably be acceptable in small amounts. However, purine pairs would not be acceptable and they would certainly terminate a helical region.

At elevated temperatures RNA molecules become partially unfolded. This state is reflected in increased absorbance at 260 nm, reduced rotation of polarised light and increased specific viscosity. Their thermal stability can be represented by their melting temperature (Tm). Disruption of RNA secondary structure by removal of hydrogen bonding also occurs with variation in ionic strength, bivalent cation concentration and addition of

denaturants, in a similar manner to thermal denaturation.

Conversely, aggregation between RNA molecules may be caused by hydrogen bonding between only a few complementary or nearly complementary nucleotide sequences. It is probably an artifact caused by certain physical conditions found during the preparation, storage and analysis of RNAs and probably does not occur in the physiological conditions prevailing in vivo.

Ishihama et al (1962), Monier et al (1962) and Hayes et al (1966) have all shown that E. coli mRNA forms aggregates with itself and with rRNA in the presence of Mg^{2+} and under certain other physical conditions, such as a pH lower than 9.5 and a temperature lower than 15° C. Similar effects have been shown for mRNA formed in E. coli after phage T2 infection (Ishihama et al, 1962; Asano, 1965). Staehelin et al (1964) have observed aggregates of mRNAs with rRNA in rat, which disaggregated in the absence of Mg^{2+}, at low ionic concentrations generally and after a short incubation at 37° C with SLS. Matus et al (1964) have shown turnip yellow virus RNA forms stable aggregates with rRNA from Chinese cabbage leaves or tobacco leaves, whilst tobacco mosaic virus RNA forms similar aggregates with Chinese cabbage leaves nucleic acids.

Certain conditions of RNA preparation have been shown to cause aggregation of eukaryotic RNAs (Parish and Kirby, 1966). Wagner et al (1967)have shown that the hot phenol - SLS method for deproteinising HeLa rRNA can give rise to non-covalently bonded aggregates of the two rRNA species. Similarly, calf crystallin mRNA (Berns et al, 1971), chicken haemoglobin mRNA (Maroun et al, 1971) and sea urchin histone mRNA (Hogan and Gross, 1972) have been found aggregated under certain extraction

conditions.

c) RNA denaturation.

Denaturation of the secondary structure of RNA molecules can occur as the result of variations in physical parameters such as ionic strength, bivalent cation concentration and temperature of the solutions in which the RNA is extracted, stored or analysed. Petermann and Pavlovec (1963) have shown that changes in ionic strength, Mg^{2+} concentration and temperature can produce interconversion of rat liver 28 S and 16 S rRNA and aggregation of the components to form more rapidly sedimenting material. Aronson and McCarthy (1961) and Rodgers (1966) have shown that the large rRNA subunit of E. coli can be reversibly converted to 16 S by exhaustive removal of Mg^{2+} with EDTA and similar changes can be produced in this rRNA by treatment at pH 9.0 (Midgley, 1965). Bishop (1966) has shown that the sedimentation of coliphage MS 2 RNA is markedly affected by changes in temperature and ionic strength.

Stanley and Bock (1965) using E. coli rRNA and Bruening and Bock (1967) using yeast rRNA have found, by heating and varying the ionic concentration of rRNA solutions, a lowering of the apparent RNA sedimentation coefficient due to the unfolding of the molecules. However, they also found that the two rRNA components were separate and non-interconvertible being formed by continuously covalently linked residues. Fenwick (1968) has observed transformations of E. coli rRNA treated with mild nuclease similar to these obtained with thermal or ionic denaturation. Thus, McPhie et al (1966) suggest the presence of labile points or 'hot spots' in the polynucleotide chain arising from a tertiary RNA structure which makes particular parts of the chain available for nucleolytic attack. Gould

(1967) and Cox (1969) further suggest that a high proportion of these points are 'hidden' owing to the secondary structure of the RNA molecules and can only be exposed on the unfolding of the polynucleotide chain.

RNA denaturation can also be brought about by the action of specific denaturants such as urea, formamide, formaldehyde and DMSO, all known to cause disruption of the RNA secondary structure by removing hydrogen bonding.

Boedtker (1967) and (1968) and de Kloet et al (1970) and Mayo and de Kloet (1971) have used formaldehyde denaturing conditions in which almost total denaturation takes place in a short time, with a minimal hydrolysis of phosphate diester bonds. Boedtker (1968) used 1.1 M formaldehyde at 63°C for 10 minutes and de Kloet et al (1970) used 7.5% formaldehyde at 65°C for 15 minutes. At these conditions, not only is denaturation almost complete, but nuclease action is effectively stopped by the formaldehyde. Any unmasking of 'hidden breaks' in the RNA can not be avoided, but Boedtker (1968) has calculated that under the conditions used less than 1 in 50,000 phosphodiester bonds would be broken so that thermal breakage resulting from the reaction of RNA with formaldehyde has a negligible effect on the molecular weight of RNAs of 2×10^6 daltons or less.

As formaldehyde chemically reacts with the amino groups of cytosine, adenine and guanine in the RNA (Hall and Doty, 1959) it is not possible to reverse the reaction. Hydrogen bonds are unable to reform on cooling as the reaction of free amino groups is rapid whereas hydrogen bonded groups react much more slowly. As formaldehyde treated RNA is almost irreversibly denatured, the recovery of biologically active RNA is impossible, (Strauss and Sinsheimer, 1968). This is a disadvantage with

this method of denaturation.

Most formaldehyde denaturation studies have been done with bacterial and viral RNAs, for example with E. coli rRNA (Tal, 1970). Denaturation with formaldehyde has also been used fairly extensively with eukaryotic cytoplasmic RNAs and have shown the existance of secondary structure in rRNAs (Strauss and Sinsheimer, 1968; Tal, 1969; Cox, 1969a). However, Staynov et al (1972) have shown that haemoglobin mRNA has no secondary structure, as formaldehyde treatment produces little or no effect on its sedimentation coefficient in aqueous gels.

There has been little research into the formaldehyde denaturation of eukaryotic nuclear RNAs although de Kloet et al (1970), Mayo and de Kloet (1971) and Peltz (1973) have used formaldehyde to denature hnRNA in yeast, mouse and sea urchin cells respectively. They have shown that on formaldehyde treatment the sedimentation coefficients of the hnRNA decrease by a considerable amount. This implies the existence of some secondary structure and/or aggregation between hnRNA molecules.

Urea denatures RNA secondary structure by breaking, not only hydrogen bonds, but also hydrophobic bonds in aqueous solution (Robinson and Jencks, 1965). Tanford (1970) has reviewed in detail the action of urea on nucleic acids. Results obtained from urea denaturation at room temperature of RNAs are very similar to those obtained by thermal denaturation. For example, Takanami et al (1961) on denaturation of E. coli tRNA with 6 M urea or heat treatment have shown that considerable loss of secondary structure of the tRNA occurs which can largely be reversed on removal of the urea by dialysis or cooling.

Similarly, the considerable hyperchromism exhibited by the r RNA subunits and their apparent molecular weights can be reduced by urea treatment. Pene et al (1968) with 5 M urea, Plagemann (1970) with 7.5 M urea and Ishikawa and Newburgh (1972) with 8 M urea have shown that the larger rRNA subunit on urea treatment undergoes an irreversible lowering of its sedimentation coefficient to roughly the size of the smaller rRNA subunit. Experiments designed to denature hnRNA with urea have been attempted but generally only low urea concentrations have been used (Hastings et al, 1968).

Formamide is an ionizing solvent having a high dielectric constant of about 110 and Helmkamp and Ts'o (1961) have shown that formamide causes the complete loss of all polynucleotide structure, both base-pairing and stacking, in RNA as well as DNA. Marmur and Ts'o (1961) have shown that the denaturation of DNA with formamide is time dependent and similar to acid and thermal denaturation. As the action of formamide on hydrogen bonding in nucleic acids is purely ionic, renaturation of nucleic acids can occur. Marmur and Ts'o (1961) have shown that D. pneumoniae DNA denatured with formamide can not only be renatured, but can also have its biological activity restored, after removal of the formamide by dialysis.

Little recent work has been done on the denaturing capabilities of formamide on RNAs, although Staynov et al (1972) have run RNA on polyacrylamide gels with formamide as the solvent in order to obtain denatured RNA so as to obtain accurate molecular weights of the RNA. The effectiveness of formamide denaturation on enkaryotic RNAs has on the whole not been determined, and certainly experiments similar to those done with formaldehyde on eukaryotic nuclear RNAs, (de Kloet et al, 1970; Mayo et al, 1971) have not previously been attempted.

The other main denaturing agent used in RNA studies is DMSO. Strauss et al (1968) have shown that DMSO is a very useful denaturing agent as it causes RNA denaturation at room temperature while it also decreases thermal chain scissions and inactivates RNase. RNA is stable in DMSO for at least several hours and has been used to denature various rRNAs. Pene et al (1968) and Plagemann (1970) using cultured cell rRNAs and Applebaum et al (1966) and Stevens and Pachler (1972) using insect rRNAs have, amongst others, observed an apparent lowering in the sedimentation coefficient of the larger rRNA component similar to that observed with other denaturing conditions. Strauss and Sinsheimer (1968) and Simmons and Strauss (1972) have used DMSO to denature viral RNAs.

Lindberg and Darnell (1970), Tonegawa et al (1970) and Acheson et al (1971) have all treated the large molecules of hnRNA transcribed in virus infected cells, that contain virus-specific RNA, with DMSO and found little difference between untreated and DMSO treated hnRNA. These results are apparently contradictory to those obtained by de Kloet et al (1970) and Mayo and de Kloet (1971) with hnRNA treated with formaldehyde. However, it is quite possible that virus-infected cells may not have the same transcriptional processes as normal cells. Certainly, Scott and Kuhns (1972) have found that hnRNA from mouse tumour cells denatures into much smaller molecules in DMSO.

Other workers have used other RNA denaturants, although there are very few reports of these. Hastings et al (1968) have used sulpholone and ethylurea which led to some dissociation of rat liver rapidly labelled RNA.

d) Heterogeneous nuclear RNA

Much of the RNA synthesized by growing and differentiated cells remains associated with the nuclear fraction and consists of rapidly labelled RNA species with high sedimentation coefficients. This heavy RNA consists of two main species. Firstly, there is the ribosomal-like RNA, which is found in the nucleolus (Penman et al, 1966), sediments with about 45 S and has a GC content of more than 60% (similar to rRNA). This type of heavy nuclear RNA has been observed in many cell types (Scherrer et al, 1963; Yoshikawa-Fukada, 1965; Rake and Graham, 1964: Attardi et al, 1966). Scherrer et al (1963), Girard et al (1964) and Weinberg et al (1967) have shown in HeLa cells the transformation of this 45 S species to smaller RNA molecules of 18 S and 28 S (the size found in ribosomes) after actinomycin D treatment, which is known to stop RNA synthesis in eukaryotes (Reich et al, 1962). They conclude that this RNA is a rRNA precursor.

The other heavy rapidly labelled RNA species is largely, if not completely, unassociated with the nucleolus (Warner et al, 1966; Penman et al, 1968). Its base ratio is quite different from rRNA but similar to DNA and sedimentation coefficients ranging from 10 S to over 200 S are reported. This RNA has been called hnRNA. HnRNA has been found by pulse labelling experiments in the nuclei of many cultured and differentiated cells.

The rapid turnover of hnRNA is indicated by the fact that during short periods of radioisotope incorporation, hnRNA reaches a high specific activity and constitutes the major part of the total RNA labelled during short pulses. Attardi et al (1966) and Scherrer et al (1966) incubated duck erythrocytes with a labelled RNA precursor and

then removed the label in order to observe what happens to the previously labelled hnRNA with time. Experiments of this type have shown that the majority of hnRNA is not transferred to the cytoplasm even hours after synthesis and 60-70% of the total radioactivity in the RNA is gradually lost, presumably back to the acid-soluble pool. If,after labelling the hnRNA, either in duck erythrocytes or cultured cells (Houssais and Attardi, 1966; Warner et al, 1966), the cells are treated with actinomycin D, the great majority (about 90%) of the hnRNA decays to acid-soluble material. Thus, it seems likely that the majority of the hnRNA never serves a cytoplasmic function and it is probable that this RNA is the rapidly turning over RNA whose presence was inferred by Harris (1963).

Many attempts to measure the base ratio of hnRNA have been frustrated by failure to separate hnRNA from the rest of the rapidly labelled nuclear RNA and to obtain enough hnRNA for analysis. However, Soeriro et al, (1966) partially separated the two rapidly labelled nuclear RNA species from HeLa cell nuclei and found that the GC content of the hnRNA was 44% GC (similar to DNA). Many other workers using different systems, including Attardi et al (1966) and Scherrer et al (1966) with duck erythrocytes, and Edström and Daneholt (1967) with Chironomus have found similar GC ratios of between 40-45% GC for hnRNA.

HnRNA contains transcripts of intermediate reiterated DNA sequences (Britten and Kohne, 1968), which in rat accounts for about 35% of the total nuclear RNA (Melli et al, 1971). Some of the reiterated sequences may be involved in the secondary structure of the hnRNA. Nevertheless, most hnRNA consists of transcripts of unique DNA sequences.

Recently, Darnell et al (1971a) and Edmonds et al (1971) using HeLa

cells have found poly A sequences in certain RNA species, including hnRNA. Edmonds and Caramela (1969), Edmonds et al (1971) and Sullivan and Roberts (1973) have shown that these poly A sequences are about 200 nucleotides long, more than 90% adenylate in composition and account for about 0.5% of the hnRNA. The poly A sequences are located at the 3′ end of the RNA chain and appear to be added after transcription of the RNA, but before the molecules are cleaved to yield mRNA (Kates, 1970; Edmonds et al, 1971; Darnell et al, 1971a and 1971b; Mendecki et al, 1972).

Molloy et al (1972) have found poly U sequences about 30 nucleotides in length and about 80% uridylate in composition in HeLa cell hnRNA. Whether these sequences are at the 3′ end of the RNA chain or not is not known. Both the poly A and poly U sequences appear to occur in the reiterated portion of the hnRNA.

Although melting experiments suggest that giant hnRNA has considerable structure in solution; Ryskov et al (1972), Jelinekand Darnell (1972) and Konenberg and Humphreys (1972) have demonstrated the existence of only a relatively small percentage of ribonuclease-stable material in hnRNA. This is thought to be intramolecularly base-paired. Jelinek and Darnell (1972) observed that the double-stranded regions made up about 3% of the hnRNA molecules. The ribonuclease-stable regions like the poly U and poly A sequences occurred in the reiterated part of the hnRNA. Thus the ribonuclease-stable regions are possibly partly made up of base-pairing between poly A and poly U sequences. Ryskov et al (1972) observed a difference between the ribonuclease-stable, or double-stranded regions, in hnRNA with large and small sedimentation coefficients. They found that the smaller hnRNA species are about 2.5% double-stranded apparently

consisting of poly A, whilst the larger hnRNA species are less than 1% double-stranded with few true poly A sequences. Kronenberg and Humphreys (1972), however, found only about 0.3% of the hnRNA was double-stranded and these sequences were associated with poly A sequences. The differences in the quantity of ribonuclease-stable regions of hnRNA obtained by these workers could be due to a variety of factors, such as the different cells and different ribonuclease incubation conditions used if the base-paired regions are imperfectly matched.

However, there is no evidence that these double-stranded regions in fact exist inside the cell. Combination with protein or other restraints on free association of the different regions of the polynucleotide chains may prevent any secondary structure from forming in vivo.

The outstanding feature of hnRNA, even in the same system, is the extreme variability of the sizes ascribed to it by different workers. This variability could be due to many possible effects. Firstly, differences in labelling times, conditions of labelling, choice of precursor or the metabolic state of the cell can all affect the type of hnRNA labelled. Another explanation lies in the different extraction procedures used as hnRNA appears to be particularly susceptible to aggregation by contaminating DNA, protein (Bramwell and Harris, 1967) and divalent ions. All methods of preparing hnRNA species should attempt to remove all traces of protein from the RNA preparation as this reduces the risk of degradation by endogeneous nucleases. Thirdly, the ionic or denaturing conditions used in the analysis of hnRNA can cause extreme variation in its apparent size.

Amongst some of the early sedimentation patterns obtained of hnRNA in animal cells are those of hnRNA sedimenting in sucrose gradients with the rRNA species and partly more rapidly than these species (Hiatt 1962; Perry, 1962) and hnRNA sedimenting more rapidly than the rRNA and up to about 50 S (Scherrer and Darnell, 1962; Scherrer et al, 1963; Brown and Gurdon, 1964; Rake and Graham, 1964; Yoshikawa-Fukada et al (1965). Although some of this large RNA is probably a direct precursor of rRNA (Perry, 1962; Scherrer et al, 1963), much of the rapidly labelled RNA has a base ratio similar to that of DNA and is probably true hnRNA (Scherrer et al, 1963; Yoshikawa-Fukada et al, 1965).

The identification of hnRNA has proved difficult due to the overlapping of this RNA with the other rapidly labelled RNA consisting of rRNA precursors. Attardi et al (1966) and Scherrer et al (1966) reduced this problem by using immature duck erythrocytes undergoing differentiation, which have a greatly reduced level of rRNA sythesis. They found much more polydisperse hnRNA, than previously found in other systems, having a continuous range of sedimentation coefficients from about 10 S to 80 S (Attardi et al, 1966) and up to 60 S (Scherrer et al, 1966). Houssais and Attardi (1966) have also obtained very large hnRNA molecules sedimenting at ranges between 10 S and more than 100 S in exponentially-growing HeLa cells, showing that giant hnRNA is not only found in differentiated cells like duck erythrocytes.

Marked variation in size can be brought about by changing the physical conditions. Bramwell and Harris (1967) have shown that under normal conditions, labelled HeLa cell hnRNA shows extreme heterogeneity, with much of it sedimenting more rapidly than 28 S in sucrose gradients. However,

at low ionic concentrations and after removal of Mg^{2+} with EDTA, the hnRNA sediments as a single peak at about 16 S. Bramwell (1972) has further shown that HeLa cell hnRNA denatured in low ionic conditions, can under normal ionic conditions again behave as high molecular weight RNA and give a pattern virtually identical to untreated hnRNA. These results suggest that hnRNA exists as aggregates of much smaller molecules.

Aronson (1972) has shown that the hnRNA of sea urchin embryos exists in the form of very large aggregates, which is possibly due to an association of the hnRNA with chromatin and/or membrane fragments.

Tamaoki and Lane (1967), using mild nuclease treatment on pulse-labelled RNA from L cells, also suggest that hnRNA is not synthesised as giant molecules which are then cut to size, but as shorter chains which are linked together to form polymeric structures. Riley (1969), also using mild nuclease treatment, obtains similar results with hnRNA of E. coli and HeLa cells.

The use of denaturants has been little used in the determination of the state of aggregation of hnRNAs and workers using denaturing conditions have obtained conflicting results. de Kloet et al (1970), Mayo and de Kloet (1971) and Peltz (1973) using formaldehyde treated hnRNA and Scott and Kuhns (1972) using DMSO treated mouse tumour cell hnRNA have shown that hnRNA denatures or 'disaggregates' into much smaller molecules. However, Holmes and Bonner (1973) find that rat ascites hnRNA does not denature under formaldehyde and DMSO denaturing conditions and, using partially denaturing urea conditions, Granboulan and Scherrer (1969) have shown in the electron microscope hnRNA molecules with high molecular

weights in the range of 5 - 10 x 10^6 daltons.

The actual size and state of heterogeneity of hnRNA is thus open to considerable debate. There are two main schools of thought concerning the size of this RNA. Scherrer et al (1966), Houssais and Attardi (1966), Warner et al (1966) and Holmes and Bonner (1973) claim that hnRNA is a mixture of covalently bound molecules of very high molecular weight; whilst Parish and Kirby (1966), Bramwell and Harris (1967) and de Kloet et al (1970) consider that the molecular weight of hnRNA molecules is much less than supposed, due to aggregations between hnRNA molecules which are possibly due to preparative artifacts.

The discrepancy between these two viewpoints is difficult to explain, although in different systems the absolute structure of hnRNA molecules may be different. Some other workers such as Fujisawa and Muramatsu (1968) consider the actual situation lies between these extremes.

e) Messenger RNA.

Cytoplasmic mRNAs have a heterogeneous size of 8 to 30 S, a DNA-like base composition, are associated with polysomes and are rapidly labelled. The properties of mRNAs and their relationship to other cellular RNAs have been reviewed by Darnell (1968). Individual mRNAs, after isolation on sucrose gradients or polyacrylamide gels, have been identified by their ability to direct the synthesis of a particular protein in a cell free system (Laycock and Hunt, 1969; Lockard and Lingrel, 1969; Pemberton et al, 1972) or in Xenopus oocytes (Gurdon et al, 1971; Lane et al, 1971).

Generally, these mRNAs have unexpectedly large sizes for the protein

sequences they code for. For example, Gaskill and Kabat (1971) have determined that the mRNA for rabbit haemoglobin has about 650 nucleotides, of which only about 440 nucleotides would be expected to code for the haemoglobin chain.

Darnell et al (1971a), Edmonds et al (1971) and Lee et al (1971) have shown, by digestion with nuclease, the presence of poly A sequences in mRNAs. These poly A sequences are up to 200 nucleotides in length and more than 90% adenylate. As, so far, poly A sequences have been identified in all eukaryotic mRNAs except histone mRNAs (Adesnik et al, 1972), it is probable that these poly A sequences and the other additional nucleotide sequences perform a regulatory function in protein synthesis.

f) Relationship between heterogeneous nuclear RNA and messenger RNA.

Although hnRNAs have some properties in common with cytoplasmic mRNAs, such as their similar GC content and their extreme heterogeneity, there are many quite striking differences between these RNA species. As we have seen hnRNA ranges in sedimentation coefficient from about 10 S to greater than 200 S, although the hnRNA may be an aggregate of smaller molecules. The cytoplasmic mRNA is much smaller in size, ranging from 8 to 30 S. Whilst mRNA is found on the polyribosomes in the cytoplasm, hnRNA is confined to the nucleus where no protein synthesising structures have been found after fractionation. Reports of haemoglobin synthesis inside the nucleus have been recorded (O'Brien, 1960; Hammel and Bessman, 1964), but these are probably due to cytoplasmic contamination of nuclear material. The rate of labelling of the majority of hnRNA is much greater than that of cytoplasmic mRNA. From studies on the formation and turnover of hnRNA, Attardi et al (1966) and Penman et al (1968) suggested that the

two species could not easily be regarded as having a precursor-product relationship. However, Penman et al (1968) showed that on labelling HeLa cells there was approximately a 15 minute delay until linear emergence of mRNA into the cytoplasm occurred, which suggests the existence of a pool probably located in the nucleus. This interpretation is further strengthened as the emergence of mRNA continued for approximately 15 minutes following the addition of high concentrations of actinomycin D.

Evidence is now accumulating which indicates that mRNA is related to hnRNA. Although, the majority of hnRNA molecules turn over in the nucleus and thus can not be mRNA precursors, this does not necessarily exclude the possibility that mRNA molecules are derived from a fraction of metabolically active hnRNA. A satisfactory test of this possibility requires some chemical tracer to identify the same sequences in the two classes of RNA.

Such a chemical tracer is available in the form of virus-specific nuclear and cytoplasmic RNA species. Cells transformed by small DNA viruses contain viral DNA, covalently integrated into cellular DNA, which is the template for synthesis of virus specific mRNA, (Benjamin, 1966; Sambrook et al, 1968). In the nucleus, the virus-specific RNA is part of large hnRNA molecules, but in the cytoplasm the molecules are smaller and uniform in size (Wagner and Roizmann, 1969; Lindberg and Darnell, 1970; Acheson et al, 1971). These results suggest that virus-specific mRNA in transformed cells is derived from hnRNA.

Several speculative models have been suggested for the structure of the hnRNA transcriptional unit and its relationship to the cytoplasmic RNAs. Generally, these models postulate that the hnRNA molecules are transcribed as large molecules (Georgiev, 1969; Darnell et al, 1971b)

which are processed to form the cytoplasmic mRNAs (Scherrer et al, 1966) by analogy with the nucleolar precursor to rRNA (Scherrer et al, 1963; Weinberg et al, 1967).

Georgiev (1969) has postulated that the transcription unit in eukaryotes consists of two kinds of sequences, namely, acceptor cistrons mainly located near the promoter and structural cistrons located distal to the promoter. The acceptor cistrons would contain operators with which regulatory proteins could interact and probably also terminators, sequences recognised by specific ribonucleases and protectors. If the RNA polymerase does not meet obstacles on its way along the template, the transcription of both informative (mainly in the 3' part) and non-informative (mainly in the 5' part) sequences takes place. It is further suggested that the non-informative part of the hnRNA is degraded and the informative part transferred into the cytoplasm. Results obtained by Georgiev et al (1972) are in good agreement with this model.

Darnell et al (1971b) further suggest that after the transcription of giant hnRNA molecules the attachment of poly A sequences, probably at the terminus of the hnRNA molecule, occurs. The attachment is apparently required for the processing of the hnRNA molecule. One or more nucleases recognise and cleave off the mRNA sequence plus poly A unit and then destroy the remainder of the hnRNA or, simply destroy all the hnRNA except for this unit. The mRNA sequence could be contained in a somewhat larger unit than the cytoplasmic mRNA molecule in order to protect it on its passage from the nucleus to the cytoplasm.

The relationship between hnRNA and mRNA has been investigated using competition - hybridisation to DNA (Birnboim et al, 1967; Shearer and

McCarthy, 1967; Soeiro and Darnell, 1970). The results show a certain amount of similarity between the two types of molecule. However, Melli and Pemberton (1972) suggest that the conditions used in most of these experiments allowed only the hybridisation of the reiterated fraction of DNA or RNA. It is possible therefore that the competing sequences are similar but not identical and in any case they may represent only a small fraction of the total sequences. Scherrer et al (1970) using conditions which might allow the hybridisation of some of the slow sequences of DNA and which would give meaningful competition, conclude that there is a precursor-product relationship between hnRNA and cytoplasmic mRNA. Melli and Pemberton (1972) have approached this hybridisation problem by using a method which overcomes the difficulty of hybridising RNA to the extremely complex DNA of higher organisms. They synthesised a labelled RNA complementary to the haemoglobin mRNA (antimessenger) of duck erythrocytes using Micrococcus lysodeikticus RNA polymerase and then studied the annealing of this molecule back to RNAs which might contain messenger sequences. Although their results suggested the presence of sequences complementary to the antimessenger in the hnRNA of duck erythrocytes, the hnRNA they used could have contained cytoplasmic mRNA as a contaminant.

Poly A sequences have been found not only in the hnRNA (Darnell et al, 1971a; Edmonds et al, 1971) but also in rapidly labelled polyribosomal RNA, presumably mRNA (Darnell et al, 1971a; Edmonds et al, 1971; Lee et al, 1971). The presence of these sequences in both hnRNA and mRNA molecules has been used to determine if at least some of the mRNA arises from specific processing of hnRNA. The experiments of Edmonds and Caramela (1969) and Edmonds et al (1971) are quite suggestive in this respect because they have shown that the adenylate-rich sequences from hnRNA and

mRNA of HeLa cells are approximately equal in size and in base composition (more than 90% adenylate in both cases). However, Sheiness and Darnell (1973) have shown that nuclear poly A sequences are in fact longer than cytoplasmic poly A sequences, which become shorter with age.

Penman et al (1970) have described for HeLa cells a reduction in the appearance of mRNA labelled with uridine in polysomes of cells treated with cordycepin (3'-deoxyadenosine), although this drug had little effect on incorporation of uridine into hnRNA. Darnell et al (1971b) labelled HeLa cells with labelled adenosine with and without prior treatment with cordycepin, which blocks the synthesis of poly A sequences, then prepared the hnRNA and mRNA from the cells and assayed for poly A in the RNA species. From the results of these experiments, they concluded that the poly A in hnRNA becomes labelled before that in mRNA and this labelling occurs on recently formed molecules without further DNA-mediated transcription. Moreover, the blockage of poly A addition by cordycepin may explain the decrease in synthesis of mRNA caused by the drug.

Gurdon et al (1971) have developed techniques which make it possible to identify very small amounts of mRNA by microinjection into Xenopus oocytes, in which exogenous mRNA is translated with very high efficiency. These techniques have been used by Stevens and Williamson (1972) to detect immunoglobulin sequences in the hnRNA of myeloma cells and by Williamson et al (1973) to detect haemoglobin mRNA sequences in the hnRNA of 14 day embryonic mouse liver. Furthermore, Stevens and Williamson (1973) have shown that a short-lived nuclear pre-mRNA isolated from myeloma cells codes for the immunoglobulin heavy chain on its injection into oocytes. However, Lane et al (1973) have criticised the experiments of Stevens and Williamson (1972) and Williamson et al (1973) on the grounds that the

hnRNA injected into the oocytes could have contained mature cytoplasmic mRNA as a contaminant and that the nuclei may normally contain functional mRNA (O'Malley et al, 1972).

The hybridisation techniques have been improved by Imaizuma et al (1973) who transcribed haemoglobin mRNA from ducks with RNA-directed DNA polymerase from oncornaviruses into anti-messenger DNA. They found this DNA could be annealed with hnRNA forming a relatively stable hybrid and calculated that 0.3-1.5% of the hnRNA consisted of haemoglobin mRNA sequences.

Although most of the simple hybridisation experiments discussed above are not precise enough to produce any firm conclusions concerning possible hnRNA-mRNA relationships; the experiments of Stevens and Williamson (1972 and 1973) and Williamson et al (1973) involving the microinjection of hnRNA into oocytes in order to detect the synthesis of specific proteins, and the experiments of Imaizuma et al (1973) hybridising DNA complementary to specific mRNAs with hnRNA, are in principle capable of resolving this problem. However, it must be stressed that even these experiments are not conclusive as there is always the problem of isolating hnRNA which is entirely free of contaminating cytoplasmic mRNAs.

g) Approach used in this thesis

The patterns of hnRNA and cytoplasmic mRNA synthesis were analysed in immature duck erythrocytes undergoing differentiation in order to find some relationship between them. Immature erythyocytes are produced in the peripheral blood of the duck during phenylhydrazine induced anaemia. These cells are particularly favourable for the detection and study of non-

ribosomal components due to the greatly reduced level of rRNA synthesis, and to restriction in the spectrum of genetic information expressed during their differentiation (Attardi et al, 1966; Scherrer et al, 1966). In fact, at the maximum state of anaemia, these cells produce about 80-90% of their protein in the form of haemoglobin (Scherrer et al, 1966).

The size and heterogeneity of hnRNA in anaemic duck erythrocytes was determined in various denaturing conditions in order to determine the actual size of this RNA species. The interrelationships between cytoplasmic haemoglobin mRNA and hnRNA were then studied by hybridisation of these RNA species with poly U and DNA complementary to haemoglobin mRNA.

MATERIALS AND METHODS

a) Materials

1) Buffers and solutions

Bovine serum albumen. Albumen (fraction V) from bovine serum was dissolved at a concentration of 5 mg/ml in 100 mls distilled water.

Butyl PBD - toluene 2 - ethoxycthanol counting fluid. Butyl PBD - toluene was made up by dissolving 8 g of butyl PBD in 1 litre of analar toluene. The counting fluid was made up with 600 mls of butyl PBD - toluene plus 400 mls of 2 - ethoxyethanol.

Column buffer. 0.3 M NaCl, 0.01 M sodium acetate pH 5.0.

NKM. 0.13 M NaCl, 0.005 M KCL, 0.0075 M Mg Cl_2.

NTE. 0.1 M NaCl, 0.01 M tris, 2mM EDTA.

Nuclease S_1. Nuclease S_1 was prepared from Takadiastase powder and assayed for its nuclease activity as described by Sutton (1971).

Pancreatic RNase. 5 times crystallised RNase A from bovine pancrease was dissolved in 2 X SSC pH 4.5 at 10 mg/ml and boiled for 5 minutes in a boiling water bath to inactivate any contaminating DNase. The solution was chilled and the pH adjusted to 7.5 with 0.1 M NaOH.

Redistilled formamide. Formamide was redistilled under reduced pressure twice

to decrease the amount of ultra-violet absorbing contaminants.

SSC. 0.15 M NaCl, 0.015 tri-sodium citrate. The pH was adjusted as required with concentrated HCl.

TE. 0.01 M tris, 2 mM EDTA.

TK. 0.05 M tris (pH 7.5), 0.03 M KCl.

TKM. 0.05 M tris (pH 7.5), 0.03 M KCl, 0.002 M $MgCl_2$.

Toluene - PPO POPOP counting fluid. Contains 3g PPO (2, 5 - diphenyloxazole) scintillation grade and 300 mg dimethyl POPOP (1, 4 - bis - 2 - (4 - methyl - 5 - phenyloxazolyl) - benzene) scintillation grade in 1 litre of analar toluene.

2) Source of materials

Except for those detailed below, all materials were obtained from British Drug Houses Ltd.

Bio-Rad. Chelex 100.

Eastman. Tri-iso- propylnaphthalene sulphonic acid sodium salt.

Falcon Plastics. 250 ml Falcon plastic flasks.

Flow Laboratory. Minimum essential medium for suspension cultures, glutamine.

Intertechnique. Butyl PBD, plastic scintillation vials.

Packard. Dimethyl POPOP (1, 4 bis - 2 - (4 - methyl - 5 - phenyloxazolyl) - benzene), POP (2, 5 - diphenyloxazole).

Pharmacia. Dextran sulphate, sephadex SPC - 50.

Radiochemical Centre, Amersham. All radioisotopes.

Sartorius. Nitrocellulose membrane filters SM 11306.

Sigma. RNase, tris.

Whatman. 2.5 cm glass fibre discs GF/83.

b) Phenylhydrazine treatment and bleeding of ducks.

Female Aylesbury ducks were made anaemic by phenylhydrazine treatment. They were injected intramuscularly with 1 ml of neutralised 1% phenyhydrazine hydrochloride on each of 5 to 6 successive days, and used one day after the last injection. The ducks were bled through the heart with 0.1% heparin in NKM as anticoagulant. Usually about 60 mls of blood were taken from a duck at each bleeding. The blood cells were spun down in the Sorvall at 2,500 rpm for 5 minutes and then washed by centifruging them 3 times in NKM (10,000 rpm, 10 minutes). In some cases the cells were lysed directly to extract unlabelled RNA or stored at -20°C until required. In other cases they were first labelled by incubation in vitro with ^{3}H uridine to obtain labelled RNA.

c) Incubation of anaemic duck blood cells with ^{3}H uridine.

1 to 2 mls of packed duck blood cells were incubated in sterile conditions with 10 mls of MEMS, supplemented with non-essential amino acids and 2 mM glutamine; and containing 50 μg/ml streptomycin sulphate, 30 μg/ml penicillin, 10^{-4}M ferrous ammonium sulphate, 10^{-2}M nucleosides, 1.5% $NaHCO_3$, 1 ml of dialysed duck serum and 300 μc ^{3}H uridine. The incubation was done in 250 ml Falcon plastic flasks for 2 hours at 37°C in an atmosphere of 5% CO_2 in air. Figure 1 shows a typical ^{3}H uridine incorporation pattern into RNA with time. The incubation was stopped by addition of cold NKM.

d) Prevention of RNA degradation.

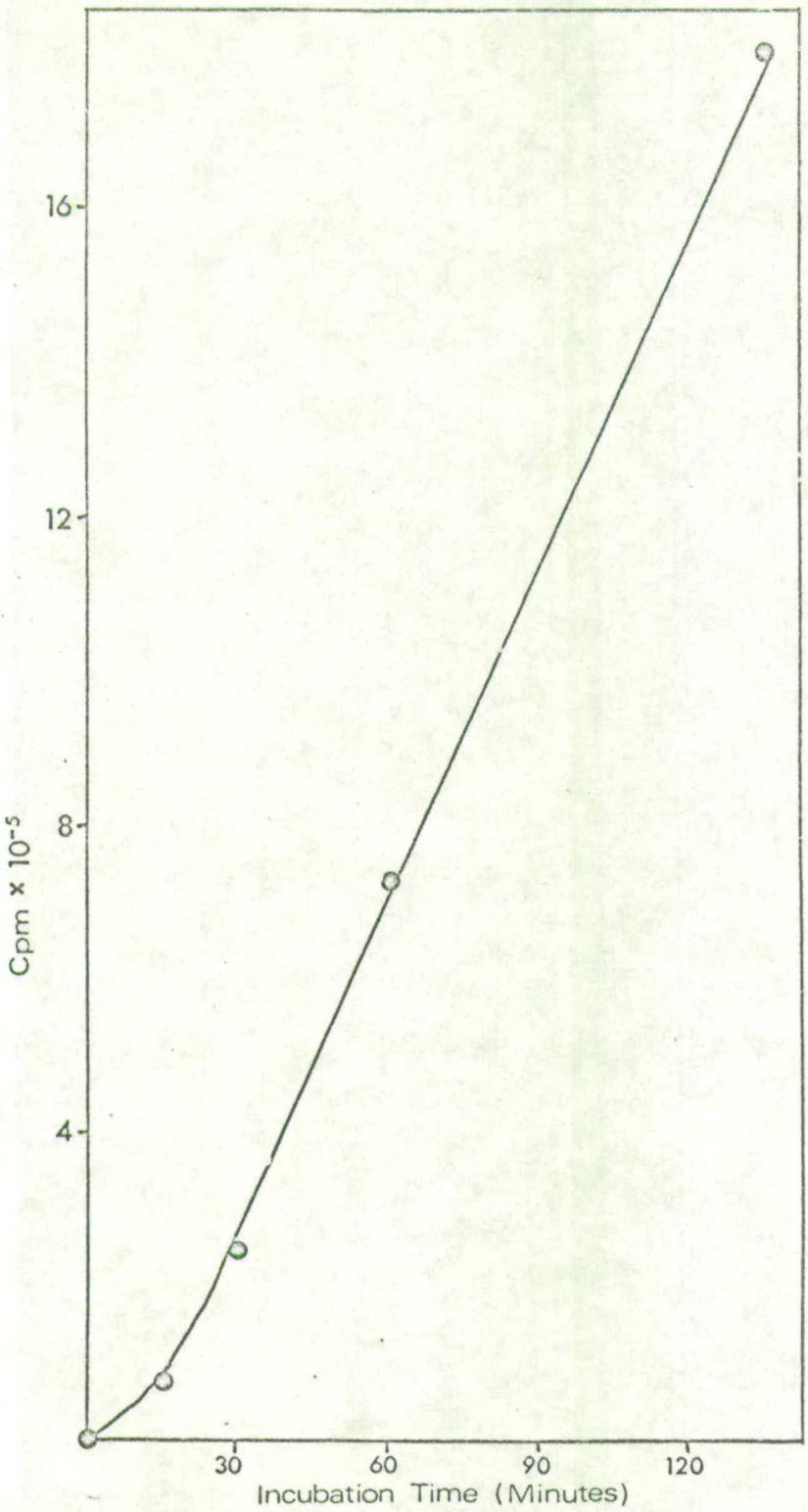
Nucleic acids are susceptible to the action of nucleases and thermal degradation. DNAs are protected to a certain extent from such degradation by virtue of their stable double-helical structure, whilst RNAs are much more susceptible as their polynucleotide chains have long single-stranded regions. As the determination of the absolute sizes of RNAs was important in this work, special precautions were taken during the preparation and analysis of RNAs in order to minimise these problems. All solutions, glassware and equipment were sterilised wherever possible before use. During the preparation and analysis of RNAs special care was taken to ensure that the RNA containing solutions were kept cold in an ice bath to minimise thermal or nucleolytic degradation, unless the RNA was in solutions containing SLS (a protein inhibitor) in which case the solutions were kept at room temperature. The effectiveness of these measures to prevent RNA degradation is discussed in the Discussion.

Legend to figure 1

Incorporation with time of ^{3}H uridine into the RNA of duck erythrocytes.

At selected times during incubation of duck erythrocytes with 300 μc ^{3}H uridine, 100 λ samples were removed from an incubation flask containing 10 mls of incubation mix and 2 mls of packed cells, in order to follow the incorporation of labelled uridine into RNA with time. The samples were put on to 2·5 cm paper discs, dried, and washed thoroughly with 3 changes of 5% TCA. Then, the discs were washed with absolute alcohol and ether, dried, and counted in scintillation vials containing 15 mls toluene - POP POPOP counting fluid in a Packard Tri Carb liquid scintillation counter.

FIGURE 1



e) Preparation of nuclear RNA.

To lyse the cell membrane of blood cells, 4 volumes of cold TK, 50 µg/ml dextran sulphate, were added to the packed blood cells with stirring for at least 3 minutes. 0.6 volumes of cold 2 M sucrose in TK was added to stop the lysis of the cell membrane and the suspension was stirred. The nuclei from the lysed cells were pelleted by centrifuging them in the cold in the Sorvall (10,000 rpm, 10 minutes). The supernatant, containing the cytoplasmic fraction, was kept and used to prepare cytoplasmic RNA.

The nuclear pellet was resuspended in an equal volume of cold TKM, and then contrifuged in the Sorvall (10,000 rpm, 10 minutes). The pellet was then resuspended in an equal volume of cold 0.5% Tween 40 and left in ice for 15 minutes before being centrifuged (10,000 rpm, 10 minutes) in the Sorvall. The nuclear pellet was then washed again with an equal volume of cold TKM (10,000 rpm, 10 minutes). Next, the pellet was suspended in 3 volumes of cold TKM and 1 volume of cold sodium deoxycholate (1 part), 10% Tween 40 (2 parts). The suspension was homogenised by hand, then centrifuged (10,000 rpm, 10 minutes).

10 volumes of 0.5% SLS, 6% p-amino salicylate, 1% NaCl, 1% tri-iso propylnaphthalene sulphonate, 6.6% water saturated phenol were added to the pellet and the mixture blended until the viscosity dropped. An equal volume of water saturated phenol : chloroform (1 : 1) was added and the mixture shaken for 30 minutes. Then the mixture was centrifuged (10,000 rpm, 20 minutes) and the aqueous phase carefully removed with a Pasteur pipette.

The phenol phase and interphase were re-extracted with 5 volumes of

0.5% SLS, 6% p-amino salicylate, 1% NaCl, 1% tri-isopropylnaphthalene sulphonate, 6.6% water saturated phenol and shaken for 30 minutes, then centrifuged (10,000 rpm, 20 minutes). The aqueous phase was removed and pooled with the first aqueous phase.

The RNA was precipitated from the aqueous phase by the addition of 1/10th volume 0.1 M sodium acetate pH 5.0 and 2 volumes of absolute alcohol : m-cresol (9 : 1) and stored at 4°C for at least 12 hours.

The RNA precipitate was spun down from the alcohol (10,000 rpm, 10 minutes), then resuspended in 1/10th volume 0.1 M sodium acetate pH 5.0 and 2 volumes absolute alcohol : m-cresol (9 : 1) were added. This was homogenised by hand and then centrifuged (10,000 rpm, 10 minutes). The nuclear pellet was resuspended in 3 M sodium acetate (pre-treated with 0.01% diethyl pyrocarbonate to inhibit nuclease action), homogenised by hand, then left in ice for 15 minutes. The RNA pellet was spun down (10,000 rpm, 10 minutes) and the DNA supernatant discarded.

The procedure, described in the previous paragraph, was repeated twice. Then the pellet was washed once with absolute alcohol (10,000 rpm, 10 minutes) and stored under absolute alcohol at -20°C until required.

f) Preparation of cytoplasmic RNA using phenol.

The supernatant, kept from the lysis of the cells from the preparation of nuclear RNA was used to extract cytoplasmic RNA. The usual method of extracting cytoplasmic RNA was by phenol extraction.

Four volumes of 0.5% SLS, 6% p amino salicylate, 1% NaCl, 1%

tri-isopropylnaphthalene sulphonate, 6.6% water saturated phenol were added to this supernatant, together with an equal volume of water saturated phenol : chloroform (1 :1). This mixture was shaken for 30 minutes, then centrifuged (10,000 rpm, 20 minutes). The aqueous phase was carefully removed by pipetting with a Pasteur pipette and re-extracted with phenol : chloroform as above. The aqueous phase was again collected with a Pasteur pipette. The RNA was precipitated from this phase by the addition of 1/10th volume 0.1 M Na acetate pH 5.0 and two volumes of absolute alcohol : m-cresol (9 : 1) and stored at 4°C for at least 12 hours.

The RNA precipitate was pelleted from the alcohol (10,000 rpm, 10 minutes). The pellet was resuspended in 1/10th volume 0.1 M sodium acetate pH 5.0 and two volumes of absolute alcohol : m cresol (9 ; 1) were added. This was left in ice for 30 minutes, then centrifuged (10,000rpm, 10 minutes). This step was repeated twice and the RNA was then washed once with absolute alcohol (10,000 rpm, 10 minutes). The RNA was stored under alcohol at -20°C.

g) Preparation of cytoplasmic RNA using proteinase K.

RNA was also extracted from the cytoplasmic fraction, obtained from the lysis of the blood cells during the preparation of nuclear RNA, using proteinase K (Mach et al, 1973)

For each 100 optical density units at 260 nm of RNA, 2 mls of TK containing 0.5% SLS were added together with 400 µg of proteinase K at 0°C. The proteinase K and pellet dissolved simultaneously. Incubation at 0°C produces a decrease in RNase action but allows proteinase K action

to continue. After 10 minutes incubation at 0°C the solution was incubated at 25°C for 30 minutes. Then the solution was made 0.1 M with sodium acetate pH 5.0, two volumes of absolute alcohol : m-cresol (9 : 1) were added and the RNA was precipitated from alcohol after 12 hours at 4°C.

h) Oligo dT-cellulose columns.

Oligo dT-cellulose has been shown to selectively bind poly A sequences (Edmonds and Caramela, 1969; Sheldon et al, 1972; Sullivan and Roberts, 1973) and oligo dT-cellulose columns were used to purify or identify RNA containing poly A sequences. 120 mg oligo dT-cellulose, a gift from Dr J.O. Bishop, were used in columns 2 cms in length by 0.45 cms diameter. Before use columns were washed at the rate of 2 mls per minute with the following : 5 X1 mls water, 2 X 2 mls 0.1 N NaOH, 5 X 2 mls water, 5 X 1 mls binding buffer (10% glycerol, 0.1% SLS, 1mM EDTA, 0.01 M tris pH 7.6, 0.4 M NaCl). The RNA loaded on to a column was suspended in binding buffer and absorbed on to the column at the rate of 1 ml per 2 minutes. 1 ml fractions of the buffer were collected. The column was then washed with 5 X 1 ml fractions of binding buffer at the rate of 1 ml per minute. The column bound RNA sequences were eluted at the rate of 1 ml per 2 minutes in eluting buffer (0.1% SLS, 1 mM EDTA, 0.01 M tris pH 7.6) in 8 X 0.5 mlfractions. All the fractions were kept and aliquots from the fractions analysed.

i) Melting curves of rRNA.

3.0 ml samples of rRNA solutions containing about 40 µg/ml of RNA (about 1 OD at 260 nm) were placed in quartz cuvettes with a 10 mm light path. The solution used to suspend the RNA was varied for each experiment. The melting experiments were performed in a Unicam SP 800 spectrophotometer

fitted with an SP 825 sample changer and a heat block controlled by an SP 876 temperature programme controller. The cuvettes were placed in the block and the ultra violet absorption spectrum of each sample recorded from 320 to 230 nm at room temperature. The spectrum of each sample was retaken after a 5 minute interval to ensure than an accurate spectrum had been taken. Then the temperature of the heating block was raised by 5 or 6°C using the SP 876 temperature programme controller and again two spectra were taken for each sample. This procedure was repeated until a whole melt had been obtained for each sample.

The percentage absorbance of RNA at a given temperature was calculated as the extinction at 260 nm at that temperature, expressed as a percentage of the extinction at 260 nm for totally denatured RNA. Similarly, the percentage denaturation of RNA at a given temperature was calculated as the increase in absorbance at 260 nm at that temperature from its native value, expressed as a percentage of the extinction at 260 nm of totally denatured RNA. The melting curve of the RNA was obtained by plotting either the percentage absorbance or the percentage denaturation of the RNA against temperature in °C.

Cooling curves of rRNA samples (optical renaturation) were obtained in a similar way to the melting curves.

j) Sucrose gradient centrifugation and analysis

A sample of each RNA preparation to be analysed was carefully layered on to the appropriate neutral linear sucrose gradient. The different sucrose gradients used were a) 5-30% sucrose, NTE, spun at 1°C and called 'cold sucrose' gradients; b) 5-30% sucrose, 0.5% SLS, NTE or 15-30% sucrose, 0.5% SLS, NTE, spun at 25°C and called 'sucrose-SLS'

gradients; c) 5-30% sucrose, 8 M urea, NTE, spun at 25°C and called 'urea' gradients; d) 2-10% sucrose, 85% formamide, TE, spun at 25°C and called 'formamide' gradients. Specially pure SLS, urea and redistilled formamide were used for the gradients. In all cases the gradients were spun at 24K in the 6 X 16 rotor of the MSE superspeed 65 centrifuge. 16 ml nitro-cellulose centrifuge tubes were used for the 'cold sucrose', 'sucrose-SLS' and 'urea' gradients. 14 ml polyallomer centrifuge tubes were used for the 'formamide' gradients.

The gradients were fractionated by upward displacement of the gradient with 50% sucrose pumped at a constant speed through an LKB peristaltic pump. The ultraviolet absorption at 257 nm of the gradients were read automatically on passage through an Isco uv optical unit connected to an Isco uv flow analyser. Gradient fractions were collected automatically and optical density traces of the gradients obtained using a Bryans chart recorder.

However, the optical density traces obtained for 'formamide' gradients by this method had considerable background absorbance, as at 257 nm even redistilled formamide has considerable absorbance (Helmkamp and Ts'o, 1961). So to obtain an optical density trace for formamide gradients, suitable dilutions of aliquots of each gradient fraction were read manually in a Beckman spectrophotometer against a blank containing the same quantity of formamide as the sample.

The sedimentation coefficients of the various RNA components in sucrose gradients were estimated on the basis of their relative migrations from the meniscus using duck rRNA as a marker. Due to the secondary structure of most RNAs, sedimentation coefficients of RNAs in sucrose gradients can vary slightly, and can thus only be considered to be

approximations. For example, Pemberton and Baglioni (1972) have found that the sedimentation coefficients of duck rRNAs are 26 S and 18 S, whilst Attardi et al (1966) and Scherrer et al (1966) suggest they are 28 S and 18 S. Similarly, there is also a variation ~~of~~ between 9 and 10 S in the sedimentation coefficient assigned to duck haemoglobin mRNA in sucrose gradients.

For sedimentation coefficients to have any significance it was important to check that all the gradients were in fact linear. This was done by measuring the refractive index of aliquots from each gradient fraction in a refractometer and plotting refractive index against gradient fraction. A straight line plot indicated the gradients were linear.

The sedimentation coefficient of the large HeLa rRNA subunit has been accurately determined as 28 S from polyacylamide gel electrophoresis (Weinberg et al, 1967) and this value was used to calibrate the sedimentation coefficients of the duck rRNA species. Figure 2 shows the pattern of unlabelled duck rRNA and labelled HeLa 28 S rRNA (gift from John Morton) on a 'sucrose-SLS' gradient. The sedimentation coefficients obtained for the duck rRNA species were about 18 S and 27 S and these values were used to calibrate all other RNAs in sucrose gradients. In all the sucrose gradients run, a marker RNA of known sedimentation coefficient was run in parallel. The marker RNA used was usually duck rRNA.

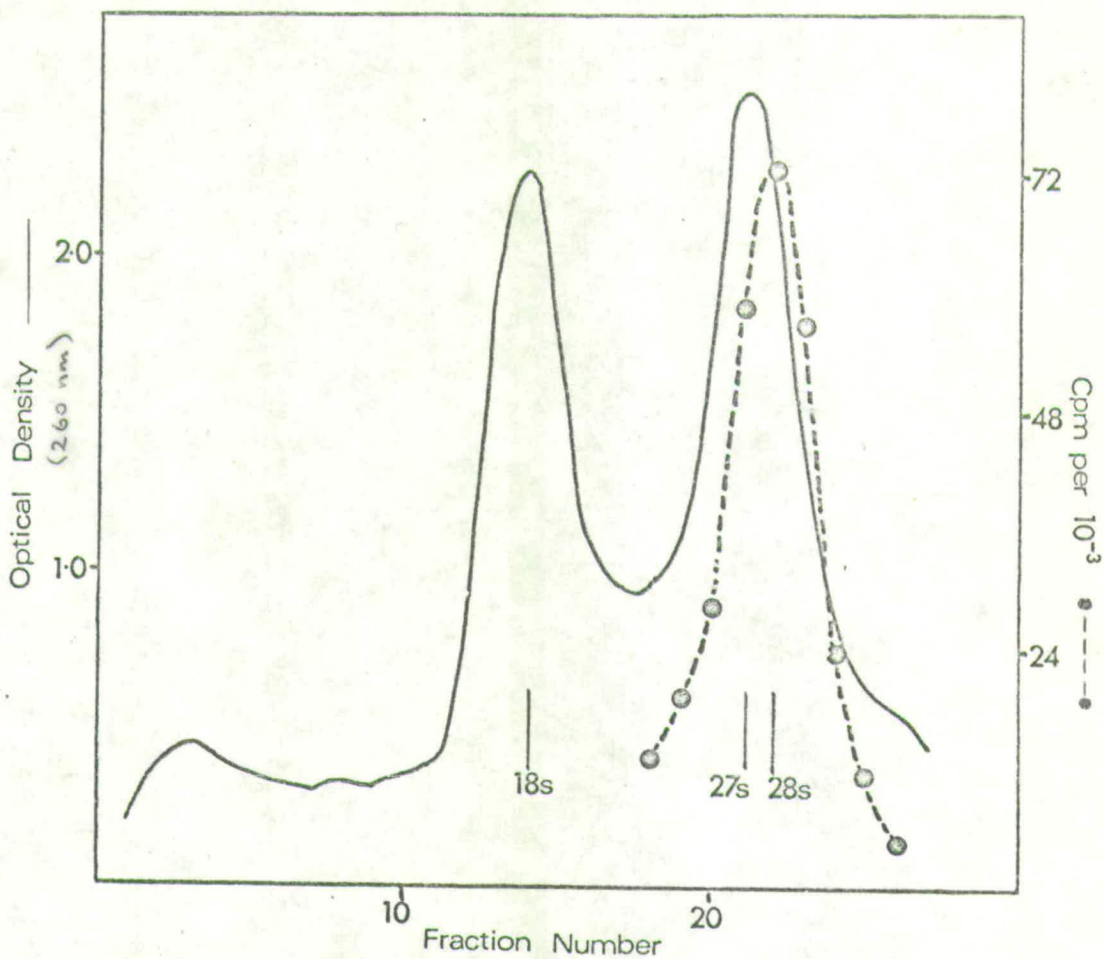
The radioactivity of an RNA species in a gradient was determined as follows. 50 λ samples from each gradient fraction were pipetted into tubes containing 1 ml 1 X SSC in an ice bath. The RNA species were precipitated by the addition of 0.25 mls 50% TCA with 50 λ BSA as a carrier. After shaking the tubes thoroughly they were left in an ice bath for 20 minutes. The samples were collected on 2.5 cm GF/83 filters, which were dried under

Legend to figure 2

Unlabelled duck rRNA and labelled HeLa 28 S rRNA run in a 'sucrose - SLS' gradient.

0.5 mgs unlabelled duck cytoplasmic RNA (consisting largely of rRNA) and ^{3}H uridine labelled HeLa 28 S rRNA were spun in a 16 ml 5-30% sucrose, 0.5% SLS, NTE gradient for 15.0 hours at 25°C, 24 K. 50 λ aliquots from the appropriate gradient fractions were counted.

FIGURE 2


Optical Density (260 nm)
2·0
1·0
72
48
24
Cpm per 10^{-3}
18s
27s
28s
10
20
Fraction Number

reduced pressure at 80°C for 20 minutes. The filters were counted in scintillation vials containing 15 mls toluene - PPO POPOP counting fluid in a Packard Tri Carb liquid scintillation counter. However, samples from gradients containing SLS were collected on 2.5 cm Millipore filters and counted in scintillation vials containing 15 mls butyl PBD - toluene 2 ethoxyethanol counting fluid to increase the counting efficiency.

k) Labelled poly U hybridisation to RNA

Labelled poly U was made by the polymerisation of ^{3}H labelled uridine diphosphate at 37°C with a suitable concentration of polynucleotide phosphorylase for 60 minutes. The reaction was stopped by adding 2 parts 2 M sodium acetate pH 5.0 : 1 part 10% SLS and then the mix was shaken for 30 minutes with an equal volume of phenol : chloroform (1 : 1). This mix was centrifuged (10,000 rpm, 10 minutes), the aqueous phase kept and the phenol phase re-extracted with an equal volume of water. The aqueous phases were pooled and $^{1}/10$th volume of 10 X column buffer added, then passed through a Sephadex SP 50 column and 0.5 ml fractions collected. Column buffer is 0.3 M NaCl, 0.01 Na acetate pH 5.0. The concentration and specific activity of each fraction was determined.

The approximate number of poly A sequences expected in a given RNA preparation was calculated and hence the quantity of poly U sequences required to bind to these poly A sequences calculated. The reaction of poly A and poly U sequences is relatively fast and specific. The hybridisation experiments were done in poly U excess.

Aliquots from each fraction containing unlabelled RNA were hybridised with an appropriate concentration of labelled poly U. The hybridisation mixes were made up to a final salt concentration of 2 X SSC

and a final volume of 1 ml. The mixes were shaken and incubated at 37°C for 30 minutes in order to allow time for complete hybridisation, and then chilled in ice and an equal volume of 2 X SSC containing 40 µg/ml RNase added to digest the unhybridised sequences. The mixes were incubated in ice for 20 minutes. For each series of experiments two control mixes containing no RNA were set up, one of which was given no RNase treatment.

50 λ of 5 mg/ml BSA and 0.5 mls 50% TCA were added to each tube to precipitate the RNA. After 20 minutes in ice, the mixes were filtered through 2.5 cm Millipore filters (for 'sucrose-SLS' gradients) and 2.5 cm GF/83 filters (for 'formamide' gradients) and dried at 80°C under reduced pressure for 20 minutes. The Millipore filters were counted in scintillation vials containing 10 mls Butyl PBD and the GF/83 filters in vials containing 10 mls toluene PPO POPOP counting fluid.

The weight of poly U hybridising with each RNA sample was calculated from the cpm from each hybridisation experiment remaining after RNase treatment. By direct proportion the weight of poly A sequences in the RNA in each fraction was obtained.

1) Hybridisation of labelled DNA (complementary to haemoglobin messenger RNA) to RNA.

Labelled DNA complementary to haemoglobin mRNA (cDNA) was prepared from a template of the 9.5 S RNA fraction from the cytoplasmic fraction of duck erythrocytes by Miss M. Richardson using the method of Bishop and Rosbash (1973). This 9.5S RNA has been shown to contain mRNA for duck haemoglobin (Pemberton et al, 1972). cDNA was synthesised from this 9.5 S RNA template using RNA-dependent DNA polymerase (reverse transcriptase) from avian myeloblastosis virus, in the presence of oligothymidylic acid

$(pT)_{10}$ which presumably acts as a primer of cDNA synthesis. The cDNA was freed of 10 S RNA, protein, and substrates by treatment with alkali and subsequent passage through a SP -sephedex (C - 50) and Chelex 100 column.

This cDNA has been shown to be simple-stranded and be less than 300 nucleotides in length of which on average about 40 nucleotides are poly T presumably in the 5' position (Bishop and Freeman, in press). This suggests that the cDNA transcripts are from the 3' end of the haemoglobin mRNA molecule. As the haemoglobin mRNA is about 630 nucleotides long (Pemberton et al, 1972), the cDNA transcript is complementary to less than half of the haemoglobin mRNA.

The approximate quantity of haemoglobin mRNA sequences expected in a given RNA preparation was calculated, and hence the quantity of cDNA sequences required to bind to these haemoglobin mRNA sequences calculated. The reaction of cDNA with haemoglobin mRNA sequences is slow and complete hybridisation does not occur under the conditions of incubation used. Hybridisation experiments were done in cDNA excess.

For each hybridisation experiment 10 λ of cDNA (about 2×10^4 cpm per ml, 3 ng per ml) was mixed with 10 λ of the appropriate dilution of unlabelled RNA from a given sample, to give a final concentration of 0.1% SLS, equimolar phosphate buffer (0.12 M NaH_2PO_4, 0.12 M Na_2HPO_4) and 1 mM EDTA pH 7.0 in each mix. The mixes were incubated at 70° C for 20 hours in small siliconised tubes in which the samples were covered with liquid paraffin. The hybridisation reaction was stopped by addition of 250 λ of a mix containing 6.25 μg E.coli denatured DNA, 0.02 M NaCl, 0.05 N acetic acid, 6×10^{-4} M $ZnSO_4$, 0.03 M sodium acetate pH 4.5. The tubes were shaken thoroughly in order to allow complete mixing. After the

paraffin had settled to the top of the tubes, 200 λ of the hybridisation mix was removed from each tube and incubated at 50°C for 30 minutes with the correct amount of nuclease S_1 to allow complete digestion of all the unhybridised cDNA sequences. Nuclease S_1 is capable of removing all single-stranded regions from reassociated DNA molecules without extensively degrading the reassociated duplex regions, (Sutton, 1971).

For each set of experiments two control mixes were set up, one containing no RNA and the other given no nuclease S_1 treatment. 2 mls 10% TCA and 50 λ 5 mg/ml BSA were added to each tube to precipitate the RNA. After 20 minutes in ice the mixes were filtered through 2.5 cm GF/83 filters and dried at 80°C under pressure for 20 minutes. The filters were counted in scintillation vials containing 10 mls toluene PPO POPOP counting fluid.

The weight of cDNA hybridising with each RNA sample was calculated from the cpm from each hybridisation remaining after RNase treatment.

m) Poly dT - sepharose and DNA (complementary to haemoglobin messenger RNA) - sepharose columns

0.2 mls of poly dT - sepharose and 0.3 mls of sepharose were used for each poly dT - sepharose column and 0.2 mls of cDNA - sepharose and 0.3 mls of sepharose used for each cDNA - sepharose column. The columns had a diameter of 0.45 cms and were surrounded by a water jacket with a thermostat to regulate the temperature. Before use the columns were washed with about 10 mls of 4 X SSC pH 5.5, 0.25% SLS at 20°C at a slow rate.

The polynucleotides or RNA to be analysed were suspended in a small volume of 4 X SSC pH 5.5, 0.25% SLS and passed very gently on to the column at 20°C. The column was then washed with 1 ml of this solution. The polynucleotides or RNA were eluted from the column in a low salt concentration of 1 X SSC pH 5.5, 0.25% SLS in small aliquots at 20°C, 35° or 40°, 60° and 70°C respectively. Any remaining sequences were eluted from the column at 70°C with 1 mM tris pH 7.5, 0.25% SLS. All washing and elution fractions were kept and samples from these fractions were analysed.

RESULTS

a) Effect of denaturants on ribosomal RNA.

The study of hnRNA in the past has been complicated by the large differences in molecular weight found under different ionic and denaturing conditions. Thus, in this work denaturing conditions which remove all aggregations and destroy any secondary structure of hnRNA were determined, so that the absolute denatured state of the hnRNA could be found. As the degree of hyperchromicity of RNA is a measure of its secondary structure, the ultra violet absorption of RNA under various melting conditions were determined.

Duck cytoplasmic RNA, containing mainly rRNA, was used for the following experiments. Bramwell and Harris (1967) have shown that under the same conditions, the melting profiles of HeLa cell hnRNA and rRNA are identical. However, the hnRNA they used appeared to contain some rRNA, presumably from cytoplasmic contamination. Nevertheless, the results obtained from rRNA melts were assumed to hold for hnRNA as well.

The secondary structure of rRNA, caused by a certain amount of double helical structure, is lost upon melting at temperatures approaching 100°C. Figure 3 shows the melting curve of rRNA in NTE(0.1 M NaCl, 0.01 M Tris, 2mM EDTA) at 260 nm. Considerable hyperchromism occurs upon melting, which is more or less completely recovered upon cooling. In the presence of 85% formamide in TE (0.01 M Tris, 2mM EDTA) (figure 3), the melting temperature is greatly reduced and virtually total absorbance is found at 30°C. Melting of rRNA in 8 M urea and NTE (0.01 M NaCl, 0.01 M Tris, 2mM EDTA) also occurs at lower temperatures. At 25°C about 91% of full absorbance is found (figure 3). Full absorbance occurs at about 70°C in

Legend to figure 3

Melting curves of duck rRNA in i) NTE, ii) NTE, 8 M urea, iii) TE, 85% formamide.

3.0 ml samples of rRNA (about 40 μg/ml) were suspended in i) NTE, ii) NTE, 8 M urea, iii) TE, 85% formamide, and melting curves obtained as described in the Materials and Methods section. Cooling points (at about 25°C) of the samples were also obtained.

■——■ Melting curve of rRNA in NTE

●——● Melting curve of rRNA in NTE, 8 M urea

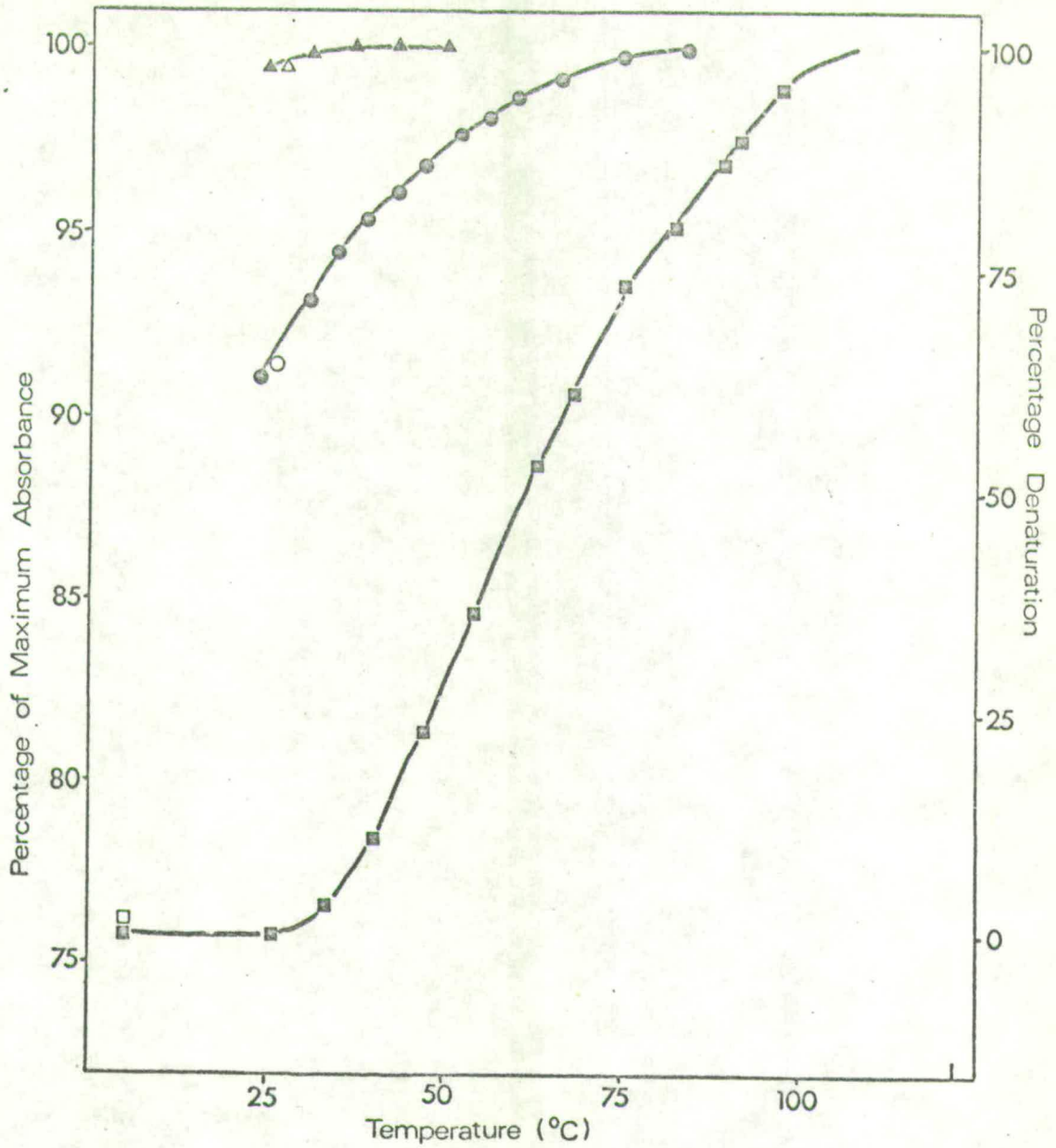
▲——▲ Melting curve of rRNA in TE, 85% formamide

□ Cooling point of melt of rRNA in NTE

○ Cooling point of melt of rRNA in NTE, 8 M urea

△ Cooling point of melt of rRNA in TE, 85% formamide

FIGURE 3


100
95
90
85
80
75
Percentage of Maximum Absorbance
100
75
50
25
0
Percentage Denaturation
25
50
75
100
Temperature (°C)

the presence of 10% formaldehyde in NTE (0.1 M NaCl, 0.01 M Tris, 2mM EDTA), but in this case the loss of secondary structure is essentially irreversible since little (about 3%) loss in absorbance occurs upon cooling (figure 4). The results of these experiments are summarised in table 1.

rRNA was run in sucrose gradients that had been made with and without denaturants. The concentrations of the denaturants used in the sucrose gradients were the same as were used in the rRNA melts (figures 3 and 4) and the purpose of these spins was to confirm the results of the melts, concerning the effectiveness of the denaturants.

Figure 5a shows a typical pattern of rRNA run in a 'sucrose-SLS' gradient. The 18 S and 28 S rRNA components of Attardi et al (1966) and Scherrer et al (1966) and the 18S and 26 S components of Pemberton and Baglioni (1972), in fact sediment at about 18S and 27 S (figure 2) in the approximate proportion of 1 : 2. Following denaturation with 10% formaldehyde the rRNA components showed two main peaks in a 'sucrose - SLS' gradient (figure 5b) sedimenting at about 14 S and 21 S. A much smaller peak was found in the 6 - 10 S region. It appears that the 18 S rRNA subunit was transformed by removal of its secondary structure to about 14 S whilst the 28 S rRNA subunit was transformed to about 21 S. However, the 1 : 2 ratio expected for the 14 S : 21 S RNA was not found and instead in most cases the 14 S component was found in larger quantities. Apparently, some of the 28 S rRNA had been 'nicked' at precise points during its preparation or analysis and on denaturation molecules of about 14 S and smaller molecule(s) of about 6 - 10 S were obtained. Similar results have been observed to greater or lesser extents by various workers, (Aronson and McCarthy, 1961; Petermann and Pavlovec, 1963; Midgley,

Legend to figure 4

Melting curves of duck rRNA in i) NTE ii) NTE, 10% formaldehyde.

3.0 ml samples of rRNA (about 40 µg/ml) were suspended in i) NTE, ii) NTE, 10% formaldehyde, and melting curves obtained as described in the Materials and Methods section. A cooling curve of the NTE, 10% formaldehyde sample was also obtained.

■———■ Melting curve of rRNA in NTE

▲———▲ Melting curve of rRNA in NTE, 10% formaldehyde

△- - - - - -△ Cooling curve of rRNA in NTE, 10% formaldehyde

FIGURE 4

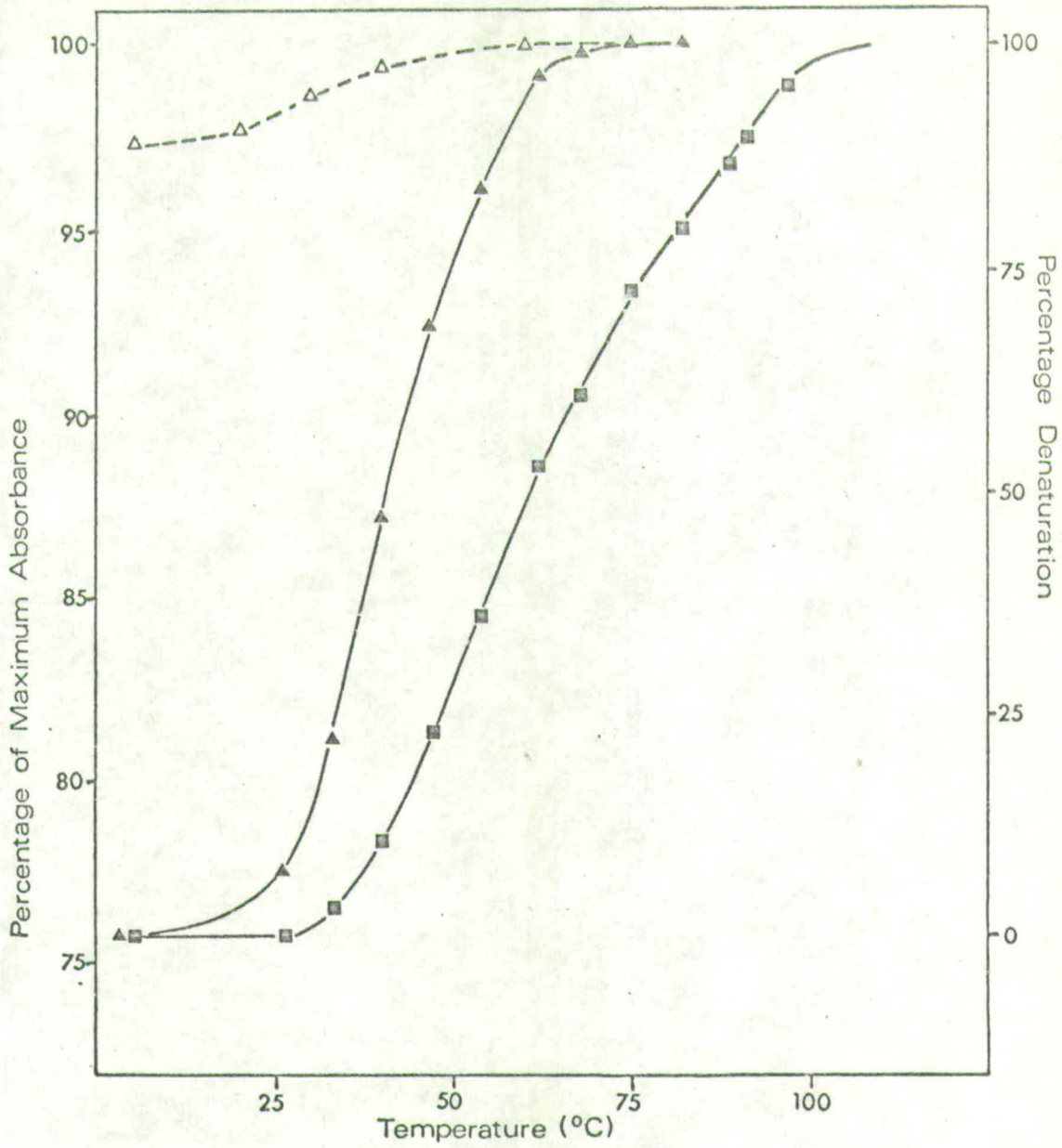

100
95
90
85
80
75
Percentage of Maximum Absorbance
100
75
50
25
0
Percentage Denaturation
25
50
75
100
Temperature (°C)

Table 1

Amount of rRNA denaturation under different melting conditions.

Melting Condition	Percentage denaturation at 25°C	Temperature required for total denaturation	Percentage denaturation remaining after cooling to 25°C
NTE	Zero	105 - 110°C	2
NTE, 8 M urea	64	75 - 80°C	65
TE, 85% formamide	98	30 - 35°C	98
NTE, 10% formaldehyde	7	65 - 70°C	93

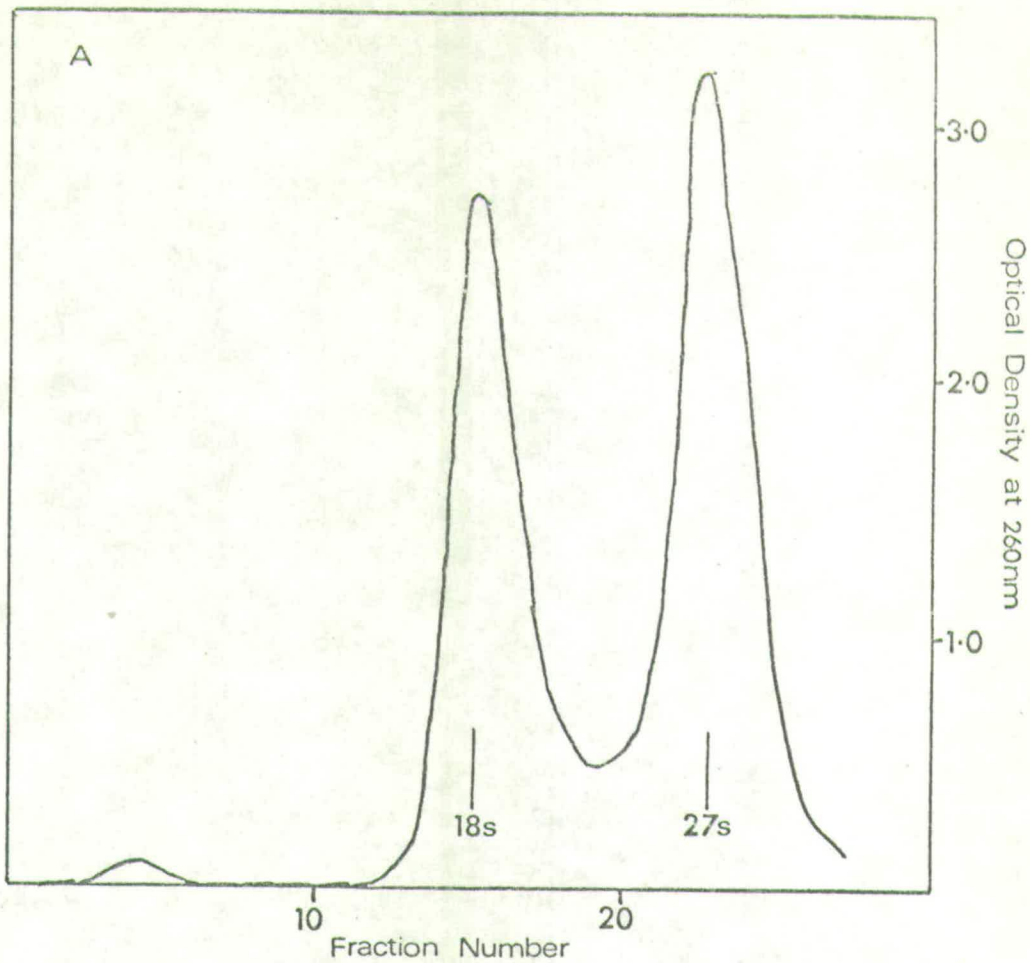
Legend to figure 5

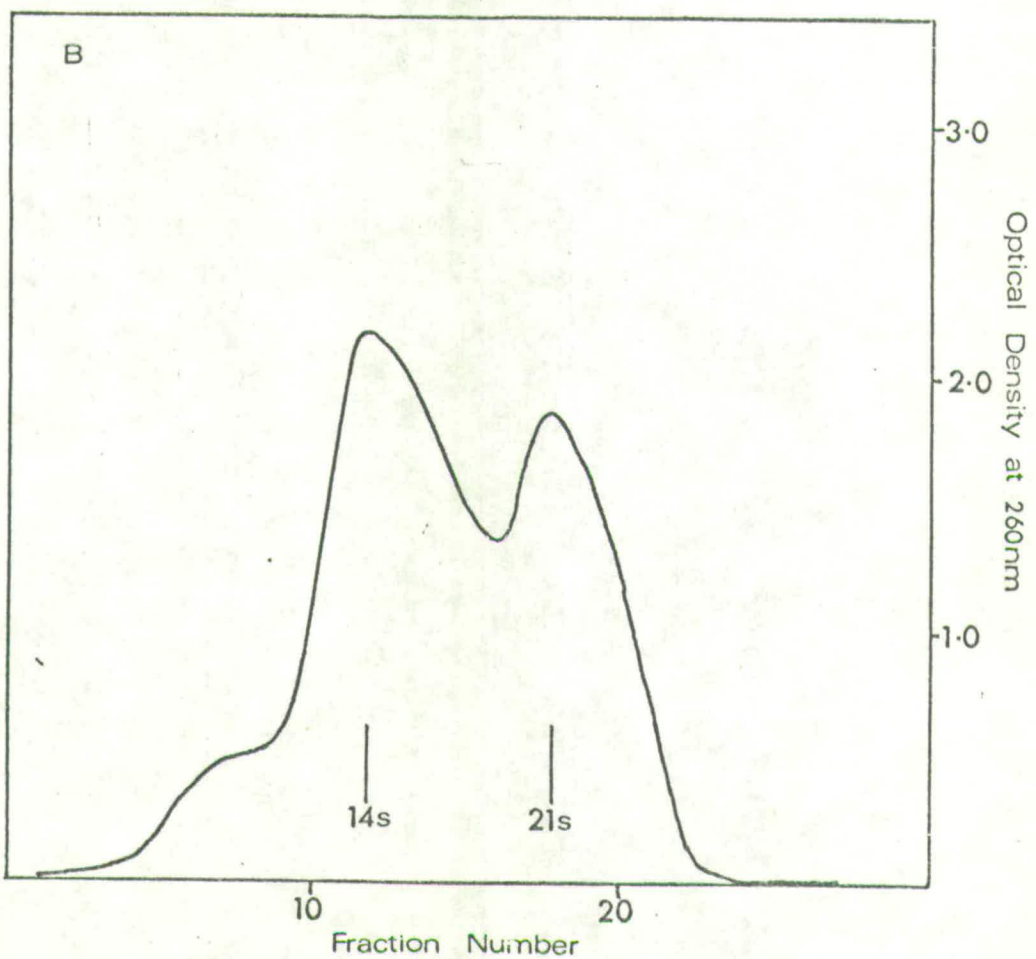
Cytoplasmic RNA run in 'sucrose - SLS' gradients with and without formaldehyde pretreatment.

a) Unlabelled cytoplasmic RNA (0.5 mg) was spun in a 16 ml 5-30% sucrose, 0.5% SLS, NTE gradient for 16.0 hours at 25°C, 24 K.

b) Unlabelled cytoplasmic RNA (0.5 mg) was spun in a 16 ml 5-30% sucrose, 0.5% SLS, NTE gradient for 16.0 hours at 25°C, 24 K. The RNA was pretreated with 10% formaldehyde at 67°C for 20 minutes.

FIGURE 5


A
3·0
2·0
1·0
Optical Density at 260nm
18s
27s
10
20
Fraction Number


B
3·0
2·0
1·0
Optical Density at 260nm
14s
21s
10
20
Fraction Number

1965; Rodgers, 1966).

Formaldehyde treated rRNA run in 'sucrose - SLS', 'formamide' and 'urea' gradients (figures 5b, 6b and 7b respectively) produced similar absorption profiles with two main peaks at 14 S and 21 S, as formaldehyde produces practically total RNA denaturation in all these gradients. Thus, rRNA treated with formaldehyde was used to calibrate sedimentation coefficients in 'urea' and 'formamide' gradients.

In 'formamide' gradients the sedimentation patterns of formaldehyde treated and untreated RNA were almost identical showing two peaks at about 14 S and 21 S (figure 6) This result confirms that rRNA completely looses its secondary structure under these formamide conditions.

In 'urea' gradients, however, the formaldehyde treated and untreated rRNA sedimentation patterns were different (figure 7). Untreated rRNA in the 'urea' gradients did not denature to the same extent as the formaldehyde treated rRNA, as considerable amounts of 18 S and 28 S components were still present. The urea conditions used (producing about 67% denaturation) were inadequate to completely disrupt the rRNA secondary structure.

b) Calculation of the molecular weights of RNAs from their sedimentation coefficients.

The reliability of molecular weight estimates of RNA from their sedimentation coefficients in sucrose gradients has been questioned due to undefined conformational differences found in RNA molecules, (Gesteland and Boedtker, 1964; Strauss and Sinsheimer, 1967). The

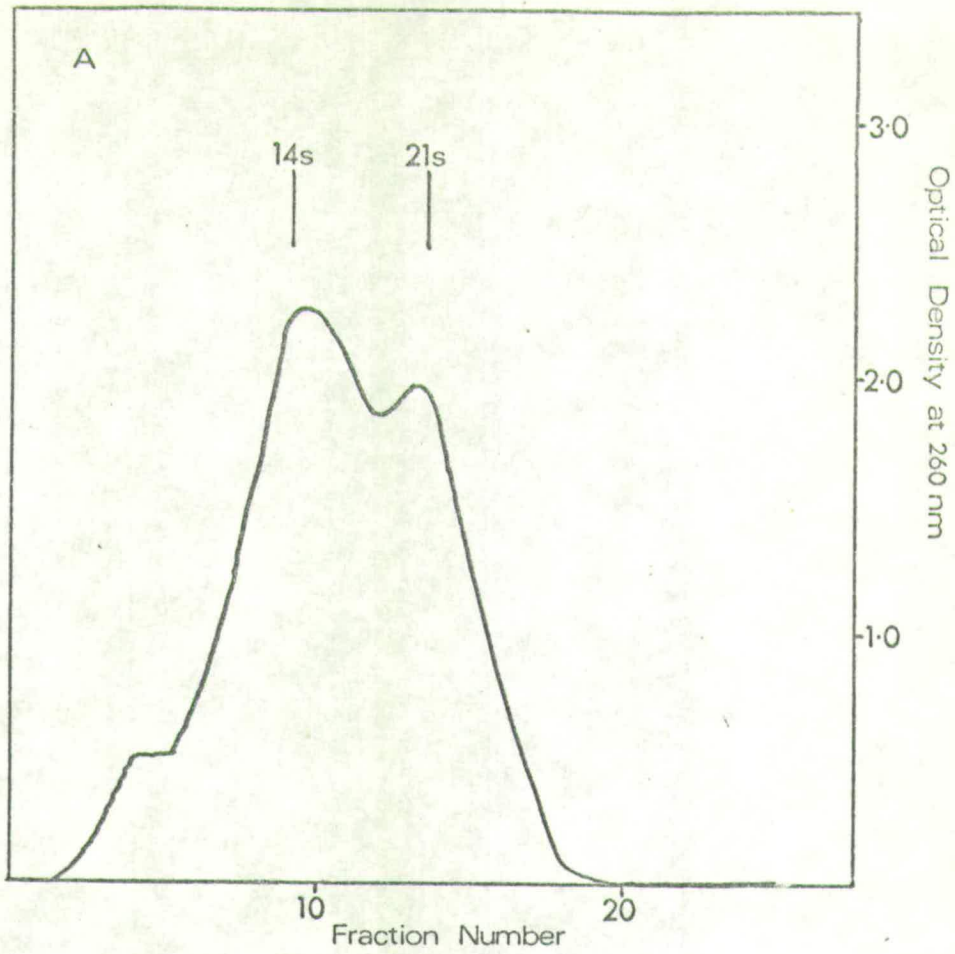
Legend to figure 6

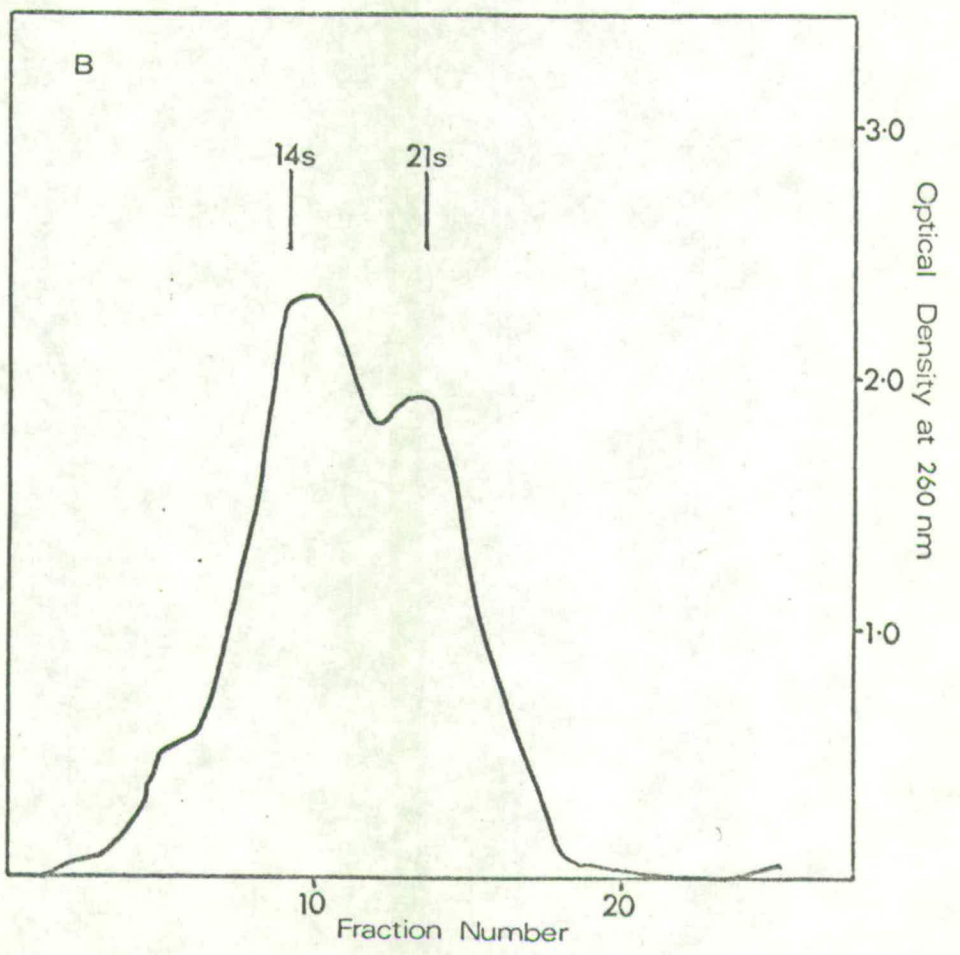
Cytoplasmic RNA run in 'formamide' gradients with and without formaldehyde pretreatment.

a) Unlabelled cytoplasmic RNA (0.5 mg) was spun in a 14 ml 2-10% sucrose, 85% formamide, TE gradient for 41.6 hours at 25°C, 24 K.

b) Unlabelled cytoplasmic RNA (0.5 mg) was spun in a 14 ml 2-10% sucrose, 85% formamide, TE gradient for 41.6 hours at 25°C, 24 K. The RNA was pretreated with 10% formaldehyde at 67°C for 20 minutes.

FIGURE 6


A
14s
21s
3·0
2·0
1·0
Optical Density at 260 nm
10
20
Fraction Number


B
14s
21s
3·0
2·0
1·0
Optical Density at 260 nm
10
20
Fraction Number

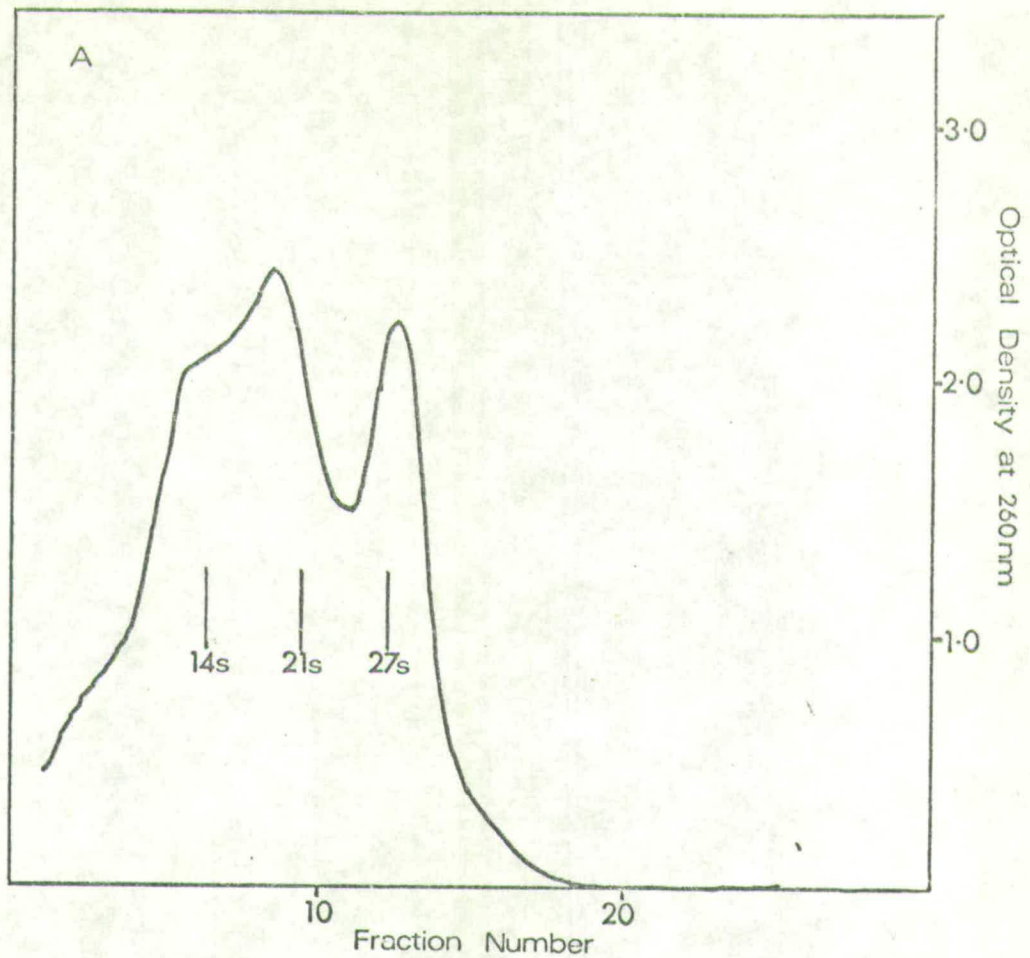
Legend to figure 7

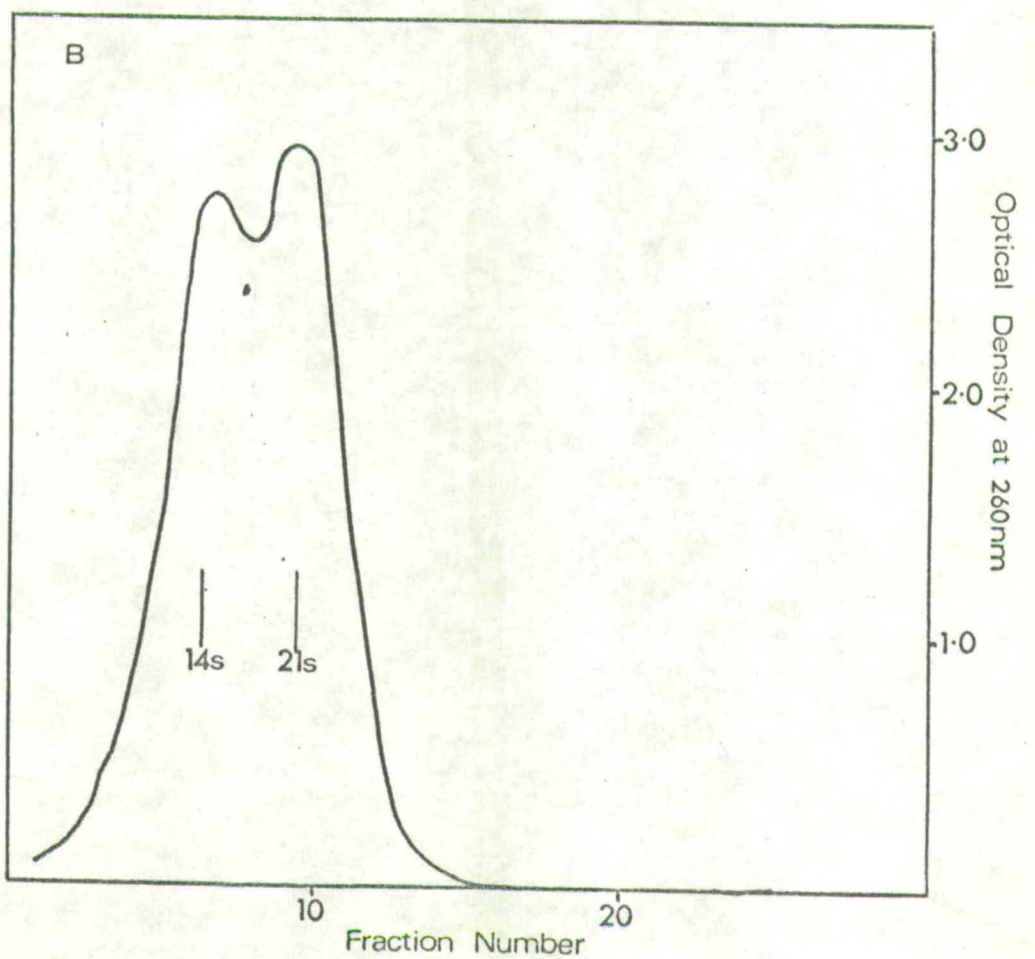
Cytoplasmic RNA run in 'urea' gradients with and without formaldehyde pretreatment.

a) Unlabelled cytoplasmic RNA (0.5 mg) was spun in a 16 ml 5-30% sucrose, 8 M urea, NTE gradient for 31.2 hours at 25°C, 24 K.

b) Unlabelled cytoplasmic RNA (0.5 mg) was spun in a 16 ml 5-30% sucrose, 8 M urea, NTE gradient for 31.2 hours at 25°C, 24 K. The RNA was pretreated with 10% formaldehyde at 67°C for 20 minutes.

FIGURE 7


A
3·0
2·0
1·0
Optical Density at 260nm
14s
21s
27s
10
20
Fraction Number


B
3·0
2·0
1·0
Optical Density at 260nm
14s
21s
10
20
Fraction Number

equations of Gierer (1958) and Spirin (1961) only give rough estimations of RNA molecular weights from their sedimentation coefficients.

In the 'formamide' gradients used, complete denaturation of the RNA occurred (table 1). The secondary structures of the RNAs were eliminated under such conditions and the log molecular weight of RNA had a linear relationship with the sedimentation distance in a similar manner as described for DMSO gradients (Sedat et al, 1969; Mc Guire et al, 1972).

The sedimentation coefficients of HeLa rRNA and duck haemoglobin mRNA and their molecular weights are shown in table 2. A plot of log molecular weight against log sedimentation coefficient is shown in figure 8. The molecular weights of RNAs spun in 'formamide' gradients were calculated from figure 8.

c) Incorporation of radioactivity into RNA

The bulk of radioactivity incorporated in vitro by duck blood cells after incubation in the presence of ^{3}H uridine remains associated with the nuclear fraction (Attardi et al, 1966; Scherrer et al, 1966). The data presented in table 3 is that from a typical incorporation experiment of a 2 hour incubation of duck blood cells with ^{3}H uridine. About 90-95% of the radioactivity and very few optical density units at 260 nm of RNA were found in the nuclear fraction, whilst only 5-10% of the radioactivity but most of the optical density units at 260 nm of RNA were associated with the cytoplasmic fraction.

d) Effects of denaturants on labelled cytoplasmic RNA.

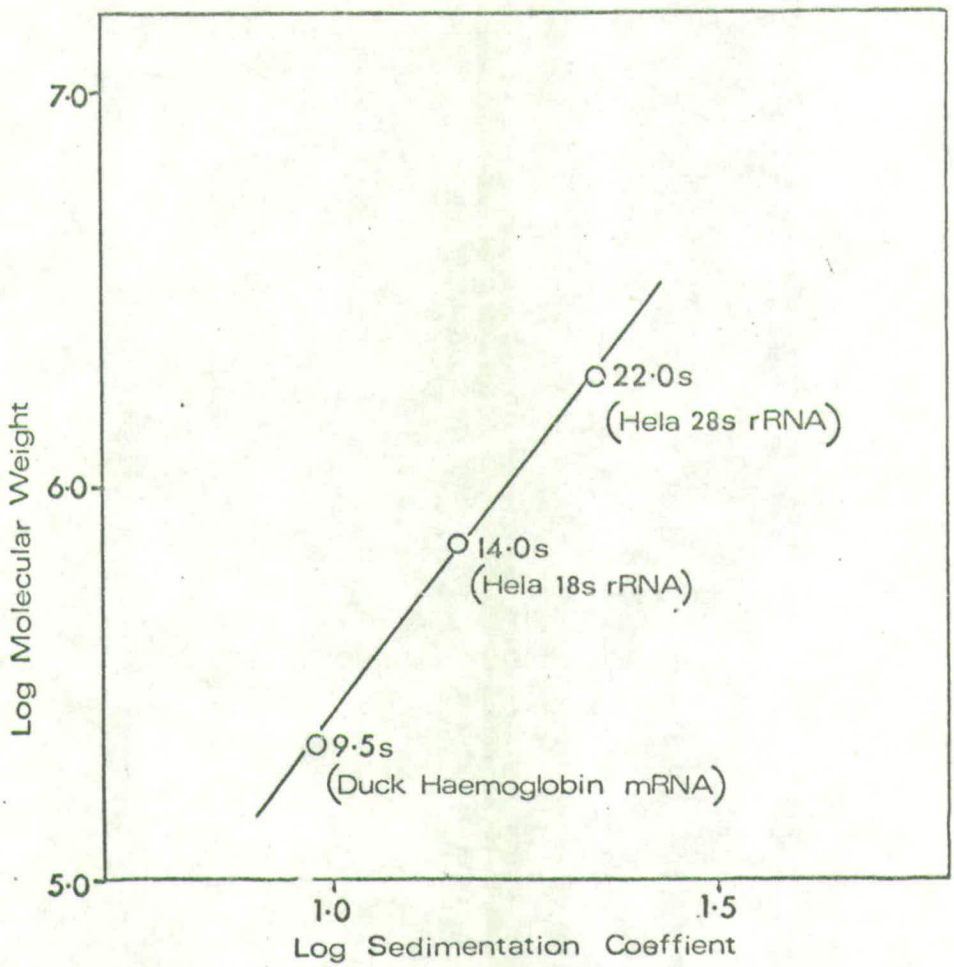
Table 2

Sedimentation coefficients of HeLa rRNAs in formamide gradients compared with their molecular weights.

	Sedimentation coefficient in formamide	Molecular weight
HeLa 18 S rRNA	14.5 S	0.71×10^6 daltons
HeLa 28 S rRNA	22.0 S	1.90×10^6 daltons
Duck haemoglobin mRNA	9.5 S	0.22×10^6 daltons

The HeLa rRNA was labelled with ^{3}H uridine (gift from John Morton), and their sedimentation coefficients in formamide gradients calculated using unlabelled duck rRNA as marker in a similar manner as in figure 2. The accurate molecular weights of HeLa rRNA were obtained from Attardi and Smith (1962) and McConkey and Hopkins (1969), and of duck haemoglobin mRNA from Pemberton et al (1972).

FIGURE 8



The molecular weights of the RNAs and their sedimentation coefficients in 'formamide' gradients are quoted in table 2.

Table 3

Recovery of cytoplasmic RNA and hnRNA after labelling with ^{3}H uridine (30 μc per ml) for 2 hours.

Cytoplasmic RNA	hnRNA
3.3 mg/ml packed cells	Unmeasurably small weight/ml packed cells.
30,000 c/m per ml packed cells	570,000 c/m per ml packed cells.

The incubation procedure for the duck cells and the methods of extraction of cytoplasmic RNA and hnRNA are described in the Materials and Methods.

Figure 9a shows the sedimentation profile of ^{3}H uridine labelled cytoplasmic RNA spun in a 'sucrose - SLS' gradient, whilst figure 9b shows the sedimentation profile of formaldehyde pretreated ^{3}H uridine labelled cytoplasmic RNA in a 'sucrose - SLS' gradient.

In the untreated RNA gradient (figure 9a) a major non - rRNA peak of radioactivity occurs in the 9 - 10 S region and smaller peaks of radioactivity occur at about 18 S and 27 S. The 18 S and 27 S peaks correspond to the positions of the rRNA components and the 9 - 10 S peak corresponds to the position of the globin mRNA. This pattern is very similar to that obtained by other workers for duck cytoplasmic RNA (Attardi et al, 1966). On denaturing the cytoplasmic RNA with formaldehyde pretreatment (figure 9b) the large peak of label is again found at 9 - 10 S and the smaller peaks occur at about 14 S and 21 S. The smaller peaks are associated with the denatured rRNA components.

There is relatively little change in the labelling pattern of total cytoplasmic RNA under normal or denaturing conditions. In both cases there is considerable labelling in the region of 9 - 10 S, the position of the globin mRNA. As there is very little or no difference in the sedimentation values between normal or denaturing conditions for this 9 - 10 S peak, it implies that there is very little secondary structure in these species. In the rRNA associated label peaks there appears to be some secondary structure, as on denaturation the sedimentation values of the peaks show a decrease in value.

e) Effects of denaturants on labelled heterogeneous nuclear RNA.

Figures 10a and 11a show typical sedimentation profiles of ^{3}H labelled hnRNA spun in 'cold sucrose' and 'sucrose - SLS' gradients respectively.

Legend to figure 9

Labelled cytoplasmic RNA run in 'sucrose - SLS' gradients with and without formaldehyde pretreatment.

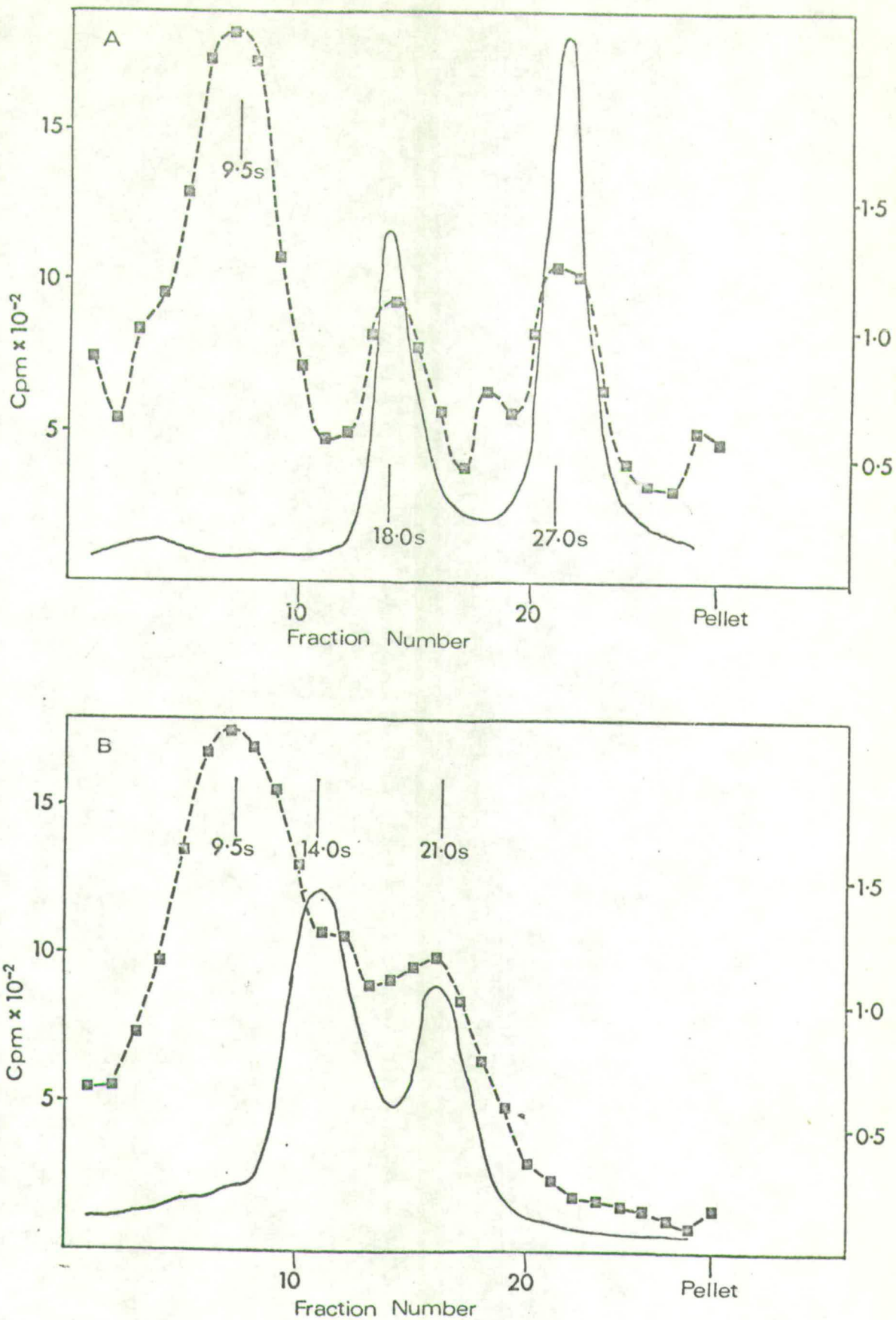
a) ^{3}H uridine labelled cytoplasmic RNA (0.5 mg) was spun in a 16 ml 5-30% sucrose, 0.5% SLS, NTE gradient for 15.65hours at 25°C, 24 K. 50 λ aliquots from each gradient fraction were counted.

b) ^{3}H uridine labelled cytoplasmic RNA (0.5 mg) was spun in a 16 ml 5-30% sucrose, 0.5% SLS, NTE gradient for 15.65 hours at 25°C, 24 K. The RNA was pretreated with 10% formaldehyde at 67°C for 20 minutes. 50 λ aliquots from each gradient fraction were counted.

■- - - - - - ■ cpm per fraction

———————— ODS.

FIGURE 9


A
9·5s
18·0s
27·0s
Cpm x 10⁻²
15
10
5
1·5
1·0
0·5
10
20
Pellet
Fraction Number
B
9·5s
14·0s
21·0s
Cpm x 10⁻²
15
10
5
1·5
1·0
0·5
10
20
Pellet
Fraction Number

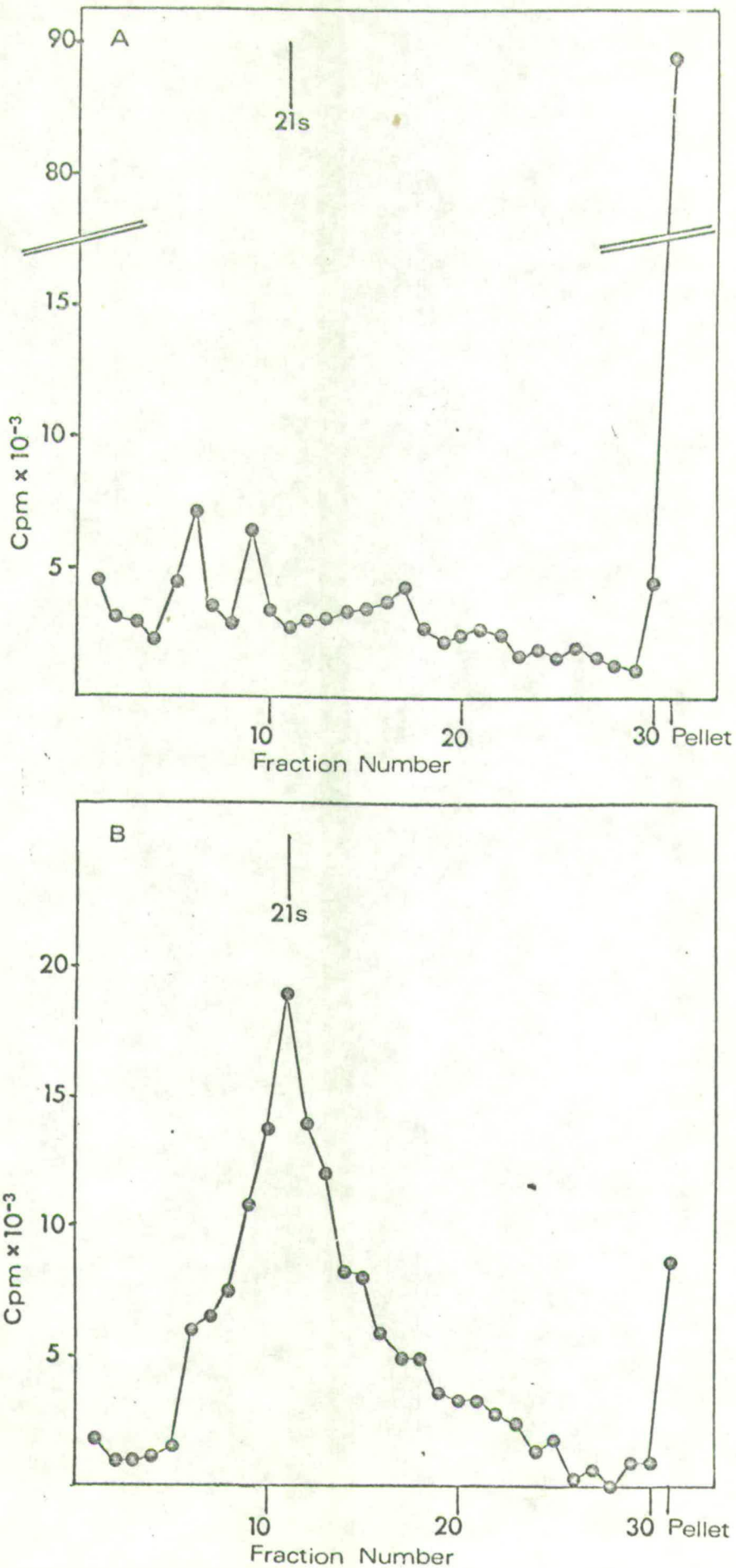
Legend to figure 10

Labelled hnRNA run in 'cold sucrose' gradients with and without formaldehyde pretreatment.

a) ^{3}H uridine labelled hnRNA was spun on a 16 ml 5-30% sucrose, NTE gradient for 17.45 hours at 1°C, 24 K. 50 λ aliquots from each gradient fraction were counted.

b) ^{3}H uridine labelled hnRNA was spun on a 16 ml 5-30% sucrose, NTE gradient for 17.45 hours at 1°C, 24 K. The hnRNA was pretreated with 10% formaldehyde at 67°C for 20 minutes. 50 λ aliquots from each gradient fraction were counted.

FIGURE 10


A
21s
90
80
15
10
5
Cpm x 10⁻³
10
20
30
Pellet
Fraction Number
B
21s
20
15
10
5
Cpm x 10⁻³
10
20
30
Pellet
Fraction Number

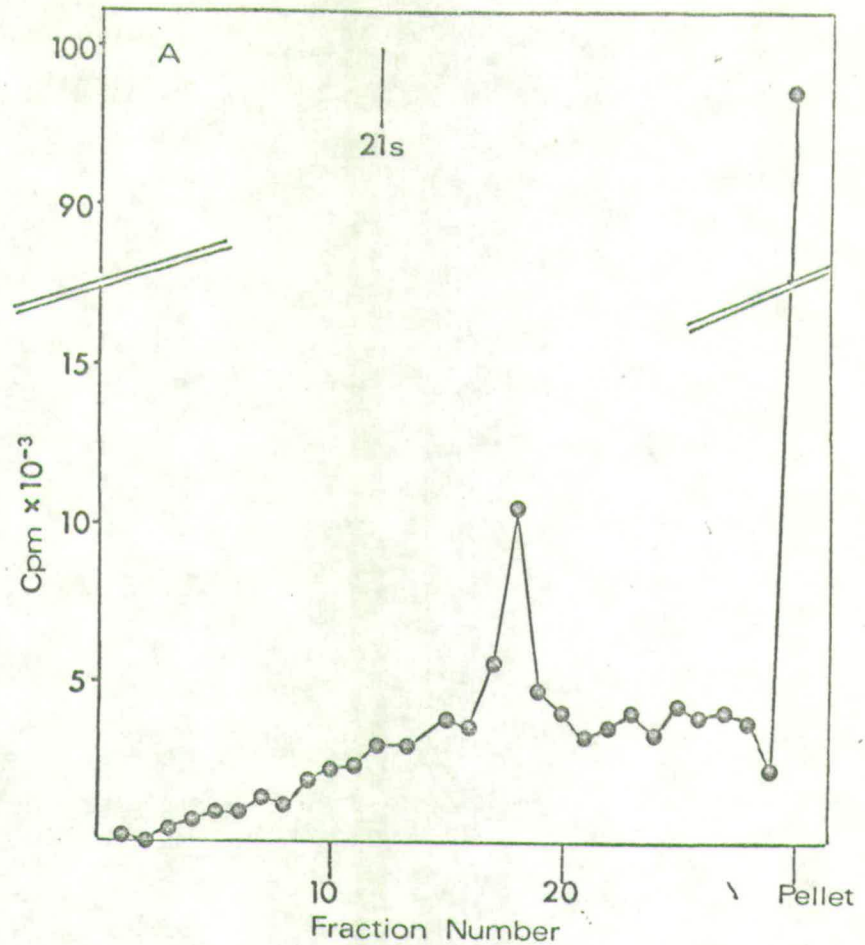
Legend to figure 11

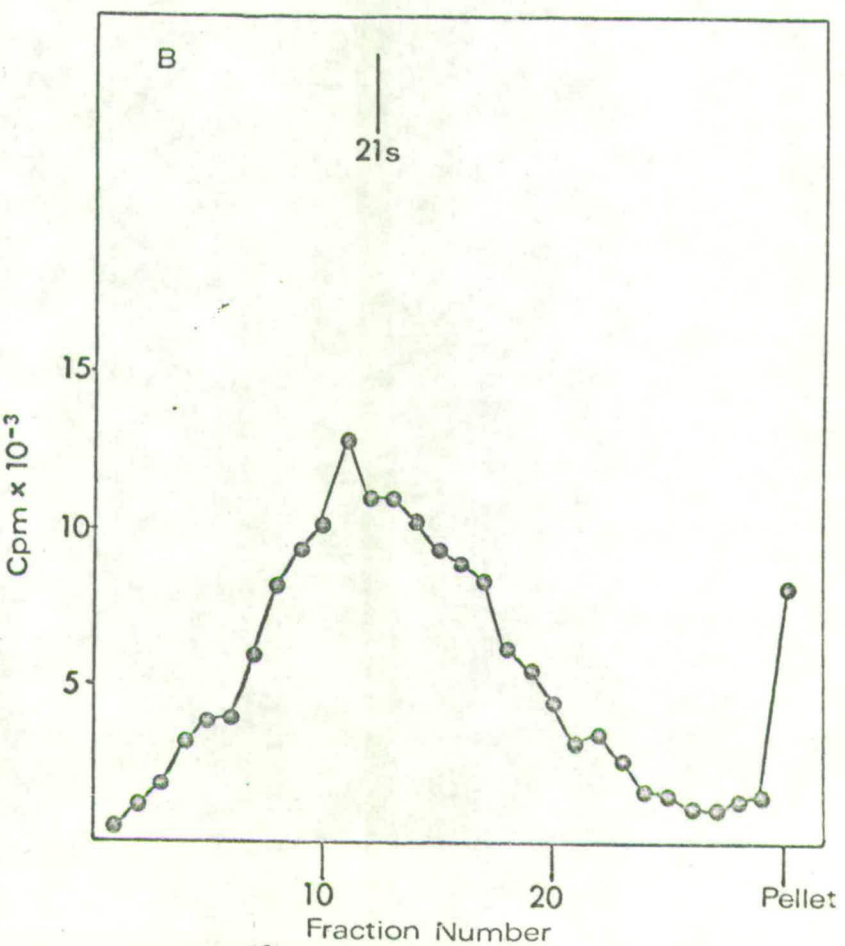
Labelled hnRNA run in 'sucrose - SLS' gradients with and without formaldehyde pretreatment.

a) ^{3}H uridine labelled hnRNA was spun on a 16 ml 5-30% sucrose, 0.5% SLS, NTE gradient for 10.6 hours at 25°C, 24 K. 50 λ aliquots from each gradient fraction were counted.

b) ^{3}H uridine labelled hnRNA was spun on a 16 ml 5-30% sucrose, 0.5% SLS, NTE gradient for 10.6 hours at 25°C, 24 K. The hnRNA was pretreated with 10% formaldehyde at 67°C for 20 minutes. 50 λ aliquots from each gradient fraction were counted.

FIGURE 11


A
21s
100
90
15
10
5
Cpm × 10⁻³
10
20
Pellet
Fraction Number


B
21s
15
10
5
Cpm × 10⁻³
10
20
Pellet
Fraction Number

SLS is known to remove weak aggregations and intermolecular interactions between RNA molecules (Staehelim et al, 1964). As these figures show essentially the same sedimentation patterns for the hnRNA species, it would appear that superficial aggregations between hnRNA molecules are not involved in the hnRNA structure. In most sedimentation analyses of hnRNA, 'sucrose - SLS' gradients were preferred to 'cold sucrose' gradients, as not only does SLS disrupt any weak RNA aggregations, but also, ~~as~~ SLS even in low concentration disrupts protein structure, inhibiting the action of nucleases (Tanford, 1970).

Figures 10 to 13 show the effect of various denaturing conditions on the sedimentation pattern of labelled hnRNA. Figures 10b, 11b, 12b and 13b show the effect of treating hnRNA with 10% formaldehyde before spinning the treated hnRNA in various sucrose gradients. The effect of 8 M urea (figure 12a) and 85% formamide (figure 13a) on hnRNA is shown by spinning hnRNA in 'urea' and 'formamide' gradients respectively. In all these cases essentially the same pattern is obtained with a relatively broad peak about 18 - 21 S. However, in the urea gradient (figure 12a) larger hnRNA species occur up to about 50 S. This is presumably because urea treatment at 25°C only produces about 65% denaturation of the RNA, compared to well over 90% denaturation obtained with formaldehyde and formamide treatment, (table 1). Thus, in 8 M urea the hnRNA still has a certain amount of secondary structure which produces the heavier sedimenting species.

In the denaturing conditions used in the gradients it is seen that there is still up to 6% of the hnRNA, found in the heavy RNA species in the bottom fractions of the gradients and in the pellets, with sedimentation coefficients greater than 50 S. Nevertheless, this compares with about 50% of the hnRNA sedimenting at values greater than

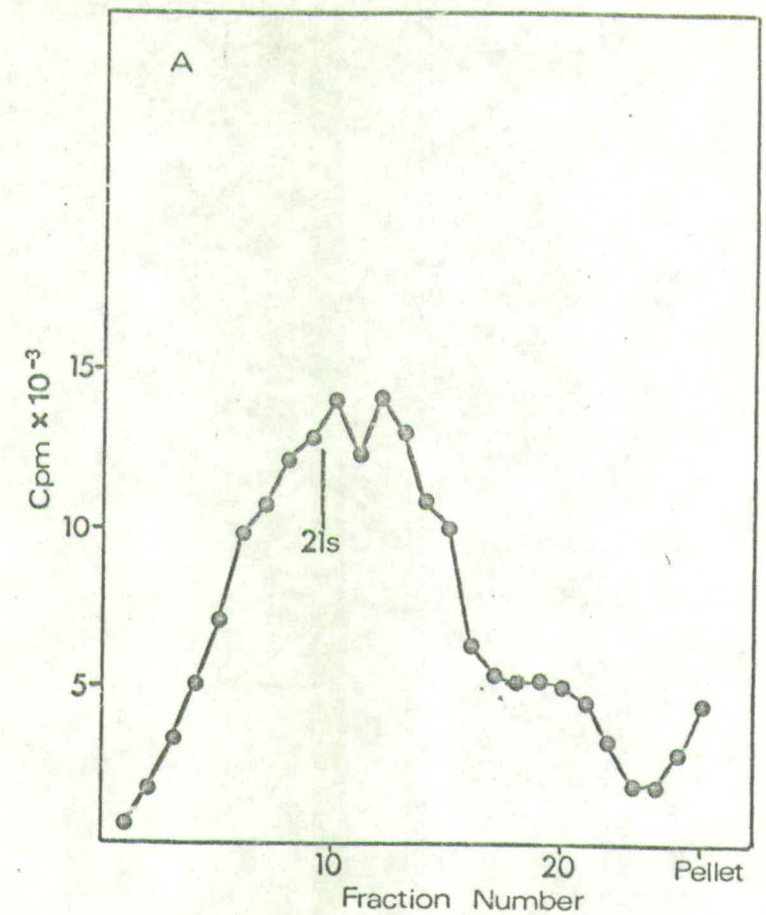
Legend to figure 12

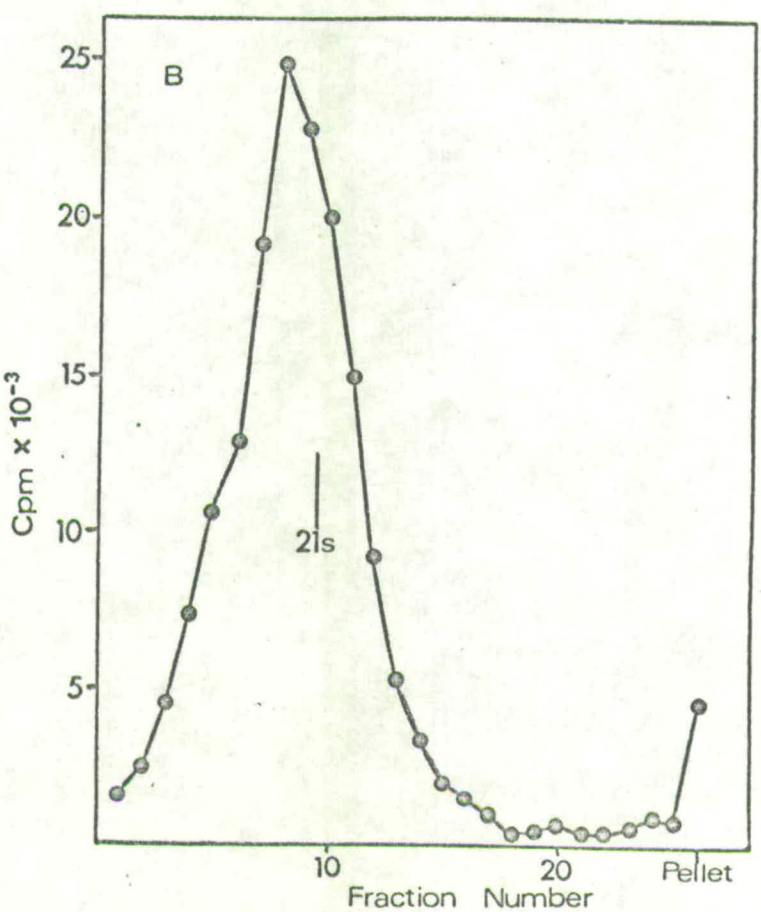
Labelled hnRNA run in 'urea' gradients with and without formaldehyde pretreatment.

a) ^{3}H uridine labelled hnRNA was spun on a 16 ml 5-30% sucrose, 8 M urea, NTE gradient for 31.2 hours at 25°C, 24 K. 50 λ aliquots from each gradient fraction were counted.

b) ^{3}H uridine labelled hnRNA was spun on a 16 ml 5-30% sucrose, 8 M urea, NTE gradient for 31.2 hours at 25°C, 24 K. The hnRNA was pretreated with 10% formaldehyde at 67°C for 20 minutes. 50 λ aliquots from each gradient fraction were counted.

FIGURE 12


A
Cpm x 10⁻³
15
10
5
21s
10
20
Pellet
Fraction Number


B
Cpm x 10⁻³
25
20
15
10
5
21s
10
20
Pellet
Fraction Number

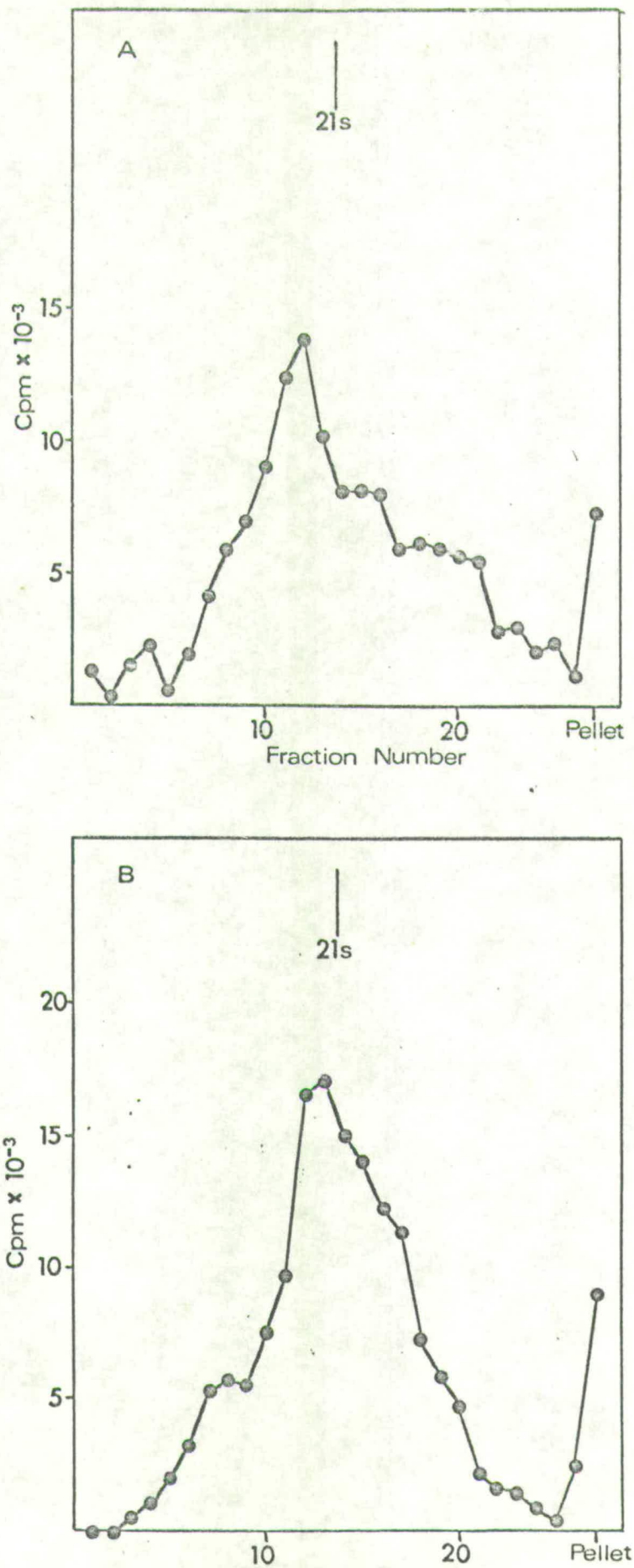
Legend to figure 13

Labelled hnRNA run in 'formamide' gradients with and without formaldehyde pretreatment.

a) ^{3}H uridine labelled hnRNA was spun on a 14 ml 2-10% sucrose, 85% formamide, TE gradient for 41.6 hours at 25°C, 24 K. 50 λ aliquots from each gradient fraction were counted.

b) ^{3}H uridine labelled hnRNA was spun on a 14 ml 2-10% sucrose, 85% formamide, TE gradient for 41.6 hours at 25°C, 24 K. The hnRNA was pretreated with 10% formaldehyde at 67°C for 20 minutes. 50 λ aliquots from each gradient fraction were counted.

FIGURE 13


A
21s
Cpm x 10-3
15
10
5
10
20
Pellet
Fraction Number
B
21s
20
15
10
5
Cpm x 10-3
10
20
Pellet
Fraction Number

50 S in the 'cold sucrose' and 'sucrose - SLS' gradients where denaturing conditions are not found (figures 10a and 11a, respectively).

It appears from the vast decrease in average sedimentation coefficient of labelled hnRNA in denaturing conditions that there is a considerable amount of aggregation of separate hnRNA molecules. However, although hnRNA appears to be aggregated in vitro, the state of hnRNA in vivo is unknown.

Renaturation experiments were attempted where denatured labelled hnRNA from denaturing gradients was spun in 'sucrose - SLS' gradients and the amount of renaturation occuring calculated by the proportion of labelled material found in the heavier parts of the 'sucrose - SLS' gradients. Figure 14 shows the results of such an experiment. Both urea denatured hnRNA (figure 14a) and formamide denatured hnRNA (figure 14b) show considerable amounts of hnRNA material sedimenting at high sedimentation coefficients after spinning in 'sucrose - SLS' gradients, compared to denaturing conditions. Although up to about 50% renaturation occurred in these experiments, the amount of renaturation occurring after denaturation varied considerably with different experiments. In most renaturation experiments a certain amount of the labelled material remained in the 18 to 21 S region of the 'sucrose - SLS' gradients.

The renaturation of formamide and urea treated hnRNA is best explained by assuming that there is considerable sequence homology in hnRNA, probably of a very non-specific type. On removal of the denaturant, secondary structure is again possible and the hnRNA secondary structure reforms to varying degrees.

RNase digestion experiments on certain ^{3}H uridine labelled RNAs were

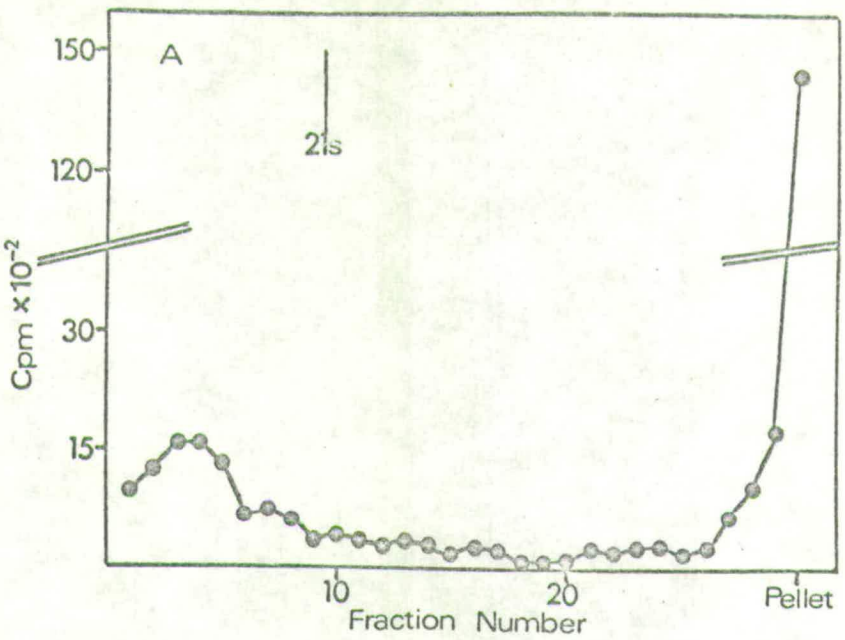
Legend to figure 14

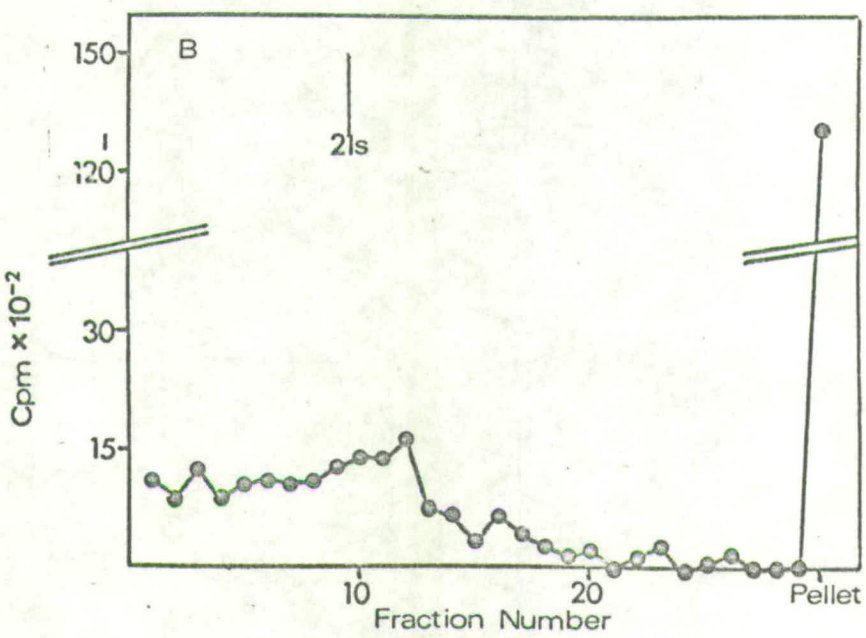
Labelled hnRNA from denaturing gradients re-run on 'sucrose - SLS' gradients.

a) ^{3}H uridine labelled urea treated hnRNA (fractions 1 - 23 of figure 12a) were precipitated for 12 hours at -20°C with 2 volumes of absolute alcohol. After pelleting this RNA at 10,000 rpm for 10 minutes, a quarter was spun on a 16 ml 5-30% sucrose, 0.5% SLS, NTE gradient for 11.3 hours at 25°C, 24 K. 50 λ aliquots from each gradient fraction were counted.

b) ^{3}H uridine labelled formamide treated hnRNA (fractions 1 - 25 of figure 13a) were precipitated for 12 hours at -20°C with 2 volumes of absolute alcohol. After pelleting this RNA at 10,000 rpm for 10 minutes, a quarter was spun on a 16 ml 5-30% sucrose, 0.5% SLS, NTE gradient for 11.3 hours at 25°C, 24 K. 50 λ aliquots from each gradient fraction were counted.

FIGURE 14


A
150
120
30
15
Cpm x 10⁻²
21s
10
20
Pellet
Fraction Number


B
150
120
30
15
Cpm x 10⁻²
21s
10
20
Pellet
Fraction Number

carried out at high salt concentrations to obtain RNase - resistant sequences. Ryskov et al (1972) and Jelinek and Darnell (1972) have obtained small amounts of RNase - resistant sequences from most RNAs at high salt concentrations, while at low salt concentrations virtually all this Rnase - resistant material is digested by RNase (Kimball and Duesberg, 1971; Darnell et al, 1971; Ryskov et al, 1972; Jelinek and Darnell, 1972). RNase digestion was done at 37°C for an hour where the reaction is essentially complete (Kimball and Duesberg, 1971; Darnell et al, 1971) and at 0°C for an hour where digestion is incomplete. Table 4 shows the results of such experiments.

27 S rRNA was used as a control and about 0.7% of this RNA was RNase - resistant at 37°C after an hours incubation in 2 X SSC, which was very similar to the results of Kimball and Duesberg (1971) with chicken, rabbit and HeLa rRNA. The number of RNase - resistant sequences in untreated hnRNA and renatured hnRNA were practically the same, producing about 3% and 7% RNase - resistant sequences at 37°C and 0°C respectively. These results are in agreement with those of Ryskov et al (1972) and Jelinek and Darnell (1972) that showed similar levels of RNase - resistance in cultured cell hnRNA after complete digestion in high salt concentrations. Jelinek and Darnell (1972) also showed that untreated hnRNA and renatured hnRNA that had been denatured with DMSO contained the same amounts of RNase - resistant sequences. If the RNase - resistant sequences are formed by intermolecular base-pairing rather than intramolecular base-pairing of complementary sequences, this could explain the high degree of aggregation seen between hnRNA molecules.

f) Hybridisation of poly U and DNA (complementary to haemoglobin messenger RNA) across gradients containing unlabelled cytoplasmic RNA.

Legend to Table 4

RNase digestion of labelled duck ~~28~~ 27 S and hnRNA.

^{3}H uridine labelled ~~28~~ 27 S rRNA, giant hnRNA (greater than 50 S) and renatured hnRNA previously denatured with urea (greater than 50 S) were suspended in 2 X SSC. Each RNA solution was divided into two tubes and at least 30,000 c/m RNA were used for each incubation.

RNase was added to each tube to a final concentration of 20 µg per ml. One of each RNA solutions was incubated at 0°C and the other at 37°C. 100 λ aliquots were removed from the tubes before incubation and after 1 hours incubation, and the radioactivity determined.

Table 4

RNase digestion of labelled duck ~~28~~ 27 S rRNA and hnRNA.

RNA species	Temperature of RNase digestion	Total RNase stable material.(Percentage of total)
a) ~~28~~ 27 S rRNA	37°C	0.7 %
	0°C	3.0 %
b) Giant hnRNA	37°C	3.0 %
	0°C	7.0 %
c) Renatured giant hnRNA (after urea denaturation)	37°C	3.0 %
	0°C	7.4 %

Hybridisation experiments between labelled poly U and the poly A sequences in unlabelled RNA samples ~~is~~ are a precise method of determining the amount of poly A sequences in an RNA sample. As nearly all mRNA species contain poly A sequences (Adesnik et al, 1972) and all other cytoplasmic RNA species lack these poly A sequences, this method is a precise way of determining the amount and the sedimentation coefficients of mRNAs in any given cytoplasmic preparation run on sucrose gradients.

The identification of haemoglobin mRNA sequences can be done using labelled cDNA (DNA complementary to haemoglobin mRNA) prepared from a haemoglobin mRNA template using reverse transcriptase. Hybridisation of this labelled cDNA with unlabelled RNA produces a very accurate method for analysing the amount and sedimentation coefficients of haemoglobin mRNAs in cytoplasmic RNA preparations run in sucrose gradients.

These two hybridisation methods were used to characterise mRNA molecules of cytoplasmic RNA in non-denaturing 'sucrose - SLS' gradients and in denaturing 'formamide' gradients. Figure 15 shows a typical ~~optical density~~ profile of unlabelled cytoplasmic RNA run in a 'sucrose - SLS' gradient. Separate aliquots from each gradient fraction were used for hybridisation with labelled poly U and labelled cDNA (DNA complementary to haemoglobin mRNA). The poly U hybridisation pattern shows a fairly broad peak at 9 - 10 S and other peaks at about 21 S, 27 S and possibly 32 S in the gradient. The cDNA hybridisation pattern shows similar peaks at 9.5 S, 21 S, 27 S and again possibly 32 S. The exact pattern of hybridisation across 'sucrose - SLS' gradients of unlabelled cytoplasmic RNA showed considerable variation in the relative sizes of these peaks, although the positions of the peaks in the gradients and the ratio of poly U to cDNA binding were essentially the same in all cases.

Legend to figure 15

Poly U and cDNA hybridisation across a 'sucrose - SLS' gradient containing unlabelled cytoplasmic RNA.

Unlabelled cytoplasmic RNA (0.5 mg) was spun on a 16 ml 5-30%, 0.5% SLS, NTE gradient for 15.65 hours at 25°C, 24 K. Poly U hybridisation across the gradient was done using 10 λ aliquots from each fraction and cDNA hybridisation across the gradient was done using a 1 in 150 dilution of each appropriate fraction. The weight of poly U and cDNA binding to their complementary sequences was plotted against fraction number.

●——————● cDNA hybridisation

o- - - - - - - - -o poly U hybridisation

FIGURE 15

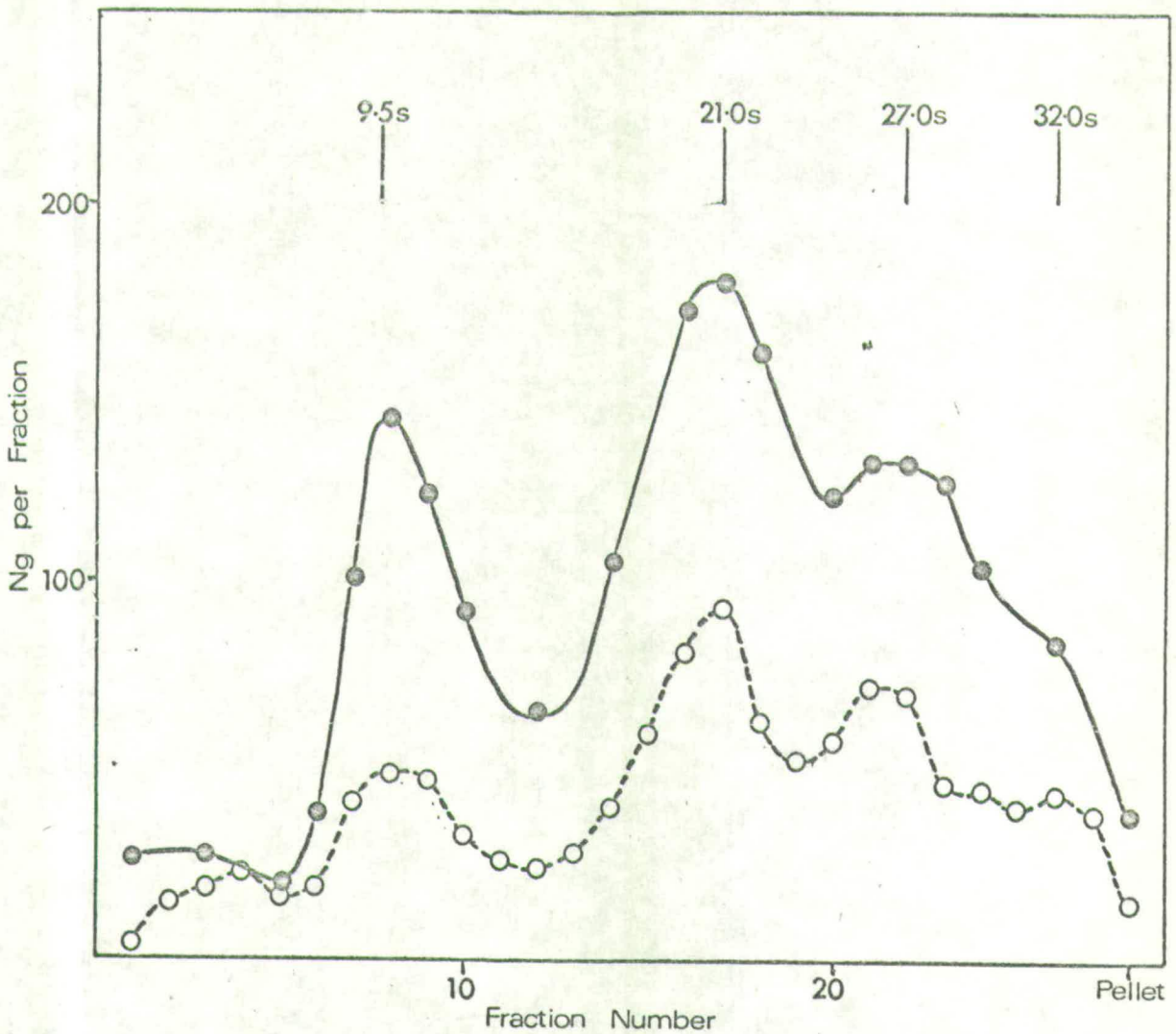

9·5s
21·0s
27·0s
32·0s
200
100
Ng per Fraction
10
20
Pellet
Fraction Number

Figure 16 shows hybridisation profiles across unlabelled cytoplasmic RNA run in a 'formamide' gradient. Aliquots from each gradient fraction were used for hybridisation with labelled poly U and cDNA. Here the poly U and cDNA hybridisation patterns across the gradient essentially follow the same pattern with a fairly precise peak at 9.5 S. No heavier hybridising species are found in such 'formamide' gradients.

The results of figures 15 and 16 imply that some of the 9.5 S haemoglobin mRNA is aggregated in complexes of 21 S, 27 S and 32 S in non-denaturing conditions, while in denaturing conditions the aggregates are destroyed. Wagner et al (1967), Berns et al (1967), Maroun et al (1971) and Hogan and Gross (1972) have shown that non-specific RNA aggregations can arise during phenol extractions of RNAs, in particular mRNAs. Figure 17 shows poly U and cDNA hybridisation profiles across 'sucrose - SLS' gradients containing enriched fractions of mRNAs prepared by proteinase K extraction (figure 17a) and phenol extraction (figure 17b). There are substantially less RNA species hybridising at sedimentation coefficients greater than 9.5 S in the proteinase K extracted RNA than the phenol extracted RNA. This result implies that mRNA aggregation occurs during the phenol extraction step in the preparation and purification of cytoplasmic RNAs. As phenol extraction procedures are widely used throughout this work in the preparation and purification of RNAs; this implies that other RNA species, in particular hnRNAs, may also be aggregated when analysed in non-denaturing sucrose gradients. This problem is discussed fully in the Discussion section.

The poly A sequences found at the 3' - terminal position of the haemoglobin mRNA molecule is about 150 nucleotides in length (Pemberton and Baglioni, 1972). It is expected that the length of poly U binding

Legend to figure 16

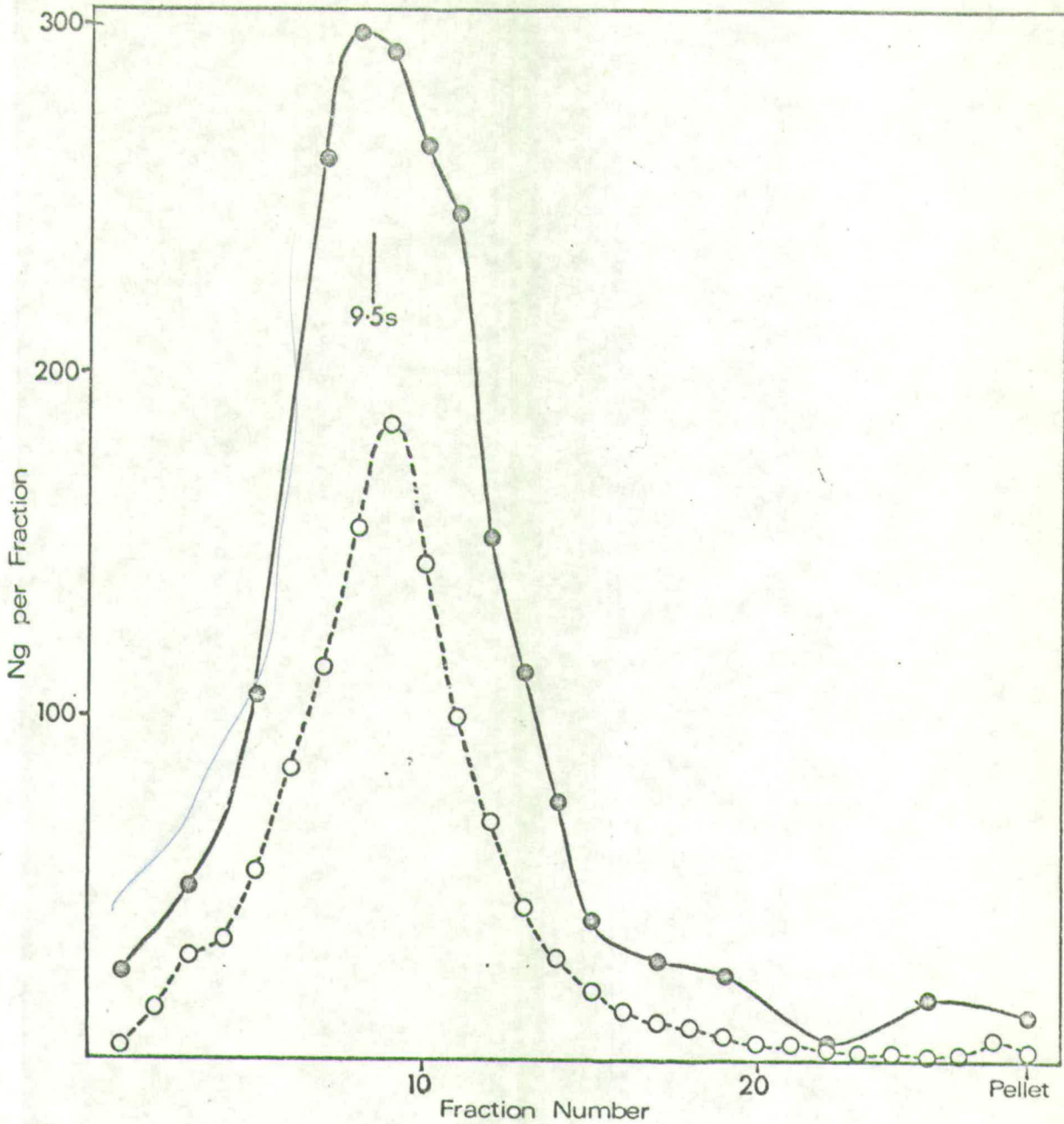
Poly U and cDNA hybridisation across a 'formamide' gradient containing unlabelled cytoplasmic RNA

Unlabelled cytoplasmic RNA (0.5 mg) was spun on a 14 ml 2-10% sucrose, 85% formamide, TE gradient for 54.6 hours at 25°C, 24 K. Poly U hybridisation across the gradient was done using 10 λ aliquots from each fraction and cDNA hybridisation across the gradient was done using a 1 in 150 dilution of each appropriate fraction. The weight of poly U and cDNA binding to their complementary sequences was plotted against fraction number.

●———————● cDNA hybridisation

○— — — — — — —○ poly U hybridisation

FIGURE 16



Legend to figure 17

Poly U and cDNA hybridisation across 'sucrose - SLS' gradients containing messenger enriched cytoplasmic RNA.

a) Unlabelled cytoplasmic RNA was prepared using proteinase K and messenger enriched RNA fractions were purified on an oligo dT - cellulose column. The RNA was then spun on a 16 ml 15-30% sucrose, 0.5% SLS, NTE gradient at 25°C, 24 K.

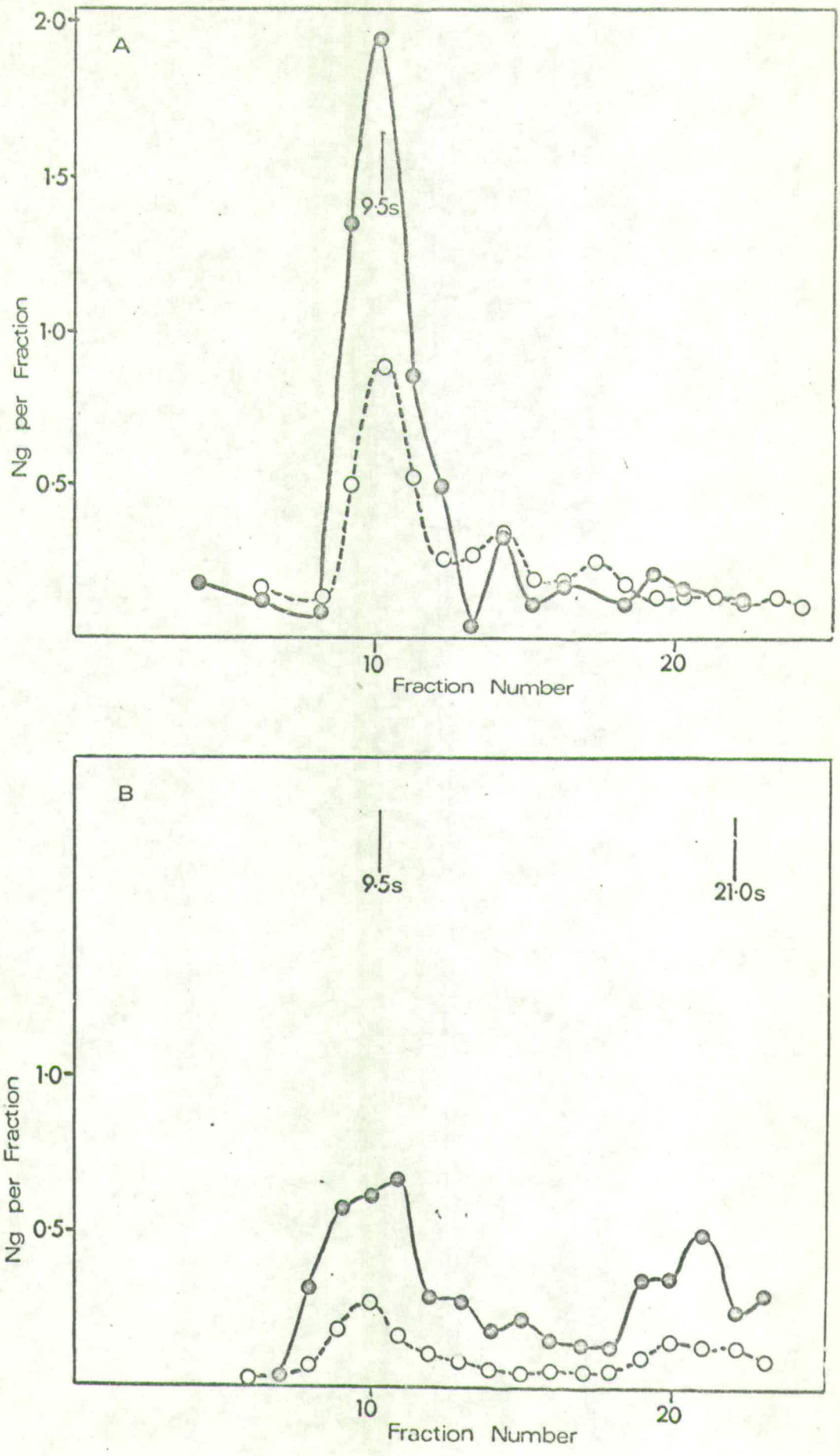
b) Unlabelled cytoplasmic RNA was prepared by phenol extraction and messenger enriched RNA fractions were purified on an oligo dT - cellulose column. The RNA was then spun on a 16 ml 15-30% sucrose, 0.5% SLS, NTE gradient at 25°C, 24 K.

Poly U and cDNA hybridisations were done across both gradients and the amount of poly U and cDNA binding to their complementary sequences was plotted against fraction number.

•————• cDNA hybridisation

o- - - - - -o poly U hybridisation

FIGURE 17


A
2·0
1·5
1·0
0·5
Ng per Fraction
9·5s
10
20
Fraction Number
B
9·5s
21·0s
1·0
0·5
Ng per Fraction
10
20
Fraction Number

to this poly A sequence would be of a similar length. As described in the Materials and Methods section, the cDNA transcript of haemoglobin mRNA is somewhat larger being just less than 300 nucleotides in length. So nearly twice the weight of cDNA compared to poly U would be expected to bind to a haemoglobin mRNA molecule.

However, other factors have to be considered concerning the amount of cDNA and poly U binding occurring in gradients of cytoplasmic RNAs. Firstly, poly U hybridisation picks up not only haemoglobin mRNA but also the other mRNAs produced in duck erythrocytes. Secondly, the hybridisation reaction between haemoglobin mRNA sequences and the cDNA transcript of haemoglobin mRNA takes place very slowly compared to poly U hybridisation and even after 20 hours incubation at 70°C the reaction is not totally complete. Therefore, the cDNA binding to haemoglobin mRNA sequences may be slightly underestimated. However, the ratio of cDNA binding to poly U binding in figures 15 to 17 is approximately 2 : 1. This implies that the majority of mRNAs produced in duck erythrocytes and hybridising with poly U are mRNAs coding for haemoglobin.

The data from figure 16 show that about 2.4 μg of cDNA binds to the RNA in this gradient. Assuming that the cDNA molecule codes for only 260 nucleotides out of the 630 nucleotides that comprise the haemoglobin mRNA molecule (Bishop and Freeman, in press), then about 5.9 μg of haemoglobin mRNA molecules are found in the gradient. As the total cytoplasmic RNA loaded on to the gradient was 0.5 mg, then about 1.2% of the total cytoplasmic RNA consists of haemoglobin mRNA.

g) Hybridisation of poly U and DNA (complementary to haemoglobin messenger RNA) across gradients containing unlabelled heterogeneous nuclear RNA.

There is considerable evidence, discussed at length in the Introduction, of the presence in small quantities of mRNA sequences in hnRNA. Hybridisation experiments with labelled poly U and cDNA were done across 'sucrose - SLS' and 'formamide' gradients containing unlabelled hnRNA to determine the quantity of these mRNA sequences and the size of molecules containing these mRNA sequences.

Figure 18 shows a typical pattern of poly U and cDNA hybridisation across an unlabelled hnRNA 'sucrose - SLS' gradient. Aliquots from each gradient fraction were used for hybridisation with labelled poly U and cDNA. The poly U and cDNA patterns across the gradient follow essentially the same pattern with most of the binding occurring in the pellet. Possibly, some aggregation of the hnRNA has occurred during the phenol extraction stage in the preparation of the RNA, in a similar manner as has apparently occurred for the mRNA species of figures 15 and 17b.

Figure 19 shows a typical pattern of the poly U and cDNA hybridisation across an unlabelled hnRNA 'formamide' gradient. Aliquots from each gradient fraction were used for hybridisation with labelled poly U and cDNA. In this case, a completely different pattern from 'sucrose - SLS' gradients is obtained. A single peak of cDNA binding occurs at about 14 S whilst the poly U peak sediments more heterogeneously in the range 10 S to 20 S with a peak about 15 S.

The data from figure 19 show that about 30 ng of cDNA binds to the

Legend to figure 18

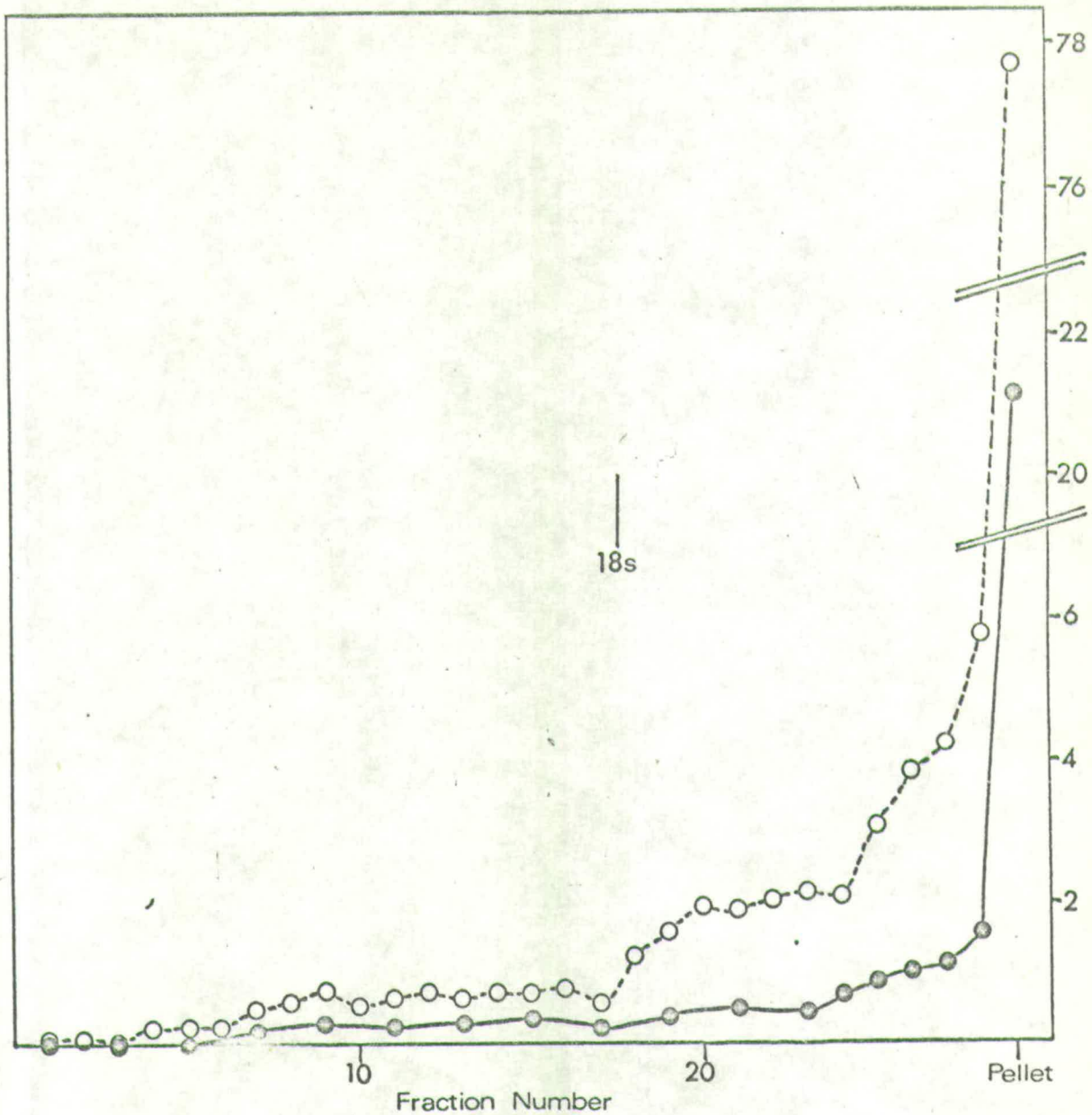
Poly U and cDNA hybridisation across a 'sucrose - SLS' gradient containing unlabelled hnRNA.

Unlabelled hnRNA (from 1.2 mls of packed cells) was spun on a 16 ml 5-30% sucrose, 0.5% SLS, NTE gradient for 15.9 hours at 25°C, 24 K. Poly U hybridisation across the gradient was done using 100 λ aliquots from each fraction and cDNA hybridisation across the gradient was done using a 1 in 3 dilution of each fraction, except for a 1 in 150 dilution for the pellet fraction. The amount of poly U and cDNA binding to their complementary sequences was plotted against fraction number.

●——————● cDNA hybridisation

o——————o poly U hybridisation

FIGURE 18



Legend to figure 19

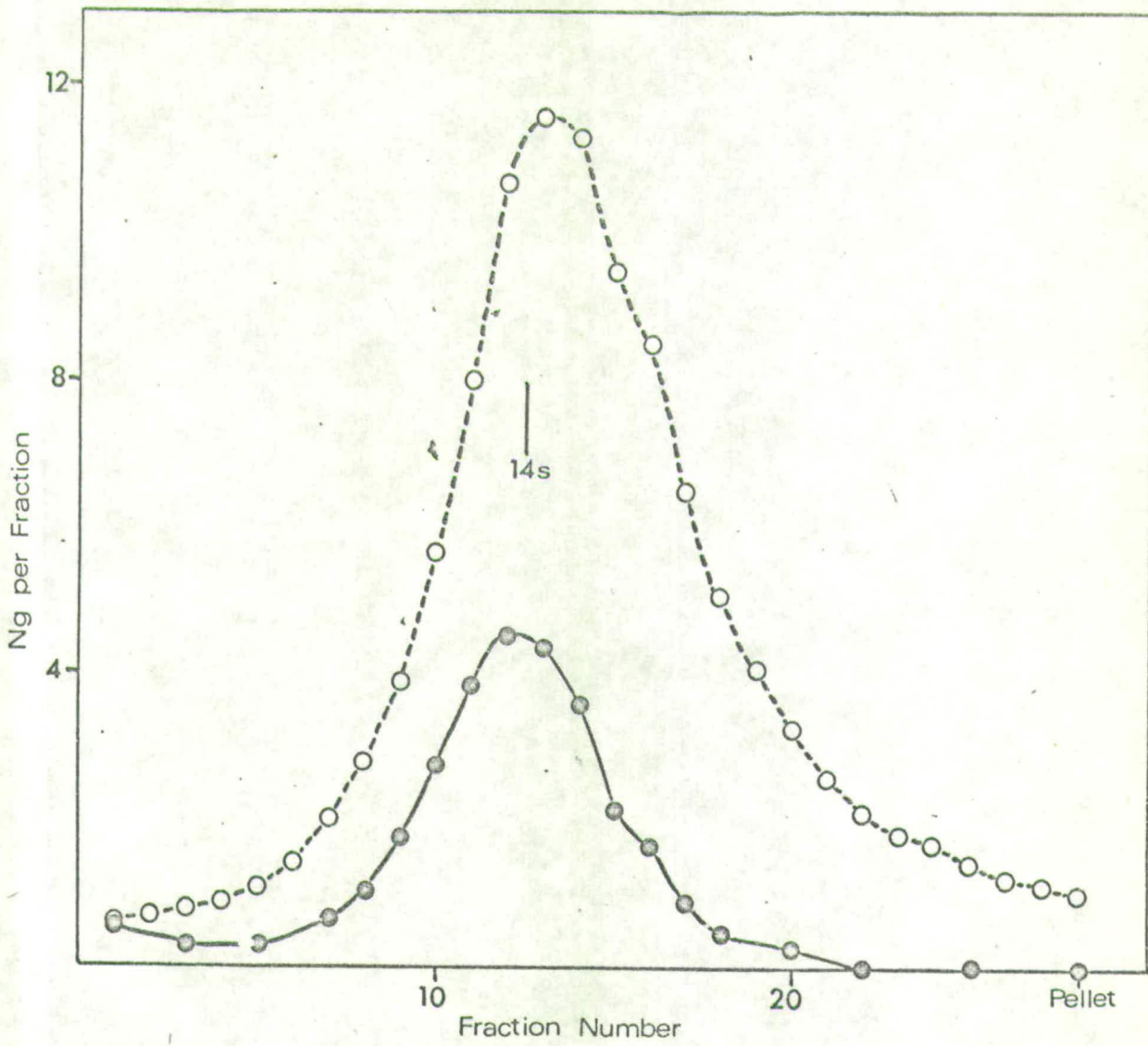
Poly U and cDNA hybridisation across a 'formamide' gradient containing unlabelled hnRNA.

Unlabelled hnRNA (from 1.2 mls of packed cells) was spun on a 14 ml 2-10% sucrose, 85% formamide, TE gradient for 54.6 hours at 25°C, 24 K. Poly U hybridisation across the gradient was done using 100 λ aliquots from each fraction and cDNA hybridisation across the gradient was done using a 1 in 3 dilution of each fraction. The amount of poly U or cDNA binding to their complementary sequences was plotted against fraction number.

●———————● cDNA hybridisation

○- - - - - -○ poly U hybridisation

FIGURE 19



hnRNA in this gradient. Assuming that the cDNA molecule codes for only 260 nucleotides out of the 630 nucleotides that comprise the haemoglobin molecule (Bishop and Freeman, in press), then about 73 ng of haemoglobin mRNA sequences are in the gradient The hnRNA loaded on to this gradient was obtained from 1.2 mls of packed cells. As described above, data from figure 16 show that about 5.9 μg of haemoglobin mRNA are found in 0.5 mg of cytoplasmic RNA. The results summarised in table 3 show that 0.15 mls of packed cells produce about 0.5 mgs of cytoplasmic RNA. Hence, by comparing the amount of haemoglobin sequences found in cytoplasmic and nuclear RNA fractions from the same quantity of packed cells, about 0.2% of the total haemoglobin mRNA sequences are found in nuclear RNA. This further implies that about 0.0002% of the total cellular RNA consists of nuclear haemoglobin mRNA sequences.

h) Characterisation of the 14 S heterogeneous nuclear RNA molecule.

Figure 20 shows a typical pattern of poly U and cDNA hybridisation across an unlabelled hnRNA 'formamide' gradient, in which aliquots from each gradient fraction were used for hybridisation with labelled poly U and cDNA. The pattern obtained is very similar to figure 19, in which the hnRNA was treated in a similar manner. In this case, however, some of the hnRNA molecules containing poly A sequences have higher sedimentation coefficients than in figure 19, but this is probably due to incomplete denaturation of the hnRNA. It is important to determine that the peak picked up by hybridisation in fact consist of RNA species and not by DNA contamination. Figure 20 shows the effect of treating each aliquot, to be used for cDNA hybridisation, with RNase before hybridising it with cDNA. No hybridisation peak is obtained after this RNA digestion treatment, showing that the hybridisation peaks consist of RNA.

Legend to figure 20

Poly U and cDNA hybridisation across a 'formamide' gradient containing unlabelled hnRNA.

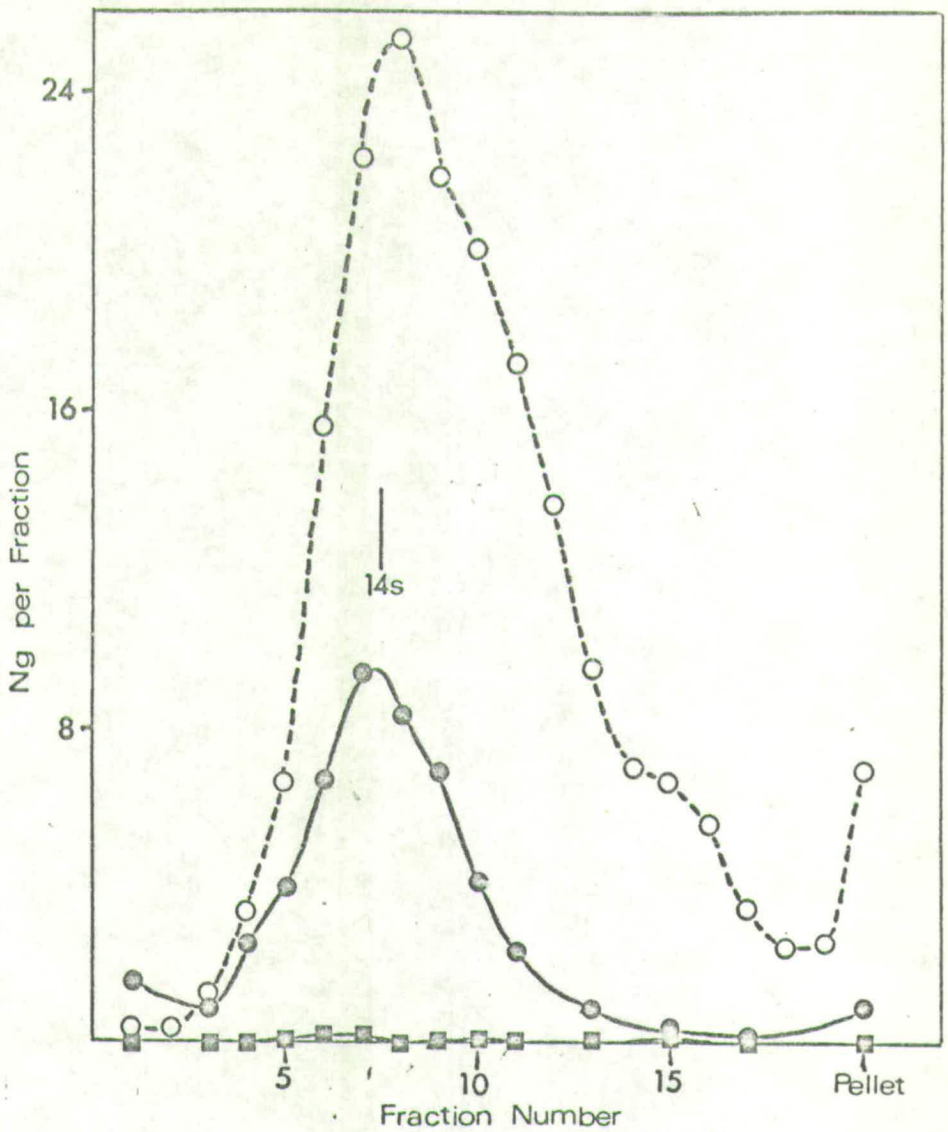
Unlabelled hnRNA (from 2.4 mls of packed cells) was spun on a 14 ml 2-10% sucrose, 85% formamide, TE gradient for 61.5 hours at 25°C, 24 K. Poly U hybridisation across the gradient was done using 100 λ aliquots from each fraction and cDNA hybridisation across the gradient was done using a 1 in 3 dilution of each appropriate fraction. cDNA hybridisation across the gradient was also done after treatment of the appropriate gradient aliquots with 20 μg/ml RNase at 37°C for 30 minutes. The amount of poly U and cDNA binding to their complementary sequences was plotted against fraction number.

●——————● cDNA hybridisation

o--------o poly U hybridisation

■——————■ cDNA hybridisation with RNase pretreatment

FIGURE 20



The results of figures 19 and 20 show that the 14 S RNA species, obtained from hnRNA after denaturation treatment, contains poly A and haemoglobin mRNA sequences. However, these results do not show that haemoglobin mRNA and poly A sequences are found in the same RNA molecules, as it is possible that these sequences could be found on different molecules of the same size.

To determine whether these sequences are in fact found in the same RNA molecules, aliquots of the 14 S hnRNA from the denaturing 'formamide' gradient (figure20) were absorbed on to an oligo dT - cellulose column in binding buffer. As oligo dT - cellulose selectively binds poly A sequences (Edmonds and Caramela, 1969; Sheldon et al, 1972; Sullivan and Roberts, 1973), any cDNA sequence could only be attached to the column if it was part of a molecule containing a poly A sequence. After washing the column with binding buffer, the poly A sequences were eluted from the column with elution buffer and the proportion of poly A and haemoglobin mRNA sequences in the various washings determined by poly U and cDNA hybridisation.

Table 5 shows the result of such an experiment. Nearly all the poly A and haemoglobin mRNA sequences are found in the elution washing, showing that haemoglobin mRNA and poly A sequences are found in the same molecule.

Table 6 shows the results of melting at low salt concentrations poly rA sequences bound to poly dT - sepharose and cDNA - sepharose columns. The poly T sequence in cDNA is about 40 nucleotides in length (Bishop and Freeman, in press) and so the poly rA binding to it would be expected to be of a similar length. A poly rA sequence of about 40 nucleotides is relatively short and the term oligo rA is more appropriate. On the other

Legend to table 5

100 λ aliquots from fractions 6-9 of the hnRNA 'formamide' gradient (figure 20) were pooled, diluted in binding buffer to give a final formamide concentration of less than 3% and passed very gently on to an oligo - dT cellulose column. The column was then washed with binding buffer and eluting buffer. Aliquots from the various washings were used in hybridisation experiments with poly U and cDNA and the percentage of poly U and cDNA binding to the RNA of each washing calculated.

Table 5

Percentage of poly U and cDNA hybridisation in different washes from an oligo - dT cellulose column loaded with 14 S hnRNA.

Washing	Percentage poly U binding activity	Percentage cDNA binding activity
Binding buffer containing the 14 S hnRNA	10.5	5.7
Binding buffer	1.4	1.2
Eluting buffer	88.1	93.1

Legend to table 6

Samples of about 10,000 c/m poly rA in 1 ml of 4 X SSC pH 5.5, 0.25% SLS were passed very gently on to cDNA - sepharose and poly dT - sepharose columns. The poly rA was eluted in low salt concentrations of 1 X SSC pH 5.5, 0.25% SLS at varying temperatures and finally in 1 mM tris pH 7.5, 0.25% SLS at 70°C. Aliquots from each washing were counted and the percentage of poly rA eluted at each temperature calculated.

Table 6

Melting of poly rA sequences from cDNA - sepharose and poly dT - sepharose columns.

Elution buffer	Temperature	Percentage of poly rA sequences in each eluate	
		cDNA - sepharose column	poly dT - sepharose column
1 X SSC pH 5.5, 0.25% SLS	20°C	0.5	1.7
1 X SSC pH 5.5, 0.25% SLS	35°C	1.2	0.7
1 X SSC pH 5.5, 0.25% SLS	60°C	75.3	11.1
1 X SSC pH 5.5, 0.25% SLS	70°C	21.5	84.2
1 mM tris pH 7.5, 0.25% SLS	70°C	1.5	2.3

hand, poly rA sequences binding to poly dT - sepharose columns are true polymers with relatively long sequences. Table 6 shows that oligo rA sequences are eluted from sepharose columns at temperatures of 60°C upwards, whilst the poly rA sequences are not eluted until much nearer 70°C. In this way oligo A and poly A sequences can be distinguished.

Table 7 shows the result of melting the 14 S hnRNA, bound to a poly - dT cellulose column in high salt, with a low salt buffer. The majority of poly A sequences were eluted at 70°C and, by comparing this result with that of table 6, it is clear that the poly A sequences of the 14 S hnRNA are present largely, if not totally, as poly A and not as oligo A. The messenger sequences (complementary to cDNA) were eluted in the same fractions as the poly A and this result confirms that of table 5, namely, that haemoglobin mRNA and poly A sequences are found in the same 14 S hnRNA molecule.

Legend to table 7

100 λ aliquots from fractions 6-9 of the hnRNA 'formamide' gradient (figure 20) were pooled, diluted in 4 X SSC pH 5.5, 0.25% SLS to give a final formamide concentration of less than 3% and passed very gently on to a poly dT sepharose column. RNA fractions were eluted in low salt concentrations of 1 X SSC pH 5.5, 0.25% SLS at varying temperatures and finally in 1 mM tris pH 7.5, 0.25% SLS at 70°C. Aliquots from the various washings were used in hybridisation experiments with poly U and cDNA and the percentage of poly U and cDNA binding to the RNA of each washing calculated.

Table 7

Percentage of poly U and cDNA hybridisation in elution fractions from a poly dT - sepharose column loaded with 14 S hnRNA.

Elution buffer	Temperature	Percentage of poly U binding activity	Percentage of cDNA binding activity
1 X SSC pH 5.5, 0.25% SLS	20°C	0.3	5.8
1 X SSC pH 5.5, 0.25% SLS	40°C	2.0	2.2
1 X SSC pH 5.5, 0.25% SLS	60°C	7.3	6.8
1 X SSC pH 5.5, 0.25% SLS	70°C	75.5	66.7
1 mM tris pH 7.5, 0.25% SLS	70°C	14.9	18.5

DISCUSSION

a) Secondary structure of hnRNA.

1) Introduction

Accurate determinations of the size and secondary structure of hnRNA molecules are important to any conclusions to be drawn concerning the relationships between cytoplasmic mRNAs and hnRNA. There are two main schools of thought concerning the size of hnRNA molecules. Scherrer et al (1966), Houssais and Attardi (1966), Warner et al (1966) and Holmes and Bonner (1973) suggest that hnRNA is a mixture of covalently bound molecules of very high molecular weight; whilst Parish and Kirby (1966), Bramwell and Harris (1967) and de Kloet et al (1970) consider that the molecular weights of hnRNA molecules are much less than supposed, due to aggregations between hnRNA molecules.

In this thesis, all the experiments designed to study the sizes of hnRNA molecules used hnRNA labelled with ^{3}H uridine. The radioactive profile of hnRNA was followed in sucrose gradients. The rate of turnover of the different RNA species in hnRNA is not the same so that the incorporation of labelled RNA precursors into hnRNA did not necessarily represent the different proportions of RNA species in hnRNA. It was important to remember this when analysing the results of the labelled hnRNA experiments.

The problem of the size of hnRNA was approached by treating labelled hnRNA preparations with three different denaturants. Such treatments produced essentially the same results with the hnRNA sedimenting in sucrose

gradients at about 18 S to 21 S instead of the much higher sedimentation coefficients found in non-denaturing gradients. On face value, these results imply that hnRNA consists of an aggregation of small molecules rather than larger molecular weight molecules. Renaturation experiments, where up to about 50% of hnRNA can form large complexes in non-denaturing conditions after previous denaturation treatment, also confirm this hypothesis. However, before such interpretations can be made, the effectiveness of the denaturants and the possibility of any RNA degradation during the preparation or analysis of hnRNA species have to be considered.

2) Denaturing conditions.

Parish and Kirby (1966), Bramwell and Harris (1967) and Tal (1969) using changes in ionic concentration; Boedtker (1967) and (1968), Cox (1969a) and Tal (1970) using formaldehyde and Strauss et al (1968) with DMSO have studied the precise denaturing conditions required to produce total denaturation of RNAs. The effectiveness of urea and formamide as denaturants of RNA has not been so accurately determined.

In the past many experiments have been designed in which only partially denaturing conditions were used, causing only partial denaturation of RNA secondary structures. However, often in these cases conclusions have been drawn which assume that total denaturation of the RNA secondary structure has occurred, even though no melting or other experiments to check the extent of denaturation had been done. In all denaturation experiments, the concentration of denaturants and the ionic conditions used are crucial. To avoid any such pitfalls in this thesis, the percentage denaturation of RNA obtained under different denaturing conditions was accurately determined by melting experiments. rRNAs were

used as controls for the melting experiments instead of hnRNA which was not easily obtainable in large enough amounts due to its extreme heterogeneity, under the assumption that the denaturing conditions required to remove the secondary structure from one RNA species were similar to those required for another RNA type. The results of Bramwell and Harris (1967) suggest this assumption is valid. Thus for the denaturing conditions used in this work, the exact percentage RNA denaturation expected was known (table 1).

The effectiveness of these denaturing conditions was confirmed using rRNA in various types of sucrose gradient. On denaturing rRNA molecules, it was found that the sedimentation coefficients of the rRNA species were transformed to much lower values than under normal conditions, caused by the unfolding of the secondary structure. Under the conditions used, formaldehyde and formamide treatment produced nearly total denaturation, whilst urea treatment produced only about two thirds denaturation (table 2)

3) RNA degradation.

A much more serious problem in the interpretation of the hnRNA denaturation experiments is the possibility of RNA degradation at some time during the extraction or analysis of hnRNA.

According to McPhie et al (1966), Gould (1967) and Cox (1969b) the structure of rRNA subunits is protected from nuclease and thermal degradation by virtue of its secondary structure. Points particularly susceptible to breakage in the polynucleotide chain are found inside the three dimensional structure. If these susceptible points are cleaved the secondary structure of the RNA is unaffected. On denaturation, however,

the secondary structure is destroyed, the cleaved points in the polynucleotide chain exposed and the molecule split into smaller fragments. As described in the Introduction, it appears in all cases that the large 27 S rRNA subunit is more susceptible to such action than the small 18 S rRNA subunit.

Results obtained, in this work, suggest a transition of the 27 S rRNA subunit to about 21 S and the 18 S rRNA subunit to about 14 S on denaturation, presumably due to the unfolding of the RNA secondary structure. However, thereis a further transition of some of the 21 S RNA to about 14 S and other small RNA species, in this case presumably caused by a nicking of the 27 S polynucleotide chain. The probable partial cleavage of the 27 S rRNA molecule, although fairly minor, raises the point that hnRNA molecules may also be cleaved to some extent with similar treatments. However, there appears to be no mRNA degradation during its preparation and analysis as the position of the labelled profile of the presumed 9.5 S haemoglobin mRNA appears exactly where expected in sucrose gradients.

Throughout this work extreme care has been taken with the RNA in order to prevent any RNA degradation. This has been done by using sterile solutions, glassware and apparatus wherever possible, using nuclease inhibitors such as SLS or keeping RNA solutions cold in ice baths in order to minimise RNA degradation. The apparent rRNA degradation observed is very small and highly specific and similarly if any hnRNA molecules had been cleaved this would be expected to be on a small scale and also at specific points. Nevertheless, if only one or two points on a long polynucleotide chain of hnRNA were cleaved by nuclease or heat, this could cause a large reduction in the sedimentation coefficients of hnRNA on denaturation producing molecules of smaller molecular weights, although in

normal sucrose gradients the hnRNA would still appear to have large molecular weights. Any large scale degradation of hnRNA would result in the appearance of low molecular weight RNA material sedimenting at the top of sucrose gradients. This has never been observed.

In all cases of hnRNA denaturation studied a profile of radioactivity appears in the 18 - 21 S region of sucrose gradients, but with a small proportion, up to about 6%, of activity remaining as very heavy material at the bottom of the gradients. These results imply either that the majority of hnRNA molecules consist of an aggregate of smaller molecules joined by hydrogen bonding or that large hnRNA molecules are cleaved at certain precise points. The minority of hnRNA molecules that do not suffer a decrease in sedimentation coefficient on denaturation must indeed be of very high molecular weight.

Furthermore, the renaturation experiments, where small denatured hnRNA molecules can under normal conditions renature up to about 50%, suggest that hnRNA molecules have many similar sequences able to bind together in a relatively non-specific fashion. Such similar sequences in hnRNA have been identified as transcripts of the intermediate reiterated DNA sequences (Britten and Kohne, 1968) making up about a third of the total hnRNA in rat (Melli et al, 1971). It is unlikely that any cleavage of hnRNA would occur randomly in these repeated regions. Any cleavage points would be expected to be found at specific points in the hnRNA molecules, as using various different denaturation techniques produce similar highly reproduceable profiles of hnRNA molecules sedimenting with a peak at 18 - 21 S.

4) <u>RNA aggregation</u>.

There is a probability that the hnRNA molecules obtained after extraction are in an aggregated state. This idea has been mentioned previously, but it is important to emphasise that the apparent very high molecular weight of hnRNA found in 'sucrose - SLS' or 'cold sucrose' gradients may possibly be an artifact of preparation observed in vitro but not in vivo. Such a result would obviously have a considerable influence on possible models of hnRNA structure.

All hnRNA in this work was prepared using phenol extraction procedures to remove attached protein. However, there is evidence that phenol treated RNA has a tendency to aggregate. In figure 17, cytoplasmic mRNAs were prepared by using a phenol extraction procedure as well as a proteinase K method for deproteinisation. The phenol extracted mRNA showed a considerable tendency to form mRNA aggregates, whilst the proteinase K extraction method produced little, if any, aggregation.

The tendency for phenol extraction procedures for RNA to cause RNA aggregation have also been observed by Wagner et al (1967) with rRNAs and Berns et al (1971), Maroun et al (1971) and Hogan and Gross (1972) with various mRNAs. Hot phenol extraction methods have been shown to produce hnRNA molecules with different properties depending upon the extraction temperature used, (Georgiev et al, 1972; Ryskov et al, 1972; Markov and Arion, 1973).

However, hnRNA is complexed with considerable amounts of protein and DNA (Bramwell and Harris, 1967), and relatively severe methods of hnRNA extraction are required in order to obtain hnRNA in an uncontaminated state. Milder methods of extracting hnRNA, without using phenol, have generally produced hnRNA complexed with considerable amounts of protein

(Samarina et al, 1968). Thus, severe deproteinising agents such as phenol must be used to obtain pure hnRNA.

5) Conclusions.

In vivo most hnRNA is transcribed and degraded extremely rapidly and is never transferred to the cytoplasm or translated (Harris, 1963). It is generally found in close association with the chromosomes and is probably bound to the DNA, if only very loosely. Bramwell and Harris (1967) and Samarina et al (1968) have shown that considerable amounts of protein, presumably of chromosomal origin are complexed with this RNA. In fact, Samarina et al (1968) and Georgiev et al (1972) have observed that these ribonucleoprotein complexes, which they called 'informofers', have a definite polysome type structure. Thus, there is no reason to suppose that the in vivo structure of hnRNA bears any relation to the observed secondary structure, found in vitro.

The size of hnRNA in vitro has been shown, in this thesis, to be very large and heterogeneous in non-denaturing sucrose gradients and to contain considerable secondary structure. This result is in agreement with those found by other workers although, as described in the Introduction, large differences in the sizes of hnRNAs have been observed by different workers depending upon the conditions used for the extraction and analysis of the hnRNA. Phenol extraction procedures were used in the preparation of the hnRNA and this possibly could have caused some of the aggregation.

Denaturing gradients were used in order to find the sizes of individual hnRNA molecules in conditions where all secondary structure was destroyed. The results show that the denatured hnRNA molecules sediment

heterogeneously with a peak about 18 - 21 S in these sucrose gradients. Three different denaturants were used and all of them produced similar results. It is not clear at present whether hnRNA molecules *in vitro* in fact consist of larger RNA molecules cleaved at specific points in their polynucleotide chains or consist of smaller heterogeneous molecules peaking at 18 - 21 S. If specific cleavage does occur during extraction or analysis of the hnRNA, this would be expected to correspond to *in vivo* processing points. This theme will be expanded later in the discussion.

b) Relationship between hnRNA and cytoplasmic mRNAs.

1) Introduction.

As discussed in the Introduction of this thesis, there is considerable evidence suggesting that a precursor - product relationship exists between some of the hnRNA and cytoplasmic mRNAs. Although extremely large amounts of hnRNA are synthesised, most of this hnRNA is degraded very rapidly in the nucleus and only a small proportion is transferred into the cytoplasm. Various functions suggested for the 90 - 95% of the hnRNA that never leaves the nucleus include a possible involvement of this RNA in the control of rRNA synthesis (Schmid and Sekeris, 1973). However, only about 5 - 10% of the hnRNA is transferred to the cytoplasm and is likely to serve a cytoplasmic function. It is this portion of the hnRNA that is of interest in the study of hnRNA - mRNA relationships.

2) Identification of haemoglobin mRNA.

In anaemic duck erythrocytes about 85 - 90% of the protein synthesis is haemoglobin (Scherrer et al, 1966), so that in the cytoplasmic RNA

samples studied, most of the mRNA codes for a single protein species, haemoglobin. The haemoglobin mRNA of duck has been identified as a molecule sedimenting at about 9.5 S in sucrose gradients with a poly A sequence of about 150 nucleotides (Pemberton and Baglioni, 1972) and able to synthesise duck haemoglobin in a rabbit reticulocyte cell free system (Pemberton et al, 1972).

In this work, hybridisation with poly U and cDNA across denaturing and non-denaturing sucrose gradients containing cytoplasmic RNA were used to identify this haemoglobin mRNA. As previously discussed some aggregation of cytoplasmic messenger-like RNA molecules had apparently occurred during their extraction, but these aggregations were removed in denaturing gradients where both poly U and cDNA hybridised to a single RNA species at 9.5 S in roughly the proportions expected for the haemoglobin mRNA molecule. This RNA species was assumed to be the haemoglobin mRNA.

Reports from some laboratories have suggested the presence of hnRNA species in the cytoplasm of some cells, (Penman et al, 1968; Spirin, 1969). These results are probably artifactual caused by either nuclear leakage or aggregations of cytoplasmic mRNAs occurring during their extraction or analysis. Certainly, no large mRNA-like species were obtained in the denaturing gradients used in this work.

3) Identification of hnRNA molecules containing mRNA sequences.

Considerable evidence has been accumulated suggesting the presence of messenger sequences in hnRNA. As described in the Introduction, these sequences have been identified by competition-hybridisation experiments with mRNA molecules to DNA, hybridisation of anti-messenger and poly U molecules to hnRNA directly and by the translation of hnRNA molecules

to produce protein in cell free systems. Such experiments have usually been attempted using hnRNA of different sedimentation coefficients from non-denaturing sucrose gradients or gels. However, precise size characterisation of the molecules containing these messenger sequences has been difficult as these sequences only represent a very small portion of the total hnRNA.

In this thesis a different approach to the problem was taken, namely, the denaturation of hnRNA in denaturing sucrose gradients to produce much smaller molecules, followed by hybridisation of poly U and cDNA molecules to this denatured hnRNA. Both poly U and cDNA hybridised to RNA molecules in the 14 S region of these gradients. Individual 14 S molecules contained both poly A and messenger sequences, as shown by passing 14 S molecules through oligo - dT cellulose and poly - dT sepharose columns. Haemoglobin messenger sequences were bound to and eluted or melted from the columns along with the poly A sequences, as shown by hybridisation of aliquots of the washings with poly U and cDNA. The melting experiments on the 14 S hnRNA molecules bound to the poly - dT sepharose column showed that these poly A sequences were relatively long (poly) rather than short (oligo).

It is important to emphasise that these 14 S hnRNA species represent only a small proportion of the poly A containing hnRNA molecules, which sediment heterogeneously in formamide gradients in the range of 10 S to 25 S with a peak about 15 S. Furthermore, the poly A containing hnRNA molecules themselves form only a small proportion of the total hnRNA molecules, which sediment heterogeneously at about 15 S to 35 S with a peak about 21 S (figure 21).

Legend to figure 21

Poly U hybridisation across a 'formamide' gradient containing labelled hnRNA

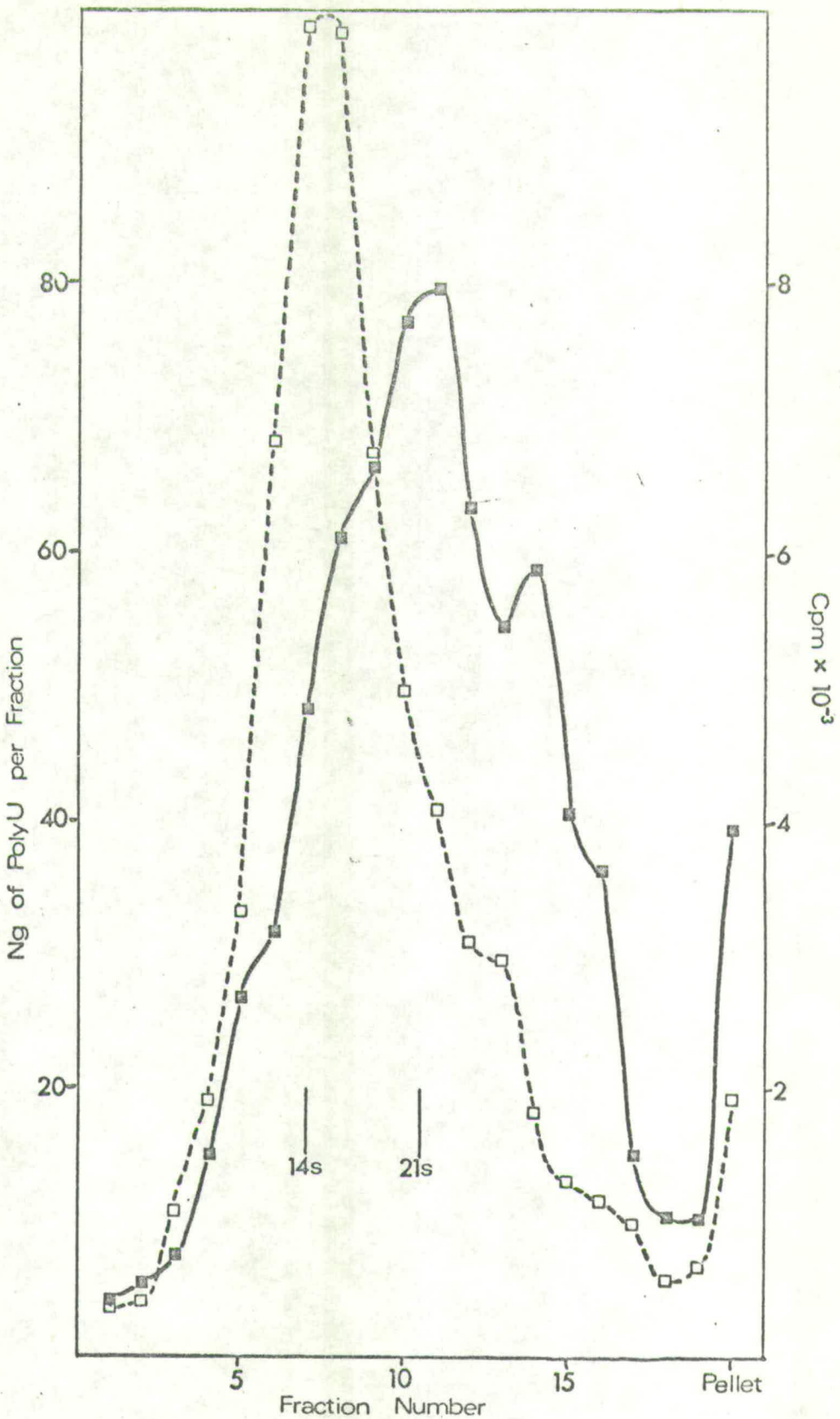
^{3}H labelled hnRNA (from 7.2 mls of packed duck blood cells) was spun on a 16 ml 2 - 10% sucrose, 85% formamide, TE gradient for 61.5 hours at 24 K, 25°C. 50 λ aliquots from each gradient fraction were counted. Poly U hybridisation was done using 100 λ aliquots from each fraction. The amount of poly U binding to its complementary poly A sequences was plotted against fraction number.

Although the hnRNA and the poly U were both labelled with ^{3}H uridine, all the labelled uridine of the hnRNA would be expected to be digested by nuclease during the poly U hybridisation procedure and would not be expected to contribute any activity to the poly U hybridisation profile.

●——————● counts per minute of hnRNA label.

□- - - - - - - - - -□ poly U hybridisation

FIGURE 21


80
60
40
20
8
6
4
2
Ng of PolyU per Fraction
Cpm x 10-3
14s
21s
5
10
15
Pellet
Fraction Number

4) Relationship between the 14 S hnRNA and cytoplasmic haemoglobin mRNA.

The haemoglobin mRNA is about 630 nucleotides long with a poly A sequence of about 150 nucleotides in length at its 3' end (Pemberton et al, 1972). The cDNA used was less than 300 nucleotides in length, or rather, allowing for about 40 nucleotides of poly T, about 260 nucleotides which are complementary to the haemoglobin messenger sequence (Bishop and Freeman, in press). Using the data of figure 7, the 14 S hnRNA species has a molecular weight of approximately 6.2×10^5 daltons, which corresponds to about 1780 nucleotides. The 14 S hnRNA furthermore contains both poly A and haemoglobin messenger sequences and although the poly A sequence probably occurs at the 3' end (Kates, 1970; Edmonds et al, 1971; Darnell et al, 1971a and b and Mendecki, 1972), the length of it and the haemoglobin messenger sequence is not known. The similarities and differences between the haemoglobin mRNA and the 14 S hnRNA are summarised in table 8. The results show that in many respects these two RNA species are very similar.

The most obvious interpretation of these results is that the 14 S hnRNA is the nuclear precursor to the haemoglobin mRNA and the large number of additional nucleotides present in the 14 S molecule are responsible for its stability and/or processing of it. It is also possible that non-functional messenger-like sequences which have diverged in evolution from the haemoglobin mRNA (Paul, 1972) are transcribed in the nucleus and are found in the 14 S hnRNA. However, at the present time, any theories concerning the structure of the 14 S hnRNA molecule must be purely hypothetical.

5) Conclusions.

Various models, discussed in the Introduction, have been presented

Table 8

Characteristics of 14 S hnRNA and cytoplasmic haemoglobin mRNA.

	Haemoglobin mRNA	14 S hnRNA
Sedimentation coefficient in denaturing gradients.	9.5 S	14.0 S
Molecular weight	About 2.2 X 10^5 daltons	About 6.2 X 10^5 dalto
Number of nucleotides	About 630	About 1780

(Georgiev, 1969; Scherrer et al, 1970; Darnell et al, 1971b) which all suggest the same basic processing mechanism of the giant hnRNA molecules to form cytoplasmic mRNAs. These models suggest that the transcription of certain DNA sequences occurs producing large hnRNA molecules which are then processed into smaller RNAs, including cytoplasmic mRNAs. However, there is some doubt as to whether intermediate conversion steps occur in this transition. Georgiev et al (1972) favour the transformation of giant hnRNA ⟶ low molecular weight hnRNA ⟶ polysomal mRNA, as they have found that the sequence homology between low molecular weight hnRNA and polysomal mRNA is higher than that between giant hnRNA and polysomal mRNA. Conversely, Scherrer et al (1970) and Darnell et al (1971b) favour a direct transformation of hnRNA to cytoplasmic mRNA without intermediary conversion steps, although they by no means rule out the existence of such steps.

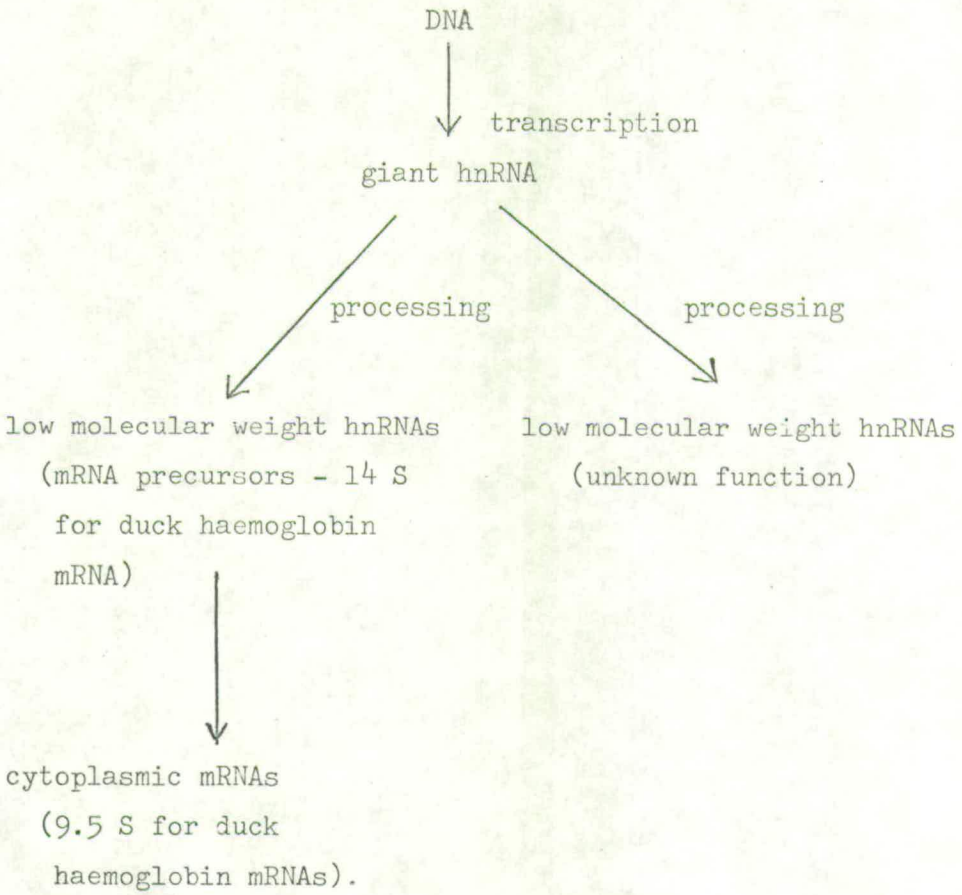
The results obtained in this thesis essentially agree with these models and the following conclusions can be made concerning the formation of mRNAs and in particular haemoglobin mRNAs in duck erythrocytes. HnRNA is transcribed from the genome as large heterogeneous molecules, the majority of which have a very high turnover rate and are degraded in the nucleus. At least some of this hnRNA is processed into smaller molecules of about 10 S to 25 S containing poly A sequences, which are probably cytoplasmic mRNA precursors. The haemoglobin mRNA precursor, identified by poly U and cDNA hybridisation, has a sedimentation coefficient of about 14 S and is about three times the size of the cytoplasmic 9.5 S haemoglobin mRNA. As no haemoglobin mRNA has been observed in the nucleus it is probable that the 14 S molecule is processed to haemoglobin mRNA on its transfer to the cytoplasm. The 14 S hnRNA species may protect the messenger sequence from nucleolytic action or be involved in the transfer

of this species to the cytoplasm. The function of the non-messenger precursor hnRNA molecules is not known. These conclusions are summarised in figure 22.

This simple model is based on studies of hnRNA molecules that have been purified by removal of protein and DNA which are closely bound to hnRNA molecules *in vivo*. Thus, the structure of this purified hnRNA obviously does not bear any close resemblance to the structure of hnRNA *in vivo* and the effect of this has not been considered in the formulation of the above model of hnRNA processing.

Figure 22

Possible model of mRNA formation in animal cells.



Addendum to Discussion

I should like to consider further the size of hnRNA in both non-denaturing and denaturing gradients which is open to considerable dispute as different workers have produced conflicting results. In particular I wish to consider further the possibility of *in vivo* processing of hnRNA molecules accounting for the small size of nuclear RNA on denaturing gradients, and the non-aggregation of 14S hnRNA molecules from formamide gradients on non-denaturing gradients.

De Kloet *et al.*(1970), Mayo and de Kloet (1971), Bramwell (1972), Scott and Kuhns (1972) and Peltz (1973) have all shown that hnRNA denatures or 'disaggregates' into much smaller molecules when spun on denaturing gradients. My results also show that in denaturing gradients hnRNA almost entirely exists as small molecules.

The earlier work of Bramwell (Bramwell and Harris, 1967) can be criticized on the grounds that breakage of RNA molecules had occurred. This invalidates their conclusions that hnRNA exists in the form of small molecules. The small size of hnRNA molecules observed in denaturing gradients by de Kloet *et al.*(1970), Mayo and de Kloet (1971), Bramwell (1972), Scott and Kuhns (1972), Peltz (1973) and myself can similarly be explained by cleavage of the hnRNA molecules. Certainly, some breakage of rRNA molecules did apparently occur in denaturing gradients in my work, (pages 51 and 112).

Holmes and Bonner (1973) and to a lesser extent Granboulan and Scherrer (1969) and Imaizumi *et al.*(1973) took stringent precautions to prevent any cleavage of hnRNA molecules during their preparation and analysis. Holmes and Bonner (1973) showed that hnRNA sedimented

through denaturing gradients of DMSO and formaldehyde still contain molecules of very high molecular weight. Similarly, Granboulan and Scherrer (1969) and Imaizumi et al.(1973) showed that in DMSO denaturing conditions, although much of the hnRNA is found as small molecules, some of the hnRNA still remains as large molecules.

These results can be best explained by assuming that the small hnRNA molecules observed in denaturing gradients are formed by cleavage. Thus, the small proportion of large hnRNA observed in these denaturing gradients would correspond to uncleaved hnRNA. However, it is unlikely that the small hnRNA molecules observed in denaturing gradients are formed by a random cleavage of larger molecules as the same profiles of small hnRNA molecules are observed using different denaturants. It is more likely that this cleavage is precise, corresponding to in vivo processing of hnRNA in a similar way as has been described for rRNA precursors, (Scherrer et al.,1963; Girard et al., 1964, and Weinberg et al., 1967).

Imaizumi et al.(1973) have further shown that some of the large hnRNA molecules on denaturing gradients contain haemoglobin mRNA sequences and are possibly cytoplasmic haemoglobin mRNA precursors. They further suggest that 'aged' or in vivo processed large hnRNA molecules have 'hidden breaks' and so appear as small molecules on denaturing gradients.

Various models have been presented, (Georgiev, 1969; Scherrer et al., 1970; Darnell et al., 1971b; and Imaizumi et al., 1973) which all suggest the same basic in vivo processing mechanism for large hnRNA (page 123). Figure 22 shows a summary of possible hnRNA processing and in particular of haemoglobin mRNA formation in duck erythrocytes.

However, aggregation of hnRNA molecules observed in non-denaturing gradients and the large amounts of DNA and protein (Bramwell and Harris, 1967) closely bound to hnRNA molecules in vivo are also important in the interpretation of any models of hnRNA structure, (pages 113-116).

Total denatured hnRNA from denaturing gradients reform large aggregated RNA in non-denaturing conditions, (figure 14 and Bramwell, 1972). However, samples of 14S hnRNA from the gradient of figure 20 rerun on non-denaturing gradients did not aggregate. This result suggests that there is insufficient complementarity between 14S hnRNA molecules for them to form aggregates with themselves and that other hnRNA molecules are required for aggregation to occur.

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