THE ISOLATION AND CHARACTERISATION OF SPECIFIC RNA MOLECULES AND RNA-DNA COMPLEXES IN ANIMAL CELLS.

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by

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1. INTRODUCTION

Genetic information is usually encoded in the base sequence of DNA. The first step in the expression of specific genes is the transcription of DNA cistrons into complementary RNA species. These then act as templates for protein synthesis. Gene expression might therefore be regulated at the levels of DNA transcription or RNA translation.

1.1 Regulation of gene expression in procaryotes.

The spectrum of proteins synthesised by procaryotes must be regulated by environmental conditions so that cellular resources are utilised efficiently.

RNA transcription

Flexible control of protein synthesis in E.coli is effected by regulation of the transcription of unstable messenger RNAs (mRNAs) coding for specific proteins; negative feed-back inhibition is mediated by repressor molecules which are synthesised by regulatory genes. These molecules combine with regulatory sequences in the DNA (Jacob and Monod, 1961a, 1961b). In some control systems, the regulatory genes synthesise inducers (Englesberg et al., 1969). The regulation of DNA - dependent RNA polymerase activity may also be a means of control at the level of transcription. Although all E.coli cellular RNA is synthesised by one RNA polymerase (Chamberlin and Berg, 1962), the activity of this polymerase may be controlled by a variety of sigma factors. These are required for the formation of a pre-initiation complex between RNA polymerase and a promoter site on the DNA. Factors with different binding

constants might vary the rate of chain initiation at different sites on the DNA (Bautz and Bautz, 1970). They might also influence RNA transcription by controlling the specificity of polymerase attachment to different DNA sites (Travers, 1969; Burgess et al., 1969). This control operates in the transcription of early genes by phage T4 (Bautz et al., 1969).

There is no evidence for permanently repressed regions in bacterial DNA. However, mRNA hybridisation (Kennell, 1968) and the visualisation of active genes (Miller et al., 1970) both show that only a small percentage of the whole E.coli chromosome is transcribed in one cell cycle.

RNA translation

There is little proof of regulation at the translation level such as proposed by Stent (1964). Delay in the formation of tryptophanase, once thought to be caused by masking of the tryptophanase messenger, is due only to the initial formation of an inactive enzyme precursor (Bilezikian et al., 1967).

No effect of inducer on β-galactokinase mRNA stability or translation could be shown (Leive and Kollin, 1967).

Coupled transcriptional and translational controls are possible through attachment of ribosomes to mRNA during its transcription (Byrne et al., 1964). In E.coli RNA polymerase - DNA - messenger RNA ribosome complexes, visualised in the electron microscope, most polyribosomes were associated with the DNA (Miller et al., 1970). Further, all lac operon messenger RNA is

associated with polyribosomes (Schwartz et al., 1970). Messenger translation by ribosomes therefore occurs while messenger is being translated by RNA polymerase.

Varying the half-lives of different messenger RNAs would be another means of regulation. The RNase responsible for messenger RNA breakdown has not been unambiguously identified. Although both $3' \rightarrow 5'$ and $5' \rightarrow 3'$ exonuclease action has been proposed, the evidence probably favours $5' - 3'$ breakdown (Morikawa and Imamoto, 1969; Morse et al., 1969).

Although most bacterial messenger RNAs have decay constants of less than five minutes (Levinthal et al., 1962), their half lives do vary within this range (Creaser, 1956). Where synthesis of a specific protein occurs throughout the cell cycle (Martinez, 1966) or in sporulating bacteria (Rosas del Valle and Aronson, 1962), the relevant messenger RNAs are stabilised.

Other factors influencing translational control might include the availability of specific initiation factors for different genes (Steitz et al., 1970) or the conformation of mRNAs (Fukami and Imahori, 1971).

1.2 Regulation of gene expression in eucaryotes

The physical organisation of even the most simple eucaryotes is more complex than that of the procaryotes. There is about a thousand times more DNA in eucaryotes. This is subdivided into separate chromosomes which contain acidic and basic proteins in close

association with the DNA.

RNA transcription

In many multicellular eucaryotes, the cells are differentiated into distinct cell types. Some examples of cellular specialisation are also found among the lower protists (Sonneborn, 1960; Bonner, 1963 ; Haemmerling, 1963). Superimposed on the flexible control of protein synthesis in non-specialised cells might be a more inflexible repression of non-functional genes in specialised cells. Such repression would more permanently restrict the range of RNAs transcribed and therefore of the proteins synthesised.

There is evidence that some DNA is relatively inactive in RNA synthesis (Littau et al., 1964; Himes, 1967).

This tightly-packed chromatin differs from the more diffuse euchromatin which is actively synthesising RNA (Frenster et al., 1963; Littau et al., 1964).

The ratio of euchromatin to heterochromatin varies in different cell types; more heterochromatin is found in differentiated cells whose range of transcription is limited (Fawcett, 1966).

Although the cytological differences between transcribing and non-transcribing DNA are known, the mechanisms both of long-term gene inactivation and of transitory changes in the levels of gene activity are still unclear.

The strongly basic intranuclear proteins, the histones, may be gene repressors (Stedman and Stedman, 1947, 1951). Their appearance in evolution coincides with cellular nucleation and differentiation

(Georgiev, 1969; Raaf and Bonner, 1968; Piéri et al., 1968).

Since RNA synthesised on a mammalian chromatin template hybridises to a smaller extent with homologous DNA than does RNA synthesised on a DNA template, the extracted proteins must restrict the transcription of RNA hybridising to repetitive DNA sequences (Paul and Gilmour, 1966 a ; 1968). A restriction of transcription by the F_1 histone has been less convincingly shown (Georgiev et al., 1966). Five main histone classes are separated by gel electrophoresis. The F2A1 fraction has been sequenced and is homogeneous. (Delange et al., 1969). The other fractions show only a small amount of microheterogeneity (Kinkade and Cole, 1966). Chemical modification such as acetylation (Allfrey et al., 1964), methylation (Paik and Kim, 1968) and phosphorylation (Kleinsmith et al., 1966) could increase the heterogeneity of a histone fraction and modify its electrostatic binding to DNA. Phosphorylation and acetylation of parts of histone IV may facilitate its dissociation from DNA prior to histone replacement by protamine in the developing rainbow trout testis (Sung and Dixon, 1970).

Most tissues examined have similar histone contents, although the range of proteins synthesised by different tissues varies widely. Some differences are found for example in wheat germ (Johns and Butler, 1962), Drosophila (Cohen and Gotchel., 1971), and in silk worm glands (Yoshida et al., 1966). In the differentiating nucleated erythrocyte, there is a decrease in the lysine - rich histone (Dick and Johns, 1969) and a minor basic histone appears (Neelin, 1964). Histone patterns vary during the embryogenesis of several species till the adult pattern is reached,

for example in chick embryo (Kischer and Hnilica, 1967) and sea urchin (Orengo and Hnilica, 1970).

It therefore seems likely that some histones do act as non-specific gene repressors, though others may serve to package the large amount of eucaryotic DNA (Mazia, 1965).

Acidic proteins are also present in chromatin. These proteins are more heterogeneous than the histones (Benjamin and Gellhorn, 1968) and have therefore more potential for specific gene regulation. The ratio of non-histone protein to DNA increases in euchromatin (Dingman and Sporn, 1964) and in DNA regions actively transcribing RNA (Beerman, 1963; Swift, 1962).

Although the histone contents of eu - and hetero - chromatin are similar, euchromatin melts at a lower temperature than heterochromatin (Frenster, 1965). Possibly, non-histone protein may antagonise histone - DNA interaction, thus lowering the Tm (Frenster, 1964).

This could account for the acidic proteins directing the formation of tissue-specific RNAs from DNA - histone - acidic protein complexes. The synthesised RNAs hybridised to different repetitive DNA fractions (Paul and Gilmour, 1968; Gilmour and Paul, 1970). There are similar differences in the RNAs synthesised from chromatins of different tissues (Flickinger et al., 1965; Paul and Gilmour, 1966 b ; Marushige and Bonner, 1966; Marushige and Ozaki, 1967).

Neither the observed changes in protein content nor the hybridisation of RNA transcribed from chromatin have yet been correlated with the

production of specific, functional messenger RNA species.

Many hormones stimulate transcription of non-ribosomal RNAs, for example, ecdysone (Clever, 1964), estrogen (Noteboom and Gorsky, 1963), erythropoetin (Krantz and Goldwasser, 1965), growth hormone (Korner, 1963) and steroid hormone precursors (Levere et al., 1967). There is no evidence that the primary site of action is transcription.

The availability of RNA polymerases might also regulate the synthesis of specific proteins. Whereas there is only one bacterial RNA polymerase, at least three polymerases have been found in sea urchins and in rat liver (Roeder and Rutter, 1969, 1970). Sea urchin polymerase I is found in the nucleolus. It may be responsible for ribosomal RNA (rRNA) synthesis. Polymerases II and III are nucleoplasmic and may transcribe mRNAs. The levels of these polymerases parallel the changes in the transcription of the relevant RNA fractions during sea urchin development (Roeder and Rutter, 1970). The intracellular levels of these polymerases might therefore regulate the transcription of different operons. Further levels of control might operate through factors similar to the sigma factor which regulates transcription in E. coli (Davidson et al., 1969).

RNA translation.

Control mechanisms in eucaryotes also operate on the processing and translation of transcribed RNAs.

The formation of rRNAs is mediated through a 45s RNA transcription product. This is sequentially broken down with loss of some sequences,

to the functional rRNAs (Review, Penman et al., 1966). The first products of non-ribosomal gene transcription have high and heterogeneous molecular weights (Scherrer and Marcaud, 1965; Soeiro et al., 1966; Attardi et al., 1966). Many of these RNAs have very short half lives and never leave the nucleus (Review, Harris, 1962). Much of the initial gene product is therefore processed to RNAs of varying half life and fate. The relationshi of mRNA to these products is still unclear.

Transcription and translation of eucaryotic mRNA are usually spatially separated. Protection of mRNA from nuclease action by association with other RNA species or with protein might be necessary. Ribonucleoprotein particles have been found in fish embryos (Spirin, 1966) and in other systems such as rat liver nuclei (Samarina, et al., 1967, 1968). These might be the form in which mRNAs are transported from the nucleus to the cytoplasm.

The 30s nuclear ribonucleoprotein particles found complexed together in rat liver nuclei, contain a specific exonuclease (Niessing and Sekeris, 1970). It is suggested that this exonuclease is responsible for the cleavage of nuclear high molecular weight RNA to nuclear DNA - like RNA.

Cytoplasmic mRNA may not be immediately utilised for protein synthesis. There is some evidence that pre-formed mRNAs in wheat seeds are activated on germination and are responsible for early protein synthesis (Marcus and Feeley, 1966). Similar storage of mRNA has been proposed in unfertilised eggs and other developing systems (Review,

Spirin 1966). The evidence for activation of pre-formed messengers is mainly based on the ability of the activated cells to synthesise proteins in the absence of detectable RNA transcription. The lack of protein synthetic activity could also be due to inactive ribosomes (Metafora et al., 1971).

The levels of cytoplasmic constituents such as glutathione could have general effects on the rate of protein synthesis (Zehavi-Willner et al., 1971). Levels of factors such as amino-acyl transfer RNA synthetases and transfer RNAs (tRNAs) could control specific protein translation (Timourian, 1967); a variation in tRNA composition has been found in differentiating plant tissues (Vold and Sypherd, 1968) and in the maturing erythrocyte (Smith and McNamara, 1971).

Other effectors may regulate the rate of formation of component polypeptide chains by the formation of completed proteins. An example is the regulation of haemoglobin synthesis by cytoplasmic heme (Levere and Granick, 1965, 1967).

Changes in the amount, conformation or nucleotide sequence of a messenger RNA could also directly affect its translation. The reduction of β chain haemoglobin in β-thalassemia is due to a defect in the β chain messenger and not to inactive ribosomes or other components of the protein synthetic apparatus (Gilbert et al., 1970; Nienhuis et al., 1971).

Further possible control mechanisms are the half-lives of functioning mRNAs. The half-lives of different eucaryotic mRNAs vary

widely within the same cell population for example, the rat liver (Pitot et al., 1965). The half-life of one messenger RNA population may vary at different stages of development, for example, in differentiating lens cells (Reeder and Bell, 1965; Stewart and Papaconstantinou, 1967).

Differential stabilisation of mRNAs is probably involved in the preferential synthesis of specific proteins in terminally-differentiated cells; the synthesis of haemoglobin in mouse yolk-sac and liver erythroid cells (Fantoni et al., 1968); the synthesis of cocoonase zymogen in the galea of the silk moth (Kafatos and Reich, 1968) and the synthesis of crystallin in lens fibre cells (Reeder and Bell, 1965; Stewart and Papaconstantinou, 1967).

1.3 DNA amplification

The control mechanisms discussed so far operate at the levels of RNA transcription or translation.

In differentiated cells, a selective amplification or loss of parts of the genome could be another means of specialisation in the production of specific proteins. Selective changes in somatic DNA content are well documented in invertebrates. Few examples are known so far among the vertebrates.

DNA content and evolution

There is a general correlation between the DNA content and degree of evolution of a genome. E. coli has fifty times the DNA content of a T2 bacteriophage. Higher plants and vertebrates have 250—1000 times

the DNA content of bacteria, with most unicellular algae and primitive metazoa having intermediate values. Within the complex phyla of the vascular plants and vertebrates, there is no simple correlation between DNA content and phylogenetic position; the vascular plants have even lost DNA during evolution.

Within the vertebrate phylum, the primitive lung fish has 30 - 100 times more nuclear DNA than more advanced teleost fishes; the salamander has over 30 times more DNA than some reptiles or mammals (Mirsky and Ris, 1951).

Some of the increase in DNA content observed between procaryotes and eucaryotes may be necessary since the eucaryotes have a more elaborate sub-cellular structure. The synthesis of new mRNAs coding for the necessary enzymes and structural proteins would therefore involve a limited increase in the content of cellular informational DNA.

This increase would only be a fraction of that observed experimentally and would not explain the differences between related eucaryotes. It has been proposed that some of the amplified DNA is quiescent (Britten and Kohne, 1968). It is also possible that some of it may be structural (Britten and Kohne, 1968). An alternative explanation is that it has a role in gene regulation (Britten and Davidson, 1969).

In bacteria, the rDNA cistrons are redundant (Kohne, 1968). Other nucleotide sequences present in multiple copies may also be present in the bacterial genome (Chiscon and Kohne, 1970). These

copies may be extrachromosomal since their number fluctuates during the cell cycle. They represent a maximum of 4-5% of total E.coli DNA.

Much eucaryotic DNA consists of repeated sequences with varying degrees of redundancy.

The degree of complexity of the vertebrate genome led Marmur et al. (1963) to predict that the rate of re-association of vertebrate DNA would be much slower than that of the relatively simple bacterial DNA. However, the fast re-association of separated complementary strands of vertebrate DNA was shown by Hoyer et al. (1964). A multiple repetition of parts of vertebrate DNA (Bolton et al., 1965) accounted for the fast renaturation rates; the greater concentration of these repetitious sequences gave a relatively high rate of renaturation.

Repeated sequences have been found in all higher organisms studied (Britten and Kohne, 1968). The size of the eucaryotic genome is therefore not due to a large number of unrelated genes. Britten and Kohne (1968) have proposed that a variable proportion consists of families of DNA sequences whose sizes range from a few to a million copies. The members of one family of recent evolutionary origin might be almost identical, such as in the mouse satellite DNA (Waring and Britten, 1966). Base mutations during evolution might reduce the similarities between members of an older family.

Repeated sequences are scattered throughout the genome (Britten and Smith, 1970b; Hennig et al., 1970). They are therefore spatially

suited for a structural or regulatory role.

Some repetitious DNA may have no function. This is inferred from differences in DNA content between related species. An example is the localised increase in dimensions of some bands in Chironomus thummi thummi polytene chromosomes compared to those in Chironomus thummi piger (Keyl, 1965a, 1965b). This difference is caused by a local DNA increase during evolution (Keyl, 1964). Variations in the satellite DNAs of related species of mice are also found (Hennig and Walker, 1970).

Repeated DNA sequences of known function.

Repetitious DNA sequences transcribing identified RNAs include those coding for rRNA. There is a general increase in the number of ribosomal cistrons with increasing genome size although there is some variation within the more complex phyla. Plants have a few thousand copies of the ribosomal cistrons (Matsuda and Siegel, 1967), whereas some amphibians have tens of thousands (Brown and Dawid, 1968; Review, Birnstiel et al., 1971).

Other repetitious DNA sequences are those coding for the tRNAs (Goodman and Rich, 1962; Giacomoni and Spiegelman, 1962) and 5s RNA (Zehavi - Willner and Comb, 1966; Brown and Weber, 1968). It is possible that these repetitions are necessary for the synthesis of adequate amounts of RNA in these more complex genomes in one cell cycle.

Some repeated sequences hybridise with transcribed RNAs. The levels of hybridisation vary with time both in the regenerating and

in the developing mouse liver (Church and McCarthy, 1967a, 1967b). This implies a role for other repetitious DNA sequences, though it is no evidence for their transcription into mRNAs.

Some repeated sequences in vertebrates are cistrons duplicated during evolution which code for functional proteins. Comparisons of common amino acid sequences in related proteins provide evidence for duplication of an ancestral gene and subsequent divergence of the gene products by independent mutations. Ingram (1961) showed that the four haemoglobin chains probably evolved from a common ancestor by gene duplication and independent mutation. A common precursor is likely for trypsin and chymotrypsin (Walsh and Neurath, 1964) and also for the cytochromes (Margoliash and Smith, 1966). There is also evidence for partial cistron duplications in the α chains of human haptoglobins (Smithies et al., 1962).

The variable carboxyl ends of the immunoglobulin light chains may be produced by thousands of duplicated genes arising from a common ancestor, differential expression then being regulated by the immune response (Cohen and Milstein, 1967; Edelman and Gally, 1967).

There is also some evidence for a limited and conserved gene duplication for the genes specifying certain sequenced proteins. Two or more proteins, with slightly varying amino acid sequences, are sometimes found in the same animal.

A sequence variation in bovine carboxypeptidase was shown by segregation to be due to allelomorphism (Walsh et al., 1966). Differences

in the amino acid at position 136 in the α chain of human foetal haemoglobin were neither caused by allelomorphism nor ambiguous translation. They are therefore probably due to gene duplication (Schroeder et al., 1968). A similar duplication is likely for the α chain of goat haemogobin (Huisman et al., 1967). The variation in the composition of the rabbit haemoglobin α chain is due to the translation of 2 distinct mRNA species (Schapira, et al., 1968) and not to ambiguous translation of mRNA by a minor tRNA species as proposed by Von Ehrenstein (1966).

A minor haemoglobin component, with several amino acid substitutions, occurs in the Irus macaque (Wade and Barnicot, 1967). Since there are no other minor species with varying substitutions, such as would be expected with ambiguous translation, it was concluded that there had been gene duplication with subsequent divergence. The high frequency of another phenotype suggested a further α chain duplication (Barnicot and Wade, 1970).

The multiple forms of α chain globin in C3H mice probably also result from gene duplication (Hilse and Popp, 1968).

Such examples of cistron duplication are unlikely to be essential to the more rapid synthesis of messenger RNAs. It is more likely that one of the reduplicated genes is quiescent, as is the case for the $^{3}\alpha$ globin locus in apes (Boyer et al., 1971).

DNA content and differentiation

Although cellular differentiation is typically stable through many cell divisions (Urspring, 1968), there is little evidence for differential loss or amplification of genes in differentiated cells.

Much chromosomal DNA is lost at certain stages in the development of the somatic cells of some worm, insect and crustacean embryos (Beerman, 1966). The lost chromosomes are not redundant, as they contain information essential for gametogenesis (Bantock, 1961). The presumptive germ cells are probably protected from chromosome diminution by cytoplasmic factors. Analogous factors might also segregate germ from somatic cells in other species. No examples of chromosome diminution have been found in autosomes, although there are some examples of selective heterochromatinization and expression of alleles.

Such diminution would presume a total commitment to one developmental pathway. However, de-differentiation followed by re-differentiation has been found in several tissues proliferating in response to injury. An example is the regeneration of the Wolffian lens in urodeles (Yamada, 1967). This therefore precludes total developmental commitment through loss of DNA in this system.

A more general conclusion is inferred from nuclei transplant experiments. The transplantation of nuclei from intestinal cells into enucleate eggs of Xenopus laevis resulted in normal development of some of the fertilised eggs (Gurdon, 1962). Since some intestinal cells are undifferentiated embryonic cells, the experiment was repeated with nuclei from monolayer cultured epithelial cells. These should contain no embryonic cells. The development of tadpoles with normal tissue types showed the differentiated cells contained all the DNA required for normal development of the whole animal (Gurdon and

Laskey, 1970).

These experiments may not preclude specific gene amplification in differentiated cells. Selective somatic DNA amplification does occur in the polytene chromosomes of sciarid salivary glands. Autonomous DNA synthesis, characterised by chromosome puffing, occurs at localised regions on the chromosomes. This extra DNA remains integrated within the chromosome. Prior to the puffing, there are orthodox replications with polytenization of all chromosomes (Crouse and Keyl, 1968). The metabolic role of the selectively-replicated DNA is unknown.

The one unequivocal case where a specific gene amplification occurs in a life-cycle is the ribosome cistron amplification in the oocytes of amphibians and insects.

In Xenopus Laevis, extra copies of the nucleolar organiser region are selectively synthesised during early meiosis (Brown and Dawid, 1968; Evans and Birnstiel, 1968; Gall, 1968). The extra copies are found in the many oocyte nucleoli, one autosomal organiser being found per nucleolus (Perkowska et al., 1968).

The extra ribosomal copies are synthesised by a cascade effect with newly synthesised rDNA also acting as a template. This is shown by thymidine incorporation in the rDNA synthesising region of Xenopus Laevis being proportional to the amount of DNA present (MacGregor, 1968). The calculated time span for a cascade synthesis is consistent with cytological estimates (Birnstiel et al., 1971).

This gene amplification is necessary for the oocyte's rapid

synthesis of the ribosomal RNAs needed after fertilisation. A specific alteration in the gene balance therefore produces an effect which could not have been mediated by gene transcription.

In Drosophila melanogaster, disproportionate rDNA duplication has also been found. In male flies partially deficient in rDNA genes, there is magnification of these genes to the wild-type level by disproportionate gene replication in the germ line cells (Ritossa, 1968). Disproportionate rDNA replication has also been shown in the wild-type Drosophila melanogaster which carries only one nucleolar organiser region (Tartof, 1971). This replication occurs during one generation and seems analogous to the amplification of rDNA in the amphibian oocyte.

Selective gene amplication might account for differential rates of rRNA synthesis in somatic cells. However, rRNA saturation hybridisation experiments to the DNAs of different tissues in Xenopus laevis (Brown and Weber, 1968), in rat (Mohan et al., 1969) and in fowl (Ritossa et al., 1966) show no evidence of any differential gene amplification.

Brown and Dawid (1968) have postulated that a specific amplification of genes outwith normal cellular controls is possible in non-dividing cells like the oocyte which is synthesising proteins for storage rather than for immediate metabolism. Terminally differentiating non-dividing cells like the erythrocyte or muscle cell whose main functions are the synthesis of specific proteins

for intracellular use might also specialise by such gene amplification.

1.4 Messenger RNA.

The function of messenger RNA.

The control mechanisms discussed mostly operate on either transcription or translation of the intermediate necessary for transfer of the information in the base sequences of DNA to the site of protein synthesis on the ribosome. This intermediate was first thought to be ribosomal RNA (Crick, 1958). However, the existence of a distinct, unstable RNA fraction serving this "messenger" function was postulated in order to account for the rapid induction and repression of bacterial enzymes (Jacob and Monod, 1961a, 1961b).

Although isolation and characterisation of a messenger RNA coding for a specific protein would be useful for analysis of the mechanisms of mRNA transcription and translation, presumptive mRNA species have only recently been isolated. This is due to the complexity of procaryotic and eucaryotic transcription products coupled with inadequate means of characterising and isolating specific mRNA species.

The appearance of a minor, unstable RNA fraction following phage infection of E.coli was documented prior to the formulation of the messenger RNA hypothesis (Volkin and Astrachan, 1956). In T2 bacteriophage infection of E.coli, the switch from production of host cell protein to phage protein was mediated solely by the synthesis of a new unstable RNA fraction (Brenner et al., 1961). A similar unstable RNA fraction was also found in uninfected cells (see Gros et al., 1961).

The RNA fractions had therefore the expected properties of mRNA species. Further proof of the messenger concept required stimulation of protein synthesis on ribosomes by the addition of exogenous RNA. Nirenberg and Matthaei (1961) investigated the ability of an RNA fraction to code for protein synthesis when the RNA was added to an E.coli ribosome preparation; the addition of ribosomal RNA and tobacco mosaic virus RNA to deoxyribonuclease-treated ribosomes stimulated only non-specific protein synthesis.

The use of synthetic polynucleotides as primers for polypeptide formation was more successful. Addition of poly U to the E.coli ribosome system stimulated the synthesis of polyphenylalanine, thus demonstrating a possible relationship between the nature of a polynucleotide "messenger" and the kind of polypeptide synthesised (Nirenberg and Matthaei, 1961). Nirenberg et al. (1963) and Speyer et al. (1963) demonstrated a similar specificity for polymers containing random sequences of bases when they were used as templates for polypeptide synthesis. The addition of fully - characterised polymers with a repeated sequence further elucidated the RNA - template protein - product relationship (Review, Khorana, 1965).

Studies with synthetic polyribonucleotides in mammalian and algal cell-free systems and on mutations in proteins of different species demonstrated that at least part of the RNA code was universal (Maxwell, 1962; Speyer et al., 1963; Sager, 1963).

Criteria for messenger RNAs.

The definitive proof that any cellular RNA is functioning as a messenger is its ability to direct the synthesis of a specific protein in a cell-free protein synthesising system from which all endogenous messenger has been removed. Since there are difficulties both in isolating a small amount of presumptive mRNA from the total cellular RNA and in satisfactory assay of its messenger activity in a cell-free protein synthesising system, several secondary criteria have been used in distinguishing mRNAs from other species.

1) Base composition.

The base composition of an mRNA fraction has been assumed to reflect that of the total DNA. However, in eucaryotes only a portion of the repetitious DNA is transcribed in any system (McCarthy and Hoyer, 1964) and some DNA may be completely non-functional in transcription (Britten and Kohne, 1968). Therefore, the base composition of total transcribed cistrons need not reflect that of total DNA. Further, the asymmetric transcription of RNA necessitated another assumption; that the two strands of DNA have equivalent base ratios.

If the base sequence of a protein is known, the probable base composition of its specific messenger could be calculated, assuming randomisation of degenerate codons.

2. Size

The size range of cellular mRNA should reflect that of the

proteins synthesised (Jacob and Monod, 1961a, 1961b). However, preliminary identification of a monocistronic mRNA coding for a protein of unusual size should be possible. An example is the identification of a 30s RNA as myosin mRNA in muscle cells (Heywood et al., 1967). RNase action during RNA preparation would make identification of a messenger by its size more difficult since
a) it would preferentially degrade the less protected mRNA during the first stages of isolation; early size estimates of mRNAs in E.coli were 6-14s. These were revised to 8-30s using improved isolation techniques (Spiegelman and Hayashi, 1963).
b) It might contaminate the designated mRNA fraction with breakdown products of other RNA species.

A further hazard in the size identification of an mRNA is the association of mRNAs with ribosomes, for example, in buffers with a high magnesium content (Millar et al., 1965).

3. Stability

The response of bacterial induction and repression mechanisms to effectors is rapid. It was deduced that bacterial mRNAs have short half-lives (Jacob and Monod, 1961a, 1961b) and therefore assumed that all mRNAs would be preferentially pulse-labelled and thus identified (Brenner et al., 1961). Most bacterial mRNAs have half-lives of less than five minutes (Levinthal et al., 1962), but there is a spectrum of stability for different messengers, for

example, in Staphylococcus aureus (Creaser, 1956).

Some examples of long-lived mRNAs are known. In sporulating bacteria, mRNAs for spore proteins are functional for several hours in the absence of further RNA synthesis (Del Valle and Aronson, 1962). Since these cells are in a terminal pattern of development, flexibility of protein synthesis in response to environmental changes is less important. Proteins needed throughout the cell division cycle also may have more stable mRNAs. An example is the mRNA for flagellin (Martinez, 1966).

The identification of messenger RNAs with a rapidly turning over RNA fraction is even less satisfactory in higher organisms. Even some unstable mRNAs have average half lives of a few hours (Trakatellis et al., 1964).

The onset of protein synthesis in the absence of detectable RNA transcription in the germination of wheat seed (Marcus and Feeley, 1966) and in the fertilised sea urchin egg (Gros, 1964) indicates the prior storage of stable mRNAs for future developmental programming.

More convincing examples of mRNA stabilisation occur during cell differentiation where mRNAs programming the necessary specialised proteins may become more stable relative to other mRNA species. Examples of presumptive mRNA stabilisation are the globin mRNA in reticulocytes (Marks et al., 1962), Keratin mRNAs in embryonic chick down feathers (Humphreys et al., 1964), mRNAs in pancreas differentiation (Wessels, 1965), cocoonase mRNA in the zymogen cells

of the galea of the silk moth (Kafatos and Reich, 1968) and the mRNAs for lens proteins in lens fibre cells (Stewart and Papaconstantinou, 1967)..

The assumption that all pulse-labelled RNA represents mRNA is also incorrect. Much is stable rRNA precursor, both in bacteria (e.g. Midgley and McCarthy, 1962) and in mammals (e.g. Scherrer et al., 1963).

4. Location.

Functional mRNA is located on polysomes during protein synthesis (Rich et al., 1963); polysome - associated RNA is rapidly-labelled; polysomes can direct protein synthesis without addition of exogenous messenger (Munro et al., 1964); polysomes are readily degraded to monosomes, possibly by the preferential breaking of an unprotected mRNA joining the monosomes together. Chantrenne et al. (1967) and Williamson et al.(1969) have used this preferential sensitivity to identify the globin mRNA.

EDTA dissociates reticulocyte polysomes into subunits with release of protein-associated mRNA (Chantrenne et al., 1967). This is a useful way of obtaining a messenger-enriched RNA fraction, provided the polysomes are initially intact and RNase is rigorously excluded.

5. Programming of protein synthesis.

Uncharacterised stimulation of amino-acid incorporation by a cell-free protein synthesising system is not a sufficient criterion

for the messenger activity of an RNA fraction; synthetic polypeptides (Khorana, 1965) and rRNA (Nirenberg and Matthaei, 1961; Maxwell, 1962) also stimulate polypeptide formation in cell-free systems.

When reticulocyte RNA is added to an E.coli cell-free protein synthesising system, the protein synthesised is similar to the protein made by the E.coli system alone (Drach and Lingrel, 1966). Similarly, addition of RNAs from different non-erythropoetic tissues to a reticulocyte cell-free system result in the stimulation of globin synthesis (Hunt and Wilkinson, 1967). The addition of these RNAs to cell-free protein synthesising systems therefore stimulates protein synthesis by the endogenous mRNA rather than the programming of new proteins by the added RNA. Proof of an RNA messenger activity would be its programming of a heterologous cell-free protein synthesising system to synthesise its specific protein.

Identification of messenger RNAs

These criteria have been used separately or together to identify possible mRNAs.

Specific protein synthesis in heterologous cell-free protein synthesising systems has been most successfully demonstrated with viral mRNAs. Nathens showed synthesis of the corresponding coat proteins by phage f2 (Nathens et al., 1962) and by phage MS2 (Nathens, 1965). The synthesis of Satellite Tobacco Necrosis coat protein was directed by its viral RNA (Clark et al., 1965).

As viruses contain only several mRNAs, the isolation of a specific messenger is simplified. Bacteria contain over 1,000 different messengers. A specific bacterial mRNA has not yet been isolated and characterised satisfactorily in a cell-free protein synthesising system.

Devries and Zubay (1967) circumvented the difficulty of isolating a specific messenger by using a ϕ 80d lac virus to direct the synthesis of the α fragment of β-galactosidase in an E.coli cell-free synthesising system. This contained the necessary components for RNA transcription. Protein synthesis was probably mediated through a messenger RNA since there was 90% reduction in synthetic ability when transcription was inhibited (Zubay et al., 1967).

There is circumstantial evidence for the existence of some bacterialmRNAs based on their kinetics of synthesis and on their size. Martin (1963) showed a 34s RNA was present in cells constitutive for the histidine operon. This RNA was not present in His^- strains. He concluded this was a polycistronic mRNA for the histidine operon.

Induction of β-galactosidase synthesis was paralleled by the appearance of a rapidly-labelled RNA component (Guttman and Novick, 1963). Further evidence for this RNA being messenger for the lactose operon was its specific hybridisation to this operon (Spiegelman and Hayashi, 1963; Attardi et al., 1963).

RNA induced in response to galactose also hybridised specifically to the galactose operon (Attardi et al., 1963).

Summary.

1. The identification, isolation and characterisation of a mammalian mRNA required choice of a cell type which synthesises only one or a few proteins. The mammalian reticulocyte synthesises globin almost exclusively. This cell type can be isolated in large quantities and has low endogenous ribonuclease activity, thus making it a suitable system for the isolation of large amounts of undegraded RNA. Preliminary experiments indicated that the reticulocyte RNA component sedimenting at 9s had many of the characteristics expected of the globin mRNAs.

2. The characterisation of an mRNA which can only be labelled to a small extent in vivo and which is only about 1% of the total RNA requires the development of large-scale isolation procedures. Preliminary experiments designed to circumvent the difficulty of labelling reticulocyte 9s RNA in vivo involved the isolation of ^{3}H-uridine-9s RNA from cultures of 14 day mouse embryo liver cells. The technique of preparative polyacrylamide gel electrophoresis was adapted for the isolation of pure 9s RNA from total 14 day mouse embryo liver nucleic acids. The 14 day mouse embryo liver may contain cell types synthesising 9s mRNAs for other proteins. Contamination of any globin 9s RNA with breakdown products of other RNA species was also likely since the livers contain some endogenous ribonuclease.

3. 9s RNA was therefore isolated from mouse reticulocytes. The development of techniques for separation of large amounts of RNA on

the zonal ultracentrifuge combined with the use of diethyl pyrocarbonate as an RNase inactivator permitted the isolation of large amounts of 9s RNA.

Preliminary experiments used the M.S.E. B-XV zonal rotor for the isolation of a fraction enriched in the 14s mRNP particle dissociated from polysomes by EDTA. The RNA isolated from the 14s mRNP particle was further purified by preparative gel electrophoresis.

A 9s RNA fraction was also isolated from total reticulocyte RNA. Since isolation of undegraded RNA was more routine using this method, conditions were developed for the isolation of a 9s RNA fraction, uncontaminated with RNAs of other size classes, after a single fractionation of the total RNA in the M.S.E. B-XIV zonal rotor.

4. The RNA was analysed for purity on 2.6% polyacrylamide gels. The microheterogeneity of the RNA in the 9s RNA region was analysed on 6% polyacrylamide gels. There were two major and several minor bands in the 9s region. There was some variation in the relative amounts of the minor bands in the 9s RNAs isolated from different batches of reticulocytes.

5. The molecular weight of the 9s RNA was determined by analytical ultracentrifugation. The value obtained was 170,000, equivalant to about seventy nucleotides greater than the theoretical estimate for RNAs coding for the α or β globins. Determination of the molecular weight by relative electrophoretic mobilities on analytical polyacrylamide gels gave a higher value, 225,000, thus demonstrating that the 9s RNA had different migration properties compared to the rRNA

marker species.

6. Isolation of large amounts of pure 9s RNA made possible the study of the hybridisation of this messenger - enriched RNA fraction to DNA. Since 9s RNA is only labelled to a small extent *in vivo*, it was chemically methylated with tritiated dimethyl sulphate. In this way, specific activities of up to 5,000 cpm/μg were obtained.

Techniques were developed for the hybridisation of dimethyl sulphate-labelled RNA to DNA. Such chemically-labelled RNA gave a higher background sticking to blank filters. This was reduced to less than 0.01% of the input RNA by hybridising at low temperatures in the presence of formamide. Background levels were further reduced by pre-incubating all filters in Denhardt's medium and by extensive purification of the RNA.

7. ^{3}H - methylated 9s RNA was hybridised to DNA by an adaption of the method of Gillespie and Spiegelman. At intermediate $C_o t$ values, the ^{3}H - 9s RNA hybridised to over 0.1% of the total DNA, although there was some variation in the saturation values for different 9s RNA preparations. The hybridisation levels were similar for total mouse embryo DNA and mouse sperm DNA. They were higher for 14 day mouse embryo liver DNA and for duck reticulocyte DNA, though within the range of values for the different 9s RNA preparations. Similar saturation values were obtained when hybridising *in vivo* - labelled 9s RNA from 14 day mouse embryo livers to total mouse embryo DNA.

Although saturation was only approached at high RNA/DNA inputs, the time course of hybridisation was rapid. This suggests that there are only a limited number of components hybridising in the 9s RNA region. Successive hybridisation of the same RNA solution to different DNA filters gave identical hybridisation levels. The hybridisation was not therefore due to the exhaustion of a minor hybridising RNA component in the RNA.

8. Various control experiments were performed. There was little hybridisation to E.coli DNA, showing the hybridisation was specific for eucaryotic DNAs. The competition with unlabelled 9s RNA was close to that predicted. Competition with unlabelled reticulocyte 18s and 28s RNAs indicated some contamination of the 9s RNA with rRNA breakdown products. Less than half the observed hybridisation could be due to rRNA hybridisation.

9. The characteristics of the ^{3}H - 9s RNA - DNA hybrids were investigated. The T_ms of the hybrids were much lower than predicted. Control experiments on the hybridisation of methylated and non-methylated ^{32}P-5s RNAs showed that methylation lowered the hybrid T_ms by about $7^{\circ}C$. However, the T_ms of the 5s and 9s RNA hybrids were still lower.

10. It is therefore improbable that the high hybridisation values are due to the hybridisation of the globin mRNAs to reduplicated globin genes. The specific hybridisation to eucaryotic DNAs might be due to the hybridisation of short stretches of the 9s RNA to homologous regions in the DNA, related either by chance or by ancestral cistron

duplications and subsequent divergence. Alternatively, the hybridisation may be between a non-coding region of the RNA and related DNA sequences. Hybridisation of the polypurine regions found in globin mRNA to DNA would account for many of the observed properties of the formed hybrids.

Derepression of the tryptophan operon initiated the synthesis of an RNA hybridising specifically to that operon (Imamoto et al., 1965a). An RNA probably transcribed from the anthranilic acid cistron was isolated (Imamoto et al., 1965b).

There are fewer simple eucaryotic induction or repression mechanisms similar to those utilised in bacteria for messenger identification. Neither are there genetic tools such as enrichment of DNA segments coding for specific mRNAs by transducing phages. Further, the size of the eucaryotic genome makes the isolation of one messenger species more difficult. By the appropriate choice of specialised cells or cells at different stages in their growth cycle, systems with a specific mRNA enrichment can be found.

Histones are the main class of proteins synthesised during the S phase of the HeLa division cycle. The amount of histone mRNA should be maximal at that time. A new class of polysomes appears in S phase, concomitant with DNA and RNA synthesis. It was postulated that histones were synthesised on these polysomes (Robbins and Borun, 1967). A rapidly-labelled RNA species, associating specifically with these polysomes during DNA synthesis, was isolated. This RNA had a half life similar to that of group A histones (Borun et al., 1967). Its size range was that calculated for monocistronic mRNAs for histones (Borun et al., 1967). Three RNA components were resolved on polyacrylamide gels (Gallwitz and Mueller, 1970).

The HeLa microsome system, synthesising electrophoretically identifiable histones, showed such synthesis was terminated on

inhibition of DNA synthesis. An RNA intermediate was necessary for resumed histone synthesis after the reactivation of DNA synthesis (Gallwitz and Mueller, 1969).

Kinetically, therefore, there is circumstantial evidence for this 9s RNA being a mixture of different histone messenger RNAs. There is no evidence that the 9s RNA specifically directs the synthesis of histone polypeptide chains in a cell-free protein synthesising system.

Heywood et al. (1967) utilised the large size of myosin (170,000 - 200,000 m.w; Kielley and Harrington, 1960) to isolate an RNA from chick embryo muscle which he identified with myosin messenger. Although muscle cells synthesise a variety of proteins, he isolated a class of polysomes containing 50-60 ribosomes which predominantly synthesised a protein co-running with myosin subunits on acrylamide gels. The size of the polysomes is comparable with the molecular weight of the protein synthesised in the cell-free protein synthesising system if a ribosome loading on mRNA equivalent to that on globin polysomes is assumed (Warner et al., 1963).

Chick embryo muscle polysomes of other size classes were shown to synthesise proteins with the same chemical properties as actin and tropomyosin (Heywood and Rich, 1968).

Total RNA extracted from the 50-60 ribosome complex directed myosin synthesis in a muscle cell-free protein synthesising system. Non-specific stimulation was excluded by using RNA extracted

from other polysomes as an experimental control (Heywood and Nwagwu, 1968). Further analysis of the polysomal RNA showed a 26s RNA component. This had a higher specific activity than rRNA when myosin was being synthesised during embryonic development (Heywood and Nwagwu, 1969). It specifically stimulated the formation of the major myosin subunit and was assumed to be myosin mRNA. This conclusion must be verified by peptide analysis of the protein formed in the cell-free system to confirm that it is myosin.

Globin messenger RNA

The terminally-differentiating erythroid cell line is a suitable system for the isolation of a specific mRNA -globin mRNA. There is now unequivocal evidence that the RNA fraction identified as globin messenger (9s RNA) in this system contains the messengers for the α and β chains of globin (Lingrel, 1971).

The reticulocyte continues to synthesise heme and protein for several days after the nucleus has been extruded (London et al., 1950); over 90% of the protein synthesised during this time is globin (Kruh and Borsook, 1956; Dintzis et al., 1958). Since there is no concomitant RNA synthesis (Marks et al., 1962; Burny and Chantrenne, 1964), the globin mRNA must be stable over a period of several days.

As actinomycin D inhibits non-globin protein synthesis in the later stages of development of mouse foetal erythroid cells (Fantoni et al., 1968), the mRNAs for other proteins synthesised at

earlier stages in maturation must be relatively unstable. It is therefore probably that in the mature reticulocyte there is a very much higher proportion of globin mRNA than in relatively young erythroid cells.

Reticulocytes can be isolated almost free from non-erythroid cell types, thus making identification of reticulocyte RNA more certain. They contain a low level of endogenous ribonuclease. Undegraded reticulocyte RNA can therefore be isolated. Contamination of small-molecular weight RNAs with ribosomal RNA breakdown products is minimised.

Early attempts to show a globin messenger function for reticulocyte RNAs tested in a cell-free protein synthesising system from E.coli gave only a non-specific stimulation of synthesis of E. coli-type proteins (Drach and Lingrel, 1966). In contrast, liver nuclear RNA and rabbit kidney and intestinal RNAs stimulated the synthesis of haemoglobin in a reticulocyte cell-free system (Kruh et al., 1964a, 1966). Attempts to demonstrate the globin messenger activity of reticulocyte RNA in the reticulocyte system (Kruh et al., 1964b, 1964c; Arnstein et al., 1964) may, therefore, only show a similar non-specific stimulation of the translation of endogenous mRNA bound to the ribosomes and not a messenger activity of the added RNA species.

The isolation of a presumptive messenger RNA on the basis of other properties has been more successful.

The predominant polysomes in reticulocytes contain 4-5 ribosomes (Marks et al., 1962; Warner et al., 1963; Gierer, 1963)

These are the polysomes most active in protein synthesis (Warner et al., 1963). Slayter et al. (1963) estimated the size of an RNA-like strand in a pentasome to be 1500A° long. Although this electron micrograph evidence is not conclusive, this is the size expected for a monocistronic globin messenger RNA.

An RNA of this size (9-10s) was first found in rabbit reticulocyte RNA (Marbaix and Burny, 1964). This RNA was isolated by scaling up the sucrose gradient preparative procedure (Burny and Marbaix, 1965; Marbaix et al., 1966; Huez et al., 1967; Williamson et al., 1971). It can have a higher specific activity than the rRNAs. (Burny and Marbaix, 1965). The preliminary GC content was different from that of rRNA (Marbaix et al., 1966). These results were incompatible with 9s RNA being a rRNA breakdown product. Scherrer and Marcaud (1965); Scherrer et al. (1966) and Attardi et al. (1966) found a similar RNA in duck erythroblasts.

There is now further circumstantial evidence for 9s RNA being the globin messenger.

a) Gros et al. (1961) showed that messenger RNAs are detached from ribosomes by lowering the divalent cation concentration. Huez et al. (1967) showed that a 9sRNA, with similar properties to that isolated from total RNA, was released from EDTA-treated reticulocyte polysomes.

b) In bacterial systems, synthetic polyribonucleotides bind to the 30s ribosomal subunit (Takanami and Okamoto, 1963; Pestka and Nirenberg, 1966). The small subunit also binds mRNA in E.coli

prior to protein synthesis (Nomura and Lowry, 1967; Guthrie and Nomura, 1968). Rapidly-labelled messenger-like RNA is associated with the small ribosomal subunit in sea urchin embryos (Spirin and Nemer, 1965) and in rat liver (Henshaw et al., 1965). Heywood (1970) has more convincingly shown the formation of an initiation complex between muscle myosin mRNA and the 40s subunits.

When rabbit reticulocyte polysomes are dissociated into subunits by ppi at a concentration such that 9s RNA is not released, subsequent analysis of the subunits shows the 9sRNA is associated with the small subunit (Holder and Lingrel, 1969). Pragnell and Arnstein, (1970) further showed a specific binding of the EDTA-dissociated 14s ribonucleoprotein particle to the small subunit of EDTA-treated rabbit reticulocyte ribosomes.

c) The amount of 9s RNA present in polysomes varies according to the size of the polysome and in a manner predicted for a messenger RNA rather than that predicted for a ribosomal component (Evans and Lingrel, 1969a).

d) Further evidence for 9s RNA being a distinct species and not a ribosomal breakdown product is that of Evans and Lingrel (1969b). They showed that ribosomal RNA is synthesised early in erythroid cell development while 9s RNA is synthesised in more mature cells.

Concomitant with the preliminary identification of 9s RNA with globin messenger, there was further characterisation of the components necessary for cell-free synthesis (e.g. Heywood, 1970). This made specific protein synthesis by exogenous messengers

more feasible. However, the unequivocal proof that 9s RNA directs globin formation has been with the combination of cell-free reticulocyte systems from one animal species and 9s RNA from another, followed by separation of the formed globin chains of each species by column chromatography. This approach combines the simplicity of using all endogenous cell-specific factors with specificity in analysis of the peptides synthesised, to discriminate between endogenous and exogenous protein synthesis.

Schapira et al. (1968) showed the fractional residues of some amino acids present in the α T14 peptide of rabbit globin were due to the translation of different globin mRNAs in two rabbit strains. The specific α T14 peptide was formed when the mRNA fraction from the corresponding rabbit strain was added to a cell-free protein synthesising system derived from the other strain. The mRNA cut used contained both 9s and 12s RNA species.

Laycock and Hunt (1969) programmed an E.coli cell-free protein synthesising system with rabbit reticulocyte 9s RNA in the presence of an N-acetyl-valyl-tRNA initiator. The protein product co-chromatographed with globin and had a similar tryptic digest pattern on Dowex 50. Proteins formed by liver mRNA showed no such similarity.

The most convincing evidence for the 9s RNA being globin messenger is the programming of mouse globin β chain synthesis on the addition of mouse 9s RNA to a rabbit reticulocyte lysate

(Lockard and Lingrel, 1969). The mouse β chain was separated from mouse α and rabbit α and β chains by chromatography on carboxymethyl cellulose. In a duck reticulocyte lysate system, the synthesis of both mouse α and β globin chains can be shown (Lingrel, 1971). Sufficient controls were introduced to prove conclusively the globin messenger activity of the 9s RNA fraction.

1.5 Hybridisation

Nature of the hybridisation reaction

The rationale behind DNA - DNA and DNA - RNA hybridisations is that when two complementary single-stranded nucleic acids are mixed, under suitable conditions, they will form a double-stranded molecule with matching of G - C and A - T (or U) base pairs.

Reassociation of DNA was first shown by Marmur and Lane (1960) and by Doty et al. (1960). The extent of association was a maximum when both DNA strands were from the same species. It was lowered when reassociation was between DNA strands from different species (Schildkraut et al., 1961). DNA - DNA hybridisation was therefore used to examine intergenomic homologies.

DNA - RNA hybrids from T2 phage showed hybridisation of RNA could distinguish between different DNA cistrons (Hall and Spiegelman 1961). Hybridisation of RNA to DNA has been extensively used to test for homologies between populations of DNA and of RNA.

The mechanisms of DNA - DNA and DNA - RNA reassociation are generally similar. DNA - DNA reassociation is a second - order process whose rate is determined by the frequency of collision of complementary

strands. The reaction rate is dependent on reaction conditions such as the ionic strength, temperature and viscosity of the medium. The reaction rate is relatively independent of pH. It is inversely proportional to the complexity of the RNA and is slightly faster for DNAs with a high GC content (Wetmur and Davidson, 1968).

The rate-determining step is postulated to be an initial nucleation between two complementary sequences. This is followed by a relatively fast association of complementary strands. The reaction is faster at high cation concentrations where the repulsion of the two negatively - charged strands is minimal (Wetmur and Davidson, 1968; Smith, 1970). The maximum hybrid formation occurs at 10-20^{o}C below the T_m (the temperature at which half of the hybrid is dissociated) of the DNA hybrid (Marmur and Doty, 1961; Wetmur and Davidson, 1968). This maximum may be a combination of two factors, the rate and the extent of hybridisation. The rate will increase as the temperature is raised; the extent will fall as mismatched hybrids are progressively eliminated. The initial rate of hybridisation may increase till 5^{o}C below the T_m of the DNA hybrid (Thrower and Peacock, 1968).

Similar results to those of Wetmur and Davidson (1968) have been obtained for DNA - RNA hybridisations (Nygaard and Hall, 1964).

The interpretation of hybridisation data depends on knowledge of

1) the degree of complementarity between two polynucleotide strands necessary for hybrid formation.

2) The sensitivity of techniques which detect non-complementary base pairs within formed hybrids.

The stability of a hybrid can be determined from its thermal dissociation profile. The T_m (the mid-point in the thermal dissociation profile for a hybrid) is sensitive to the variables determining hybrid stability.

Factors influencing hybrid stability

a) Base composition and sugar backbone

The stability of a duplex is affected by its base composition; a higher proportion of GC-rich base pairs will increase the stability (Marmur and Doty, 1962; Inman and Baldwin, 1964). The presence of substituted bases, making standard Watson - Crick base pairing impossible, will lower the stability. The base sequence of a duplex also influences its stability (Chamberlin et al., 1963). The mixed RNA - DNA hybrids have varying stabilities, depending on which bases are attached to the ribose or deoxyribose backbone. RNA - DNA hybrids have a greater range of stability than DNA - DNA hybrids (Chamberlin and Patterson, 1965) and a more gradual thermal transition (Chamberlin and Berg 1964).

b) Length of hybrid.

Thomas (1966) predicted that the minimum stable hybrid length for a simple genome would be about 12 nucleotides. Using in vitro synthesised RNAs from phages T4 and T7, sequences 10-12 residues long were shown to form stable complexes with the corresponding DNAs when incubated in 5 x SSC at optimum temperatures for hybrid

formation. No hybrids were formed with heterologous DNA. The hybrids were therefore species - specific (Niyogi and Thomas, 1967; Niyogi, 1969). Gillespie and Spiegelman (1966) partially digested E.coli rRNA and incubated oligomers of known length with DNA at various temperatures. Stable duplexes of 50 nucleotides were needed in 0.33 M Na^+ at 67°C. Only 32 and 17 nucleotides were needed for stable duplex formation at 55°C and 44°C respectively.

Mouse DNA - DNA hybrids showed increasing hybrid stability, measured by the Tm, for hybrids 20 - 200 nucleotides long (McConaughy and McCarthy, 1967). The hybrids were species - specific above chain lengths of 33. The annealing of hybrids was in 0.5 M KCl; 0.01 M tris. Interaction of B.subtilis and mouse deoxyoligonucleotides with homologous and heterologous DNA showed species - specificity in the range of 15-40 nucleotides (McConaughy and McCarthy, 1970).

c) Mismatched bases

Experiments with poly A- poly U complexes which contained known percentages of non-complementary bases, showed a depression in T_m of 0.7°C for every 1% base mismatching (Bautz and Bautz, 1964). Chemically - modified Watson - Crick base pairs affect the hybrid stability in natural and in modified polynucleotides (Kotaka and Baldwin, 1964; Laird et al., 1969). The depressionin T_m was of the same extent as that found for the polyA - polyU mismatched bases.

Oligonucleotide - DNA complexes formed by mouse DNA are always less stable than the corresponding duplexes formed by

B. subtilis DNA (McCarthy, 1967). Since the DNAs have similar GC contents, the lower stabilities are not due to a smaller proportion of GC base pairs. The lower T_ms of mammalian duplexes are probably due to mismatched regions within the duplexes (Britten and Kohne, 1968).

d) Single-stranded regions.

The presence of single-stranded ends in long DNA - DNA duplexes may not affect the T_ms of the hybrids, but stability of shorter duplexes might be lowered by single-stranded ends and also by internal loops. The minimum length for hybrid formation must be increased in compensation (Walker, 1969).

Conclusions from hybridisation data.

In viruses, RNA - DNA hybridisation can prove a specific DNA primer - RNA product relationship. Hybridisation of a specific viral messenger to viral DNA was shown with partially complete mRNAs from deletion mutants of the RIIβ cistrons of phage T4. The mutant DNAs hybridised specifically to the corresponding viral RNAs (Bautz and Bautz, 1967). Specific hybridisation of lysozyme mRNA was also found with DNA from deletion mutants of phage T4 (Kasai and Bautz, 1967). A high degree of hybrid base-pairing is shown in the electron microscope when the separated strands of phage λ are reannealed (Westmorland et al., 1969).

Specific hybridisation to distinct loci has also been shown in bacterial systems. In most cases, the disadvantage of the larger

genome, and hence a lower concentration and reactivity of complementary sequences, has been circumvented by using the selectively - amplified DNAs of transducing phages to enrich host DNA sequences.

Attardi et al (1963) showed that an RNA species, identified with galactokinase mRNA, hybridised with the gal operon in E.coli. Similarly, the synthesis of lac mRNA in response to enzyme induction was shown by a difference in the levels of hybrid formation to the lac operon in induced and non-induced cells (Attardi et al., 1963; Hayashi et al., 1963).

Specific hybridisation of different parts of the tryp mRNA molecule to the relevant parts of the tryp operon was shown with transducing coliphage ϕ 80 DNA (Immamoto and Yanofsky, 1967).

The selective amplification of ribosomal cistrons makes rRNA hybridisation to rDNA possible at low input levels of RNA (Yankofsky and Spiegelman, 1962b; Attardi et al., 1965). The redundancies of the cistrons coding for tRNA and for 5s RNA have also facilitated their hybridisation (Goodman and Rich, 1962; Giacomoni and Spiegelman, 1962; Zehavi - Willner and Comb, 1966).

There are two main features of the mammalian genome which are different from the viral and bacterial genomes and which make interpretation of hybridisation data more complex.

The first is size. An average mammalian genome contains about 3.2×10^9 nucleotide pairs, compared to the 4.5×10^6 nucleotide pairs in a bacterium. As the rate of reassociation of RNA and DNA strands depends on the concentration of specific reacting

sequences, the rate of hybrid formation in mammals should therefore be 1/700 the rate in bacteria.

The second is the varying degree of reiteration in parts of the mammalian genome. There are no recurring sequences longer than 12 nucleotides in the rII region of bacteriophage T4 (Ruger and Bautz, 1968). This is expected for a simple genome on the basis of minimum recombination lengths (Thomas, 1966).

There may be some remote base sequence homology in bacteria. This would result from the similarity of some enzyme active sites and the convergent or divergent evolution of short base sequences (Freese and Yoshida, 1965; McCarthy and McConaughy, 1968). Much internal homology might result in unequal crossing over at mitosis (Thomas, 1966).

The physical segregation of mammalian DNA into separate chromosomes would, in theory, allow greater redundancy within a genome. Experimentally, the rate of hybridisation of some mammalian DNA - DNA and DNA - RNA strands is of the same order as the rate of association of bacterial hybrids (Hoyer et al., 1963, 1964). This rapid hybridisation is between redundant sequences of mammalian DNA.

Some of the mammalian genome is composed of families of DNA sequences which are very highly reiterated and very similar in base sequences (fast fraction). A less highly reiterated fraction (intermediate fraction) with less similarity between members, is the fraction whose reassociation is observed in the hybridisation

reactions. Part of the genome is composed of unique species (slow fraction). This is not hybridised under normal conditions.

The reiteration present in a genome is conveniently shown by the plot of DNA renaturation against a composite time and concentration axis. This is expressed as log (mole nucleotides)x sec/litre, denoted by the term C_0t (Britten and Kohne, 1968). A C_0t curve for calf thymus DNA shows the rapid reassociation of highly repetitive families and the progressively slower reassociation of families with fewer members. When mammalian hybridisations are carried out under conditions used for bacterial and viral hybridisations (about C_0t 100), only the redundant portions of the genome will hybridise. No hybridisation of the unique sequences would occur.

The degree of interspecies DNA/DNA cross-reaction in bacteria is dependent on the stringency of the reaction conditions, although the DNAs may have dissimilar base sequences (Johnson and Ordal, 1968; Brenner et al., 1969). Greater stringency of reaction conditions is required for bacterial DNAs of high GC content since stable hybrids will form more easily between mismatched regions (Johnson and Ordal, 1968). The repetitive part of the mammalian genome probably consists of many families with varying degrees of similarity. The ability to distinguish between different families by hybridisation techniques will depend on the stringency of the reaction conditions.

Variation of reaction temperature does not affect the extent or the stability of hybrid formation with bacterial polynucleotides

as much as with mammalian polynucleotides (McCarthy and McConaughy, 1968; Church and McCarthy, 1968). The extent of mammalian hybrid formation with pulse-labelled RNA is a function of the temperature of incubation. Denis (1966) showed that the decrease in the saturation values for hybrids of total amphibian RNA to DNA at higher temperatures is due to a difference in the fraction of DNA titrated at these temperatures. This shows experimentally that the stringency of reaction conditions only limits the extent of cross-reaction between members of a family. Even the most stringent reaction conditions used do not give complete locus specificity. The low T_m of 55°C for hybrids of total RNA to DNA shows a high percentage of mismatched base pairs in the hybrids (Wegnez and Denis, 1970).

Although saturation hybridisations at intermediate C_0t values are carried out at high temperatures and low salt concentrations (e.g. Crippa et al., 1967; Stevenin et al., 1968; Church and McCarthy, 1967a, 1967b), they

1) overestimate true hybrid formation between complementary sequences by the annealing of related, non-identical sequences.
2) underestimate hybrid formation between unique sequences by annealing at low C_0t values (Melli and Bishop, 1969; McCarthy and Church, 1970).

Similar reservations apply to competition experiments designed to show differences between populations of RNA molecules from different tissues or from the nucleus and the cytoplasm

(e.g. Paul and Gilmour, 1968; Soeiro and Darnell, 1970).

The hybridisation of rRNA to rDNA can be carried out at low C_0t values since the rDNA cistrons are extensively and conservatively reiterated in eucaryotes (Review, Birnstiel, et al., 1971). The hybrids are locus specific (Wallace and Birnstiel, 1966). This locus specificity may be partly due to atypical sequences of bases in rDNA, cross hybridisation with other regions in the genome being therefore minimised. Mammalian rRNA - DNA hybrid formation does show less dependence on temperature and other incubation conditions than do non-rRNA hybrids. The T_ms of the hybrids are high (Moore and McCarthy, 1968; Birnstiel, et al., 1971). Transfer RNAs and 5sRNA also form specific hybrids at low C_0t values

DNA - RNA hybridisation at high C_0t values is possible using conditions of vast DNA excess (Melli et al., 1971). This technique has been used to hybridise a 9s RNA fraction probably containing mRNAs for histones of intermediate size classes (Kedes and Birnstiel, 1971). The 9s RNA hybridised to reiterated DNA sequences. The hybrids formed were very stable, indicating a high percentage of matched base pairs.

There have been several attempts to hybridise unique DNA sequences at high C_0t values by prior isolation of non-repetitive DNA and its incubation with RNA at high concentrations and for long periods of time. Although there are experimental difficulties such as the degradation of polynucleotides on prolonged incubation,

fractions of RNA have been hybridised to unique DNA sequences.

Britten and Kohne (1968) hybridised unique DNA sequences by annealing DNA at very high C_0t values.

Gelderman et al. (1967, 1971) hybridised mouse RNA to the unique fraction of DNA. The hybrids were separated on hydroxyapitite. At least 80% of the DNA was hybridised and over 70% of the RNA was homologous to the unique DNA fraction. Davidson and Hough (1969) isolated non-repetitive Xenopus Laevis DNA as the DNA fraction remaining single-stranded at a C_0t of 2500. This was hybridised to RNA at a C_0t of 2100. The hybrids had a T_m consistent with a high fidelity of base pairing and were therefore probably hybridising to unique base sequences in the DNA. Brown and Church (1971) isolated non-repetitive DNA from several mouse tissues and hybridised these fractions to the tissue RNAs. Saturation values showed 10% unique DNA hybridised to mouse brain RNA, whereas less than 2% hybridised to liver, kidney or spleen RNAs.

Rationale behind present approach.

The first aim of this project was to isolate and identify a mammalian messenger RNA. The system chosen for this required a cell type which synthesises only one or a few proteins with, preferably, characterised structures and functions. It was therefore decided to isolate globin mRNA since large amounts of cells (reticulocytes) can be isolated in which haemoglobin is almost exclusively the only protein synthesised. The isolation of large amounts of the presumptive globin mRNAs was undertaken in order to demonstrate their messenger activity and to further characterise their physical properties.

Much information can be obtained from the DNA/RNA hybridisation of an mRNA. One can determine whether the mRNA hybridises to unique or reiterated DNA and whether the mRNA contains control sequences common to many different mRNA species. The results can also determine the purity of the mRNA preparation. DNA/RNA hybridisiation experiments were therefore carried out with the globin mRNAs.

These mRNAs were also hybridised to DNAs isolated from different tissues so as to determine whether the differentiated cells synthesising large amounts of globin have a duplication of the globin genes compared to the number in a non-erythroid cell.

MATERIALS AND METHODS

2. MATERIALS AND METHODS

Materials.

Isotopes

Enzymes

Culture media

Ribonuclease inhibitors

Methods

MATERIALS

All chemicals used were supplied by British Drug Houses (AR grade), unless otherwise specified.

Isotopes

^{3}H - uridine, carrier - free ^{32}P- orthophosphate and ^{3}H-dimethyl sulphate were obtained from the Radiochemical Centre (Amersham, England).

Enzymes

Ribonuclease - free deoxyribonuclease was obtained from Worthington Biochem. Co. (Freehold, New Jersey).

Bovine pancreatic ribonuclease was obtained from Sigma Chemical Co. (St. Louis, Missouri). Ribonuclease was boiled for 10 minutes to destroy any deoxyribonuclease activity. It was stored in 0.1 ml aliquots at -20°C.

Culture media

Commercial culture media were obtained from Flow Laboratories Ltd. Trypsin was obtained from Difco Laboratories.

Ribonuclease Inhibitors

Macaloid was a gift from Texas Lead and Byrites Co. (Houston, Texas).

Diethylpyrocarbonate ("Baycovin") was a gift from Bayer Co. Ltd. (London).

METHODS

2.1 RNA estimation in DNA samples

0.5 mls 0.2 N NaOH was added to 0.5 ml DNA solution (>450µg DNA). After incubation for one hour at 50°C, the solution was cooled in ice. 0.1 ml bovine serum albumin solution (2mg/ml) was added. After mixing, 1 ml 2.5 M perchloric acid was added. After standing on ice for 15 minutes, the solution was centrifuged at 1,000 g for 10 minutes. The absorbance of the supernatant fraction at 260 mµ was read, using a 20 mM path cell.

2.2 Protein estimation in DNA samples

Protein estimation was by the method of Lowry *et al* (1951). Since DNA interferes with the reaction, it was first hydrolysed.

1 ml 10% perchloric acid was added to 1 ml DNA solution (>450 µg DNA). The solution was heated at 90°C for 10 minutes then left overnight in ice. It was centrifuged at 18,300 g for 1 hour. The supernatant fraction was decanted. 0.3 ml 1 N NaOH was added, the solution was heated at 100°C for 5 minutes then left for 30 minutes at room temperature. 0.1 ml aliquots were used in the Lowry estimation.

Lowry estimation:

Reagent A : 2% $Na_2 CO_3$.

Reagent B : 0.5% $CuSO_4 . 5H_2O$ in 1% Na citrate.

Reagent C : 1 ml B + 50 ml A, freshly prepared.

Reagent D : Folin Ciocalteau reagent; dilute 1 volume with 1.3 volumes water.

To 0.1 ml protein solution was added 1 ml reagent C. After 10 minutes, 0.1 ml reagent D was added and the solution mixed. After 30 minutes, the absorbance at 700 mμ was read. A calibration curve was prepared using bovine serum albumin solutions from 2.5 - 25 μg/ml in 1 M NaOH.

2.3 Acid precipitation of labelled RNA

All steps were in the cold. To 1 ml RNA in water or SSC was added 0.2 mls 50% trichloroacetic acid. The solution was mixed. 0.1 ml bovine serum albumin (10 mg/ml) was added. After mixing, 5 mls 2% trichloroacetic acid were added and the solution was again mixed. After 10 minutes, the solution was centrifuged at 1,000 g for 10 minutes. The pellet was dissolved in 0.3 ml 1N NaOH. 0.2 ml water and 0.5 ml 1 N HCl were added. The solution was counted in 10 mls toluene based scintillator/Triton X-100 (see section on determination of radioactivity).

2.4 Determination of radioactivity

Radioactivity was measured in a Beckman LS-100 liquid scintillation counter.

^{32}P was measured by Čerenkov radiation. The efficiency of counting was almost 30%.

Radioactive samples on filters were dried then counted in 5 mls toluene based scintillator (TBS) containing 0 .5% 2,5-diphenyl oxazolyl (PPO, scintillation grade, Nuclear Enterprises, Edinburgh) and 0.03% 1,4 bis (2-(5-(phenyl oxazolyl) benzene (POPOP, scintillation grade, Nuclear Enterprises, Edinburgh) in toluene.

Samples in aqueous solution were made up to 1 ml with water. 10 mls TBS/Triton X-100 (Lennig Chemicals, Croydon) in the ration 2:1 were added. The mixtures were shaken then counted. Corrections were made for counting efficiency using standard quench curves. The efficiency of counting was 25 - 30%.

2.5 Induction of reticulocytosis and preparation of reticulocyte RNA.

Induction of reticulocytosis and collection of reticulocytes

Reticulocytosis in animals can be induced by frequent bleeding or by repeated injection of phenylhydrazine.

Phenylhydrazine-induced anaemia is caused by the elimination of poisoned mature red blood cells from the peripheral circulation by the spleen. This causes an increased production of resistant immature erythrocytes by the bone marrow cells. The blood reticulocyte count is thus raised from <2% in normal animals to > 80% in phenylhydrazine-treated animals (Wintrobe, 1961). Phenylhydrazine-induced reticulocytes are similar to those in the normal circulation.

Method

Mice were injected subcutaneously with 0.1 ml 2.5%(w/v) phenylhydrazine hydrochloride (May and Baker, Dagenham, England) in 0.17 N NaOH. Injections were on days 1 and 5 and the blood was collected on day 7. Rabbits were injected subcutaneously with 0.3 ml phenylhydrazine solution/kg body weight on days 1-5 and blood was collected on day 7. The mice were stunned and bled from the neck; the rabbits were anaesthetised by the injection of 1 ml

10% pentothal into the ear vein. They were bled from the neck.

The blood was collected into sterile balanced salts solution (Paul, 1965) containing 0.1 ml heparin/200 ml (Pularin heparin, 5,000 I.U./ml; Evans Medical Ltd., Liverpool). The blood was kept in the cold. It was filtered through heparinised glass wool and centrifuged at 800g for 10 minutes. The plasma and buffy coat were removed by aspiration. The cells were resuspended in 2 volumes balanced salts solution and the centrifugation and aspiration steps were repeated twice more. The reticulocytes were either used immediately or stored at -70°C.

Determination of reticulocyte count

Reticulocytes are immature anucleate blood cells which still contain large numbers of ribosomes. These form a blue precipitate with brilliant cresyl blue, giving a characteristic network of blue filaments. Progressively less precipitable material is observed as the reticulocytes mature into erythrocytes.

Method

To 3 drops of filtered 1% brilliant cresyl blue (water-soluble; Gurr) in 0.6% sodium citrate; 0.17% NaCl, was added 3 volumes oxalated blood. After mixing, the solution was incubated at 37°C for 15 minutes. After a further mix, a slide was prepared (oil immersion) and the reticulocytes counted under the microscope at a magnification of 1,000 (Dacie, 1956).

Preparation of RNA

Since the 9s RNA is identified by its size, ribonuclease

breakdown products of rRNA in the 9s RNA region (Gould, 1966) must be eliminated. Mammalian reticulocytes contain little endogenous ribonuclease (Mathias et al., 1964) and most ribonuclease (RNase) activity arises from the contamination of preparative solutions and vessels. Several reagents can be used to minimise RNase action; initially, macaloid was added to all solutions. This adsorbs and therefore inactivates the negatively-charged RNase molecules (Fraenkel-Conrat et al., 1961; Singer and Fraenkel-Conrat, 1961).

The adsorption of RNase is less preferable than its irreversible inactivation. Diethyl pyrocarbonate (DEP), the diethyl ester of oxydiformic acid, inactivates enzymes by forming ethoxycarbonyl compounds with primary and secondary amino groups. These ethoxycarbonyl groups may then react with the carboxylic groups of amino acids and form inactive polymers of the enzymes (Wolf et al., 1970). Other groups in proteins are also carbethoxylated by DEP, for example the imidazole ring of histidine (Melchior and Fahrney, 1970).

DEP was first used in the phenol extraction of RNA (Solymosy et al., 1968). It has since been used in conjunction with phenol extraction in the preparation of plant RNAs (Fedorcsák et al., 1969). Since it decomposes spontaneously on standing into ethanol and carbon dioxide, its effect on RNAse is time-dependent. In this work, all solutions used were sterilised by vigorous shaking with 0.1% DEP then left for at least 24 hours at room temperature to allow

decomposition of the DEP.

Methods

The reticulocytes were lysed in the cold by addition of 2 volumes 0.001 M $MgCl_2$. Cell membranes, mitochondria and any remaining white blood cells were precipitated by centrifugation at 18,300g for 15 minutes.

a) Total RNA

The supernatant fraction after centrifugation was shaken with 0.1% DEP for 1 minute. The solution was made 6% with respect to 4-amino salicylic acid. An equal volume of redistilled 90% phenol, containing 70 mls m-cresol/500 gm phenol and 0.1% 8-hydroxyquinoline, was added (Kirby, 1965). The mixture was shaken for 20 minutes at room temperature. After centrifugation at 18,300 g for 10 minutes, the aqueous phase was removed and stored at 0°C. The interphase was re-extracted with 10 mls of both phenol and aqueous phases as before. The combined aqueous phases were re-extracted with the phenol mixture until there was no visible protein at the interface. To the final aqueous phase was added 1/5 volume 6M filtered potassium acetate (pH 7) and 2 volumes ethanol.

The RNA was precipitated by standing for 1 hour at -20°C and then pelleted by centrifugation at 18,300g for 30 minutes. The RNA was dissolved in 1mM Tris; 50 mM KCl; 1.5 mM $MgCl_2$, pH 7.5 (TKM buffer) and again precipitated as above. The final pellet was dried with 95% nitrogen/5% oxygen and dissolved in TKM buffer.

The RNA was stored at -70°C.

b) Polysomal RNA

The post - 18,300g supernatant from the lysed reticulocytes was centrifuged at 150,000g for 1 hour. The pellet was dissolved in TKM buffer and the RNA was extracted by the method used for the total RNA.

Preparation of EDTA - treated polysomes.

To obtain EDTA - treated polysomes, the polysome pellet was prepared as above. It was gently suspended by hand-homogenisation in 0.01 M tris; 0.01 M KCl, pH 7.0 at a polysome concentration of 5 mg/ml. A half volume of 0.1M EDTA, pH 7 was then added to the polysomes.

Preparation of 5s RNA

5s RNA was prepared by the method of Reynier et al. (1967).

Polysomes were prepared from the starting material (Mouse reticulocytes or cultured Landschutz cells). The polysome pellet was taken up in TKM buffer with gentle homogenisation. The solution was made 6% with respect to 4-amino salicylic acid and extracted with phenol/cresol/8-hydroxyquinoline, as in the preparation of RNA, until there was no visible protein interface. The RNA was twice precipitated with ethanol and potassium acetate as in the preparation of RNA. The final RNA pellet was taken up in 1 ml water. To this was added 1 ml 4 M sodium chloride. The solution was stored overnight at 4°C. After centrifugation at 18,300g for 10 minutes, the pellet containing 18s and 28s RNAs

was discarded. Two volumes of ethanol were added to the supernatant fraction. After two hours at -20°C, the solution was centrifuged at 18,300g for 10 minutes. The pellet was taken up in 1 ml 0.05 M KCl, pH 5.0, and passed through a 1.5 x 80 cm sephadex G-100 column (Pharmacia, Uppsala, Sweden), previously equilibrated with the buffer. 1 ml fractions were collected. The fractions from the 5s RNA peak were pooled and precipitated with ethanol and potassium acetate as above. The RNA pellet was dissolved in 0.5 ml 0.05 M KCl, pH 5.0 and again passed through a sephadex G-100 column. The 5s RNA was again precipitated with ethanol and potassium acetate. The RNA pellet was taken up in the appropriate buffer and analysed for purity on 6% polyacrylamide gels (see methods).

2.6 Culture of mouse embryo livers and preparation of RNA

The foetal liver is the site of erythropoesis from the 12th to the 17th day of gestation of the mouse embryo (Russell and Bernstein, 1966). The haemoglobins synthesised are of the adult type. The liver contains a small but varying number of liver parenchymal cells and circulating immature blood cells as well as the erythroid cells. In the erythroid series, the proerythroblasts decrease and the basophilic and polychromatic erythroblasts increase from days 13 to 15. Concomitant with this maturation is a decrease in the actinomycin D inhibition of globin synthesis (Fantoni et al., 1968). 95% of the total protein synthesised is globin by the time the cells become anucleate.

Methods

Preparation and culture of 14 day mouse embryo livers

14 day pregnant mice were killed by dislocation of the neck. The embryos were removed into sterile Hank's balanced salts solution (Paul, 1965). Livers were excised and disaggregated by overnight exposure at 4°C to Difco trypsin 1/250 volume in isotonic NaCl; sodium citrate, pH 7.8, containing 0.3% sodium carboxymethyl cellulose (Paul, 1965). Alternatively, the livers were suspended by vigorous pipetting.

The cell suspensions were set up in Waymouth's medium MB 752/1 (Waymouth, 1959) as described by Cole and Paul (1966), supplemented with 10% foetal bovine serum (Flow Laboratories Ltd) and 2 mls 6.6% Na_2CO_3. The cell concentration was approximately 10^6 cells/ml. 4μc/ml 5 - ^3H uridine was added to the culture medium. Incubation was for 20 hours at 37°C in Roux flasks gassed with 5% CO_2 in air, to a pH of 7.4.

Preparation of RNA from incubated mouse embryo livers

The cells were centrifuged at 700g for 10 minutes. The medium was decanted and the cells suspended in 10 mls 0.001 M $MgCl_2$ by hand homogenisation. After centrifugation at 18,300 g for 10 minutes, 0.6 gm 4-amino salicylic acid was added to the supernatant. This was extracted 3 times with phenol/cresol/8-hydroxyquinoline as in the preparation of reticulocyte RNA. After two precipitations with ethanol and potassium acetate, the RNA pellet was dissolved

in 1 ml TKM buffer. 20µg/ml DNase was added to the nucleotide preparation. After incubation at 37°C for 30 minutes, the solution was made 6% with respect to 4-amino salicylic acid. After two cycles of extraction with phenol/cresol/8-hydroxyquinoline as before, the RNA was precipitated twice with ethanol and potassium acetate. The final precipitate was dissolved in 1 ml TKM and stored at -70°C.

2.7 Maintenance and culture of Landschutz tumour cells; preparation of RNA.

The Landschutz cell line was used. This is derived from the mouse mammary gland and is cultured in the mouse peritoneum in vivo. Mice were innoculated directly into the abdomen with 0.1 - 0.25 ml tumour suspension. A week after injection, the mice were killed. The skin was everted, exposing the sterile abdomen wall. The cell suspension was syringed off aseptically.

The cells were set up in culture at a concentration of 200,000/ml. The medium used was 10 mls MEM amino acids; 5 mls MEM vitamins; 5 mls glutamine; 25 mls calf serum; 2.5 mls penicillin; 5 mls 20% glucose in 400 mls Tris-citrate BSS (Paul, 1965). In some runs, phosphate-free BSS was used. The pH was adjusted to 6.9 with 6% sodium bicarbonate. 5.0µc/ml ^{32}P-orthophosphate was added and the cells were incubated in stirrer cultures for 36 hours.

The cells were centrifuged at 1,000 g for 10 minutes. They were resuspended in 10 mls 0.001 M $MgCl_2$ and homogenised at top

speed (5 strokes). After centrifugation at 18,300 g for 10 minutes, the supernatant was stored and the pellet taken up in 4 mls 0.001 M $MgCl_2$ and subjected to a second homogenisation and centrifugation. The supernatants were pooled and total RNA or 5s RNA was prepared as in methods.

2.8 Separation and analysis of RNA species by centrifugation.

Density gradient centrifugation in swing-out heads

Gradients formed by a device adapted from that of Bock and Ling (Bolton et al., 1958) were stored at 4°C for several hours prior to use. The polynucleotide solutions were layered onto the gradient in a volume of 0.1 ml (3 x 5 head) or 0.4 ml (3 x 23 head). Centrifugation of RNA was for 3 hours at 100,000 g (3 x 5 head) or for 6 hours at 92,000 g (3 x 23 head). 5 ml tubes were pierced in the tube holder designed by Edwards and Mathias (1963). The M.S.E. tube holder (cat. no. 59557) was used for 23 ml tubes. Gradients were collected by upward displacement with 40% (w/v) sucrose containing methylene blue. The effluent was continuously monitored at 260 mμ by passing through a flow cell (Perkin-Elmer Ltd., London) in a Unicam Sp-800 spectrophotometer.

Density gradient centrifugation in the zonal ultracentrifuge

Zonal ultracentrifuges (Anderson, 1962) were developed for the large-scale separation of particles of different densities or molecular weights. The scale of such separations had previously been limited by the size of conventional swing-out heads. Zonal ultracentrifuges have several advantages besides capacity. They have sector-shaped

compartments for ideal sedimentation; rapid gradient formation with minimum convection; sharp sample boundaries and rapid recovery of the gradient. There is no loss of resolution by band spreading since the gradient is recovered while the rotor is rotating (Brakke, 1964).

Anderson and Burger (1962) demonstrated the sub-fractionation of a rat liver homogenate in a zonal rotor. Higashi et al. (1966) showed the improved resolution of 6-55s RNA species using a zonal rotor. The technique has been utilised in the large-scale isolation of viruses (Anderson, 1963), rat macroglobin (Fisher and Canning, 1966), Tetrahymena polysomes (Cameron et al., 1966), ribosomal RNA (Hastings et al., 1965), ribosomal subunits (Eikenberry et al., 1970) and isoenzymes (Self and Weitzman, 1970).

Zonal ultracentrifugation also facilitates the recognition and isolation of minor RNA components (Hastings et al., 1965); for example the isolation of 5s RNA from EDTA - treated polysomes (Parish et al., 1966), the preliminary fractionation of 4-7s RNA species in the isolation of 7s RNA from liver nuclei (Hodnett and Busch, 1968) and the preliminary purification of 9sRNA from rabbit reticulocytes (Huez et al., 1967).

Methods

The aluminium B-XV and the titanium B-XIV zonal rotors were used in the M.S.E. 50 and the M.S.E. 65 superspeed ultracentrifuges respectively. The construction and properties of these rotors have been described by Anderson et al. (1967). Loading was carried out

according to M.S.E. technical publication no.49 and Anderson (1966).

Both loading and unloading of the rotor were at a rotor speed of 2,500 rpm. A linear with volume sucrose gradient, light end first, was introduced into the rotor through the edge pipe of the rotor feed head assembly. This was followed by a 40% (w/v) sucrose "cushion" until the rotor was full. The sample, in 2.5% (w/v) sucrose, was introduced through the centre pipe of the rotor feed head assembly and displaced to the required position in the rotor by the introduction of 40% (w/v) sucrose through the edge gradient pipe.

The rotor was sealed and centrifuged for the required time. The rotor contents were then displaced through the centre pipe by the introduction of 40% (w/v) sucrose through the edge gradient pipe. The effluent was continuously monitored at 260mμ by passing through a variable path length flow cell (Perkin-Elmer Ltd., London) in a Unicam SP-800 spectrophotometer. A servoscribe external chart recorder was linked to the spectrophotometer. The sucrose concentration of the effluent was monitored at intervals using an Abbe refractometer. Peak fractions were pooled and precipitated (see methods). The purity of each fraction was monitored on 2.6% polyacrylamide gels.

Loading Volumes

B - XV rotor (capacity: 1670 ml).

linear sucrose gradient : 1400 ml

underlay : 50 ml

sample : 20 ml

overlay : 200 ml.

B - XIV rotor (capacity: 650 ml)

linear sucrose gradient : 560 ml

underlay : 30 ml

sample : 8 ml

overlay : 52 ml

For the separation of EDTA - treated polysomes, a gradient of 15-30% (w/v) sucrose in 10 mM tris; 10 mM KCl, pH 7.0 was used. For RNA fractionation, a 5-20% (w/v) sucrose gradient in TKM buffer, pH 7.4 was used. The overlay and the 40% (w/v) sucrose underlay were made up in the appropriate buffer. Latterly, all sucrose solutions were shaken vigorously for 2 minutes with 0.1% DEP and stored for 24 hours at room temperature. After use, all apparatus and tubing was sterilised with water saturated with DEP.

Recovery of RNA from large volumes.

a) Ethanol precipitation

Messenger RNA cuts from zonal runs (about 270µg in 100 mls) were usually recovered by precipitation with 1/5 volume potassium acetate and 2 volumes ethanol. After standing at -20°C for at least 12 hours, the RNA was sedimented by centrifugation at 22,280g for 30 minutes in the Sorvall swing-out rotor, HB-4.

Larger volumes of more concentrated RNA fractions were similarly precipitated by centrifugation in 150 ml corex bottles at 15,600 g for 30 minutes in the angle head, GSA. Ethanol

precipitation was the usual method used to concentrate most of the RNA preparations.

b) Column concentration

The addition of ethanol to a nucleic acid solution permits non-ionic sorption of RNA onto modified cellulose. The variation of sorption with the species of RNA permitted Barber (1966) to separate rRNA and tRNA on a modified cellulose column. Huez et al. (1967) used the procedure to concentrate the messenger ribonucleoprotein (mRNP) fractions recovered from zonal ultra-centrifugation of EDTA - treated ribosomal subunits.

Method

The zonal mRNP fraction was made 0.5% with sodium dodedyl sulphate and 0.1 M with NaCl. 0.54 volumes of ethanol were added and the solution passed through a 10 x 1.5 cm column of Whatman column chromedia CF11 sephadex. This had previously been equilibrated with 0.1 M NaCl/35% ethanol at 4°C. The column was washed with 20 mls of the 0.1 M NaCl/35% ethanol solution. The RNA was eluted with distilled water. Peak RNA fractions were pooled and precipitated with ethanol and potassium acetate.

Analytical ultracentrifugation

Determination of RNA molecular weights on sucrose gradients may depend not only on RNA chain lengths but also on their secondary structures (Gesteland and Boedtker 1964). The sedimentation coefficient is dependent on molecular weight alone if the RNA secondary

structure is eliminated, for example by formaldehyde (Boedtker 1968).

Methods

The sedimentation coefficient of 9s RNA was determined in the presence of formaldehyde (Boedtker, 1968). 25µg/ml 9s RNA in 1.1 M formaldehyde; 0.09 M Na_2HPO_4; 0.01 M NaH_2PO_4 was heated for 15, 30 or 60 minutes at 63°C. The samples were cooled rapidly in ice and sedimentation rates analysed immediately at 20°C in a spinco Model E ultracentrifuge.

Determination of RNA sedimentation coefficients by zone ultracentrifugation

The sedimentation coefficients of RNA species in a sucrose density gradient in the B-XIV zonal rotor were calculated using the computer programme published by Oak Ridge National Laboratory (Bishop, 1966). A zonal centrifugation on total reticulocyte RNA was set up using exactly defined conditions. The effluent was monitored for sucrose concentration at 20 ml intervals and continuously at 260mµ as already described.

2.9 Separation of RNA species by polyacrylamide gel electrophoresis

Analytical polyacrylamide gels

Polyacrylamide gel electrophoresis, as adapted by Loening (1967), gives a better resolution of high molecular weight RNA species than sucrose gradients (Weinberg et al., 1967; Loening and Ingle, 1967). In 2.4% polyacrylamide gels, RNA species of up to 45s can be resolved (Loening and Ingle, 1967). Polyacrylamide gel electrophoresis was

first used to study the heterogeneity of small molecular weight RNAs (Richards, et al., 1965) and RNA breakdown products (Gould, 1966). On 15% polyacrylamide gels, species in the molecular weight range 10-50,000 and differing by 2-3 nucleotides can be resolved.

Bishop et al. (1967) showed that the logarithm of the molecular weights was linearly related to the relative electrophoretic mobility for viral RNAs of molecular weights between 2.6×10^4 and 23×10^6 in 2.4% polyacrylamide gels. Loening (1969) showed this relationship was valid for RNAs of different base compositions at moderate salt concentrations in 2-2.6% gels. The relationship between molecular weight and mobility only partially holds for higher percentage polyacrylamide gels. In 5% gels, the plot of S value is linear up to a molecular weight of 40×10^4. Heavier RNA species are retarded at the top of the gel (McPhie et al., 1966).

Methods

Acrylamide and N,N^1- methylenebisacrylamide (Eastman Organic Chemicals, New York) were recrystallised from chloroform and acetone respectively (Loening, 1967). No purification was required for the N,N, N^1, N^1 - tetramethylethylenediamine (TMED; Kodak Ltd. London) or the ammonium persulphate (May and Baker, Dagenham, England).

Characterisation of RNA species was as described by Loening (1967). Electrophoresis was in 0.5 x 7 cm siliconed glass tubes. The buffer used was 0.04 M Tris; 0.02 M sodium acetate; 0.002 M

EDTA, pH 7.8 (Loening, 1967). 2.6% gels contained 0.26gm acrylamide, 0.013 gm bisacrylamide, 0.008 ml TMED and 0.08 ml 10% ammonium persulphate in 10 mls buffer. 6% gels contained 0.6 gm acrylamide, 0.015 gm bisacrylamide, 0.016 ml TMED and 0.07ml 10% ammonium persulphate in 10 mls buffer.

Samples were made up in 10% sucrose in buffer. Gels were pre-run for 1 hour at 17 v/cm. After sample application, electrophoresis was carried out for 20 minutes at 3v/cm then for the required time at 17v/cm. Electrophoresis was at 6 ^{o}C. The gels were scanned at 260mμ with the Joyce-Loebel UV scanner using a liquid filter (0.001% p-dimethylamino-benzaldehyde, (Sigma) in methanol). Gels were stained in 0.2% toluidine blue (Williamson et al., 1961). in water for 1 hour. They were destained overnight.

Samples of polysomes were electrophoresed in sodium dodecyl sulphate (specially pure) buffer. Gels were made up as above and the compartment buffers were made 0.2% with SDS. The gels were pre-run for 1 hour. The polysome samples were made 1% with SDS. 60μg were applied/gel. Electrophoresis was for 20 minutes at 3v/cm then for 40 minutes at 7v/cm. Electrophoresis was at room temperature.

The gels were scanned as before and treated for staining by an adaption of the method of Weber and Osborn (1969). Gels were soaked overnight in 5% acetic acid; 50% methanol. After 1 hour in water, the gels were stained with 0.2% toluidine blue

in water for 1 hour. They were destained overnight.

Determination of radioactivity in gels

Gels were frozen with powdered CO_2 (Dri-cold). 1 mM slices were cut using a gel slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey). The slices were transferred to scintillation vials. For counting ^3H, 1 ml 10% (v/v) piperidine was added to each slice and the vials incubated at 60°C until the piperidine had evaporated. 1 ml water was added and after the gels had swollen, 10 mls toluene based scintillator/triton X-100 were added (Loening, 1967). ^{32}P gels were counted directly by Čerenkov radiation.

Preparative polyacrylamide gels

The RNA species separated on analytical polyacrylamide gels may be purified in large amounts by electrophoresis on preparative polyacrylamide gels (Lanyon et al., 1968). Although the maximum loading of RNA is less than one sixth that for the B-XIV zonal rotor, the resolution of all the RNA species in one gel run is much greater.

Method

A Buchler preparative polyacrylamide gel electrophoresis apparatus ("Polyprep"; Buchler Instruments Inc., Fort Lee, New Jersey, U.S.A.) was used (Lanyon et al., 1968). The buffers used were 0.04 M tris; 0.02 M sodium acetate; 2 mM EDTA, pH 7.8, or a buffer of one quarter this strength. The gel was 75 mls 3% acrylamide, 0.3% bisacrylamide containing 0.086 ml TMED

and 0.86 mls 10% ammonium persulphate.

The gel was pre-run for 1 hour at 100v/150mA. The sample was layered onto the gel in 4 mls electrophoresis buffer, 5% (w/v) sucrose. After application of the sample, the voltage was lowered to 50v till all the sample had entered the gel (2 hours). Electrophoresis was then continued at 150v/200mA. Electrophoresis was at room temperature.

The elution rate was 1 ml/minute. 5 minute fractions were collected. The eluant was monitored with a LKB Uvicord II linked to a chart recorder (Yokogawa Electric Works Ltd., Japan). 0.05 ml aliquots were taken to monitor radioactivity. Peak fractions were pooled and precipitated with ethanol and potassium acetate. The purity of each fraction was monitored by analytical polyacrylamide gel electrophoresis.

2.10 In vitro methylation of RNA with ^{3}H- dimethyl sulphate

Polynucleotides and bases in nucleic acids can be alkylated *in vitro* with alkylating agents such as dimethyl sulphate (Lawley and Brookes, 1963; Brimacombe *et al.*, 1965). RNA is methylated at N^7- guanine with dimethyl sulphate at pH 5 (Pochon and Michelson, 1967). At pH 7, the order of decreasing reactivity to methylation is N^7 of guanine, N^1 of cytosine and N^3 of adenine. All RNA size classes are methylated more or less equally (Smith *et al.*, 1967). The specific activities of different RNAs in a radioactive methylated RNA sample may therefore be unambiguously calculated (Smith *et al.*, 1967).

Methylated RNA has similar hybridisation properties to RNA labelled *in vivo* (Smith *et al.*, 1967; Davidson and Hough, 1969). Contamination of the RNA with labelled impurities gives non-specific binding to filters. This is reduced by incubating in the presence of sodium dodecyl sulphate (Crippa and Gross, 1969) or pre-treating the filters in the medium of Denhardt, 1967 (Smith *et al.*, 1967; Crippa and Gross, 1969).

Method

RNA was methylated *in vitro* by a modification of the method of Smith *et al.* (1967).

To 0.5 - 1 mg RNA in 0.2 mls 0.1 M sodium phosphate buffer, pH 7.5, was added 0.2 mls diethyl ether and 0.1 ml ^{3}H-dimethyl sulphate in benzene (167 mc/mM; batch 4, Amersham). The two phase system was shaken vigorously in a tightly - stoppered glass tube for 3 hours at room temperature. After incubation, 1 ml TKM buffer, 0.4 mls 6 M potassium acetate and 2 volumes ethanol were added. After standing at -20°C for at least 3 hours, the RNA was precipitated by centrifugation at 22,280 g for 30 minutes. The RNA was redissolved in 2 mls TKM buffer and washed twice more by successive potassium acetate and ethanol precipitations. Aliquots of each ethanol supernatant fraction after centrifugation were taken for determination of radioactivity.

The final RNA precipitate was resuspended in 0.5 mls 0.01 M sodium acetate; 0.05 M NaCl, pH 5.0 and layered on a 1 x 28cm column of sephadex G-25 (Pharmacia, Uppsala, Sweden) previously equilibrated

with the same buffer. The RNA was eluted with the 0.01 M sodium acetate; 0.05 M NaCl buffer, 1 ml fractions were collected and 0.01 ml samples were assayed for radioactivity. Peak RNA fractions were pooled and precipitated with ethanol and potassium acetate as before. The RNA was finally dissolved in the required hybridisation medium and dialysed overnight in the cold against the same medium. The final concentration and specific activity of the RNA were measured. The RNA solution was stored at -20°C.

2.11 Preparation of DNA

Preparation of crude mouse embryo DNA

DNA was prepared from 17 day mouse embryos by a modification of the method of Marmur (1961). The embryos were washed twice in Hank's balanced salts solution (Paul, 1965) then homogenised in 20 volumes of 0.005 M citric acid in an M.S.E. atomix homogeniser. The homogenate was filtered through 3 layers of fine gauze then centrifuged at 10,800 g for 10 minutes. The supernatant was discarded. The nuclei were suspended in 10 mls 0.1 M EDTA, pH8.0 and centrifuged at 10,800 g for 10 minutes. The nuclei were resuspended in 10 mls of the EDTA solution and added dropwise to a vortexing solution of 100 mls 2.2% sodium dodecyl sulphate in 1.1 x SSC. (1 x SSC = 0.15 M NaCl; 0.015 M sodium citrate, pH 7.0).

The mixture was stirred for 1 hour at room temperature. 6.6 gm NaCl were added and stirring continued till the mixture was homogeneous. An equal volume of chloroform/isooctanol (24:1) was added and the

mixture was gently shaken for 15 minutes. After centrifugation at 10,800 g for 15 minutes, the aqueous phase was recovered and the DNA spooled from it on the addition of 2 volumes ethanol, with stirring.

The DNA was dissolved in 50 mls 0.1 x SSC and extracted with an equal volume of chloroform/isooctanol. The aqueous phase was recovered and subjected to two further cycles of extraction and centrifugation. Two volumes ethanol were added to the final aqueous phase and the DNA was spooled as before. The DNA was dissolved in 50 mls 1 x SSC for RNase treatment. DNA to be purified by banding in caesium chloride was dissolved in 10 mls 0.01 M EDTA; 0.1 x SSC, pH 7.0.

Enzymic purification of DNA

2.5 mg pronase was dissolved in 2.5 mls water and pre-incubated for 2.5 hours at 37°C. 1.25 ml RNase (2mg/ml; boiled for 10 minutes to remove DNase contamination) was added to the DNA solution. This was incubated for 30 minutes at 37°C. The pronase was then added and incubation continued for a further 90 minutes.

The mixture was cooled to room temperature then extracted several times with equal volumes of chloroform/isooctanol (24:1) until there was no visible protein interface. DNA was spooled from the supernatant after the addition of 2 volumes ethanol. The DNA was redissolved in 27 mls 1 x SSC. 3 mls 3 M sodium acetate; 0.001 M EDTA, pH 7.0 were added and the DNA was spooled out by the dropwise addition of

16.2 mls isopropanol. The spooled DNA was washed with 70% ethanol then with 90% ethanol. It was dissolved in 4 mls 0.01 x SSC.

Iodoacetate inactivation of RNase

Carboxymethylation by iodoacetate of histidine residues at the active sites of several cellular ribonucleases may be used for their inactivation (Terao and Ukita, 1967). The DNA solution was adjusted to pH 5.5 with acetic acid and 0.1 N NaOH. 0.1 M iodoacetate, pH 5.5, was added to bring the iodoacetate concentration to 20 µg/ml. After incubation for 40 minutes at 40°C, the DNA was freed from iodoacetate by precipitation with ethanol. The DNA was dissolved in 0.01 x SSC; 0.15 M NaCl, pH 7.0, and stored at -70°C.

Assay for residual ribonuclease activity

To 0.05 mls DNA sample was added 1.45 mls 0.1 M tris, pH 7.4. A blank containing 20 µg RNase in 1.45 mls tris, pH 7.4, was also incubated at 37 °C. 1.5 mls of yeast RNA solution (0.5mg RNA/ml 0.1 M tris, pH 7.4) was added to each tube. After 90 minutes incubation at 37°C, the tubes were placed in ice. 3 mls ice-cold RNA precipitant solution (2.5 gm lanthanum nitrate in 125 mls 5 M hydrochloric acid and 385 mls ethanol) were added. The solutions were mixed and filtered through Whatman No. 1 filter paper. The absorbance of the filtrates was read at 260 mµ. Readings for blanks were <0.22 optical density units.

DNA purification by isopycnic banding in caesium chloride

Isopycnic caesium chloride centrifugation is used to purify

DNA. Polysaccharides and proteins are dissociated from the DNA by the high salt. They remain at the meniscus. The DNA is banded and the RNA is pelleted. Centrifugation in fixed-angle rotors has several advantages over centrifugation in swing-out rotors.

1) More DNA can be purified in one run.

2) The narrower density range of fixed-angle CsCl gradients concentrates polysaccharides and protein contaminants at the meniscus.

3) RNA sediments more quickly in fixed-angle rotors. It pellets on the outer tube wall and does not contaminate the gradient during drop collection (Flamm et al., 1966; Flamm et al., 1969).

An alternative large-scale procedure is to band the DNA in the titanium B-XIV zonal rotor (Williamson, 1970).

Methods

a) Banding of DNA in fixed-angle heads

100 gm CsCl (BDH, analytical grade) was dissolved in 60 mls 0.01 M EDTA; 0.1 x SSC, pH 7.0. The solution was filtered through a millipore filter. 8 mg DNA were added and the refractive index was adjusted to 1.4012 (1.72 gm/ml) with the buffer. 0.9 mg DNA in 15 mls caesium chloride/tube was overlaid with paraffin. Centrifugation was at 100,000g for 48 hours in the M.S.E. 8 x 2.5 aluminium head or at 190,000 g for 24 hours in the M.S.E. 8 x 25 titanium head. The running temperature was 20°C.

20 drop fractions were collected using the M.S.E. tube piercer

(cat. no. 59557). The refractive index of each fraction was read. 1 ml water was added to each tube and the absorbance at 260 mμ was measured. Peak tubes were pooled and the solution was dialysed overnight against 0.1 x SSC. Two volumes of ethanol were added. After 2 hours at -20°C, the DNA was precipitated by centrifugation at 18,300 g for 30 minutes. The DNA pellet was dissolved in 0.15 M NaCl; 0.1 M EDTA, pH 7.0 and stored at -70°C.

b) Banding of DNA in the B-XIV titanium zonal rotor

The method is that of Williamson (1970). 470 gm CsCl was dissolved in 340 mls 0.01 M EDTA; 0.1 x SSC, pH 7.0. The solution was passed through a millipore filter. Up to 20 mg DNA was added. The refractive index was adjusted to 1.4012 with buffer. The DNA solution (about 450 mls) was poured into the open B-XIV titanium zonal rotor. The sealed rotor was centrifuged at 39,000 rpm for at least 3 days.

The rotor contents were pumped out with sym-tetrabromoethane (2.96 gm/ml) at a flow rate of 1 litre/hour. 15 ml fractions were collected. The refractive index and absorbance at 260 mμ of each fraction were found. Peak fractions were pooled and DNA recovered as before.

Preparation of DNA from mouse sperm.

Induced ejaculation in mice is unreliable and the ejaculate soon coagulates. Sperm can however be obtained from the epididymis and vasa deferentia (Parkes, 1965). Mammalian sperm DNA cannot be

isolated by conventional techniques since it is protected by a keratin - like membrane surrounding the sperm head. Prior reduction of the protein - sulphur linkages in a lipid - free sample allows isolation of the DNA (Borenfreund et al., 1961).

Method

Mouse vasa deferentia were excised into balanced salts solution (Paul, 1965). The sperm were freed by gently stroking the vasa deferentia with a glass rod. The sperm were counted in a counting chamber (x 250 magnification).

Initial isolation of the DNA was according to Borenfreund et al. (1961). The sperm were centrifuged at 8,000 g for twenty minutes and washed twice with 0.15 M NaCl; 0.015 M sodium citrate, once with ethanol and once with a 1:1 ethanol, ether mixture. After each wash the sperm were pelleted by centrifugation at 8,000 g for thirty minutes. To each 100 mg sample was added 20 mls 0.15 M NaCl; 0.015 M sodium citrate; 0.25 M mercaptoethanol. The sample was incubated for 2 hours at $4^{o}C$ with gentle stirring. 10 mg trypsin were added and the incubation continued for 1 hour at room temperature.

The pellet obtained after the sample was centrifuged at 12,000 g for 1 hour was re-extracted with 10 mls buffer and 5 mg trypsin as before. The DNA was spooled from the combined supernatants after the addition of two volumes ethanol. The DNA was dissolved in 50 mls 0.1 M tris; 0.15 M NaCl, pH 7.4. 50 mls 2.2% SDS and 1.1x SSC were added and crude DNA was then prepared as in the methods.

The DNA was finally purified by banding in CsCl.

Estimation of DNA size

180µg DNA were dissolved in 0.5 ml 0.9 M NaCl; 0.1 M NaOH and layered on 5 - 25% (w/v) sucrose gradients in 0.9 M NaCl; 0.1 M NaOH. After centrifugation at 58,000 g for 13.5 hours in the M.S.E. 3 x 23 head, 1 ml fractions were collected by upward displacement with 40% (w/v) sucrose. The refractive index and absorbance at 260 mµ were measured for each fraction. The S value was determined according to McEwan (1967) and the molecular weight calculated using the formula $S = 0.0528\ M^{0.4}$ (Studier, 1965).

2.12 RNA - DNA hybridisation

The technique of RNA - DNA hybridisation has been widely used in the study of DNA primer - RNA product relationships.

Hall and Spiegelman (1961) formed hybrids by cooling denatured DNA in the presence of RNA from 67°C to room temperature. The hybrids were separated from the bulk DNA and from free RNA by CsCl centrifugation. Grossly unpaired regions of RNA still attached to DNA were eliminated by treating the hybrids with pancreatic ribonuclease (Yankofsky and Spiegelman, 1962a). Several methods were developed for immobilising the DNA prior to hybrid formation. Bautz and Hall (1962) used phospho-cellulose acetate to immobilise glucosylated phage DNAs. This method was extended to non-glucosylated DNAs by their immobilisation in cellulose or agar gels (Bolton and McCarthy, 1962).

The selective retention of denatured DNA by nitrocellulose filters allowed Nygaard and Hall (1963, 1964) to isolate hybrids by their retention on filters after a liquid annealing step. This property of denatured DNA was also used in solid-liquid hybridisations where the DNA was immobilised on a filter and then hybridised to RNA in solution (Gillespie and Spiegelman, 1965). This not only allowed rapid processing of many filters but also eliminated DNA - DNA reannealing during the incubation.

Incubation temperaturesof 67°C and above are often necessary for the formation of hybrids with a high proportion of matched base pairs. These elevated temperatures can cause RNA degradation. The properties of organic solvents such as formaldehyde to reduce the thermal stability of double-stranded polynucleotides (Marmur and TS' 0, 1961; Subirana and Doty, 1966) make possible maximum rates of hybridisation and specific hybrid formation at lower temperatures (McConaughy et al., 1969).

Methods

Loading of filters

All solutions were pre-treated with 0.1% DEP and incubated for 48 hours at room temperature. The pH of each solution was adjusted to 7.0 .

13 mM diameter nitrocellulose Sartorius membrane filters (V.A. Howe Ltd., London) were boiled in 4 x SSC for 10 minutes, soaked overnight in 4 x SSC then washed on each side with 20 mls

4 x SCC. DNA was alkali-denatured in the cold by a 10 minute incubation in 0.44 N NaOH. The pH was adjusted to 7 and the DNA solution was loaded onto the filters at a DNA concentration of 5μg/ml and in 4 x SSC. Loading was done under gravity. The filters were washed with 20 mls 4 x SSC on both sides, dried overnight at room temperature then baked at 80°C for 2 hours.

The filters were pre-incubated for 1 hour in 4 x SSC containing 0.02% ficoll (Sigma, St. Louis, Missouri), polyvinylpyridone (Sigma) and bovine serum albumin (Sigma) according to Denhardt (1967). The filters were dried at room temperature for 1 hour and were used immediately. Blank filters, containing no DNA, were taken through the washing and pre-incubation procedures.

Hybridisation procedure.

Incubations were with varying amounts of labelled RNA in 0.1 ml 2 x SSC/0.1% SDS at 67°C or in 0.2 ml 4 x SSC/50% formamide at 36.5°C, unless otherwise specified in figure legends.

13 mM filters were incubated in plastic vials. 2 blank and 2 DNA filters were incubated in each vial. 27 mM filters were rolled up and incubated in polypropylene microtubes (Glen Rothes, Fife). 1 blank and 1 DNA filter were incubated in each tube. Annealing was for 16 hours, unless otherwise specified in figure legends.

Washing procedure.

After the period of annealing, the 13 mM filters were placed in 4 x SSC at 36.5°C. All subsequent washes were performed at room

temperature. The filters were washed on each side with 20 mls 4 x SSC. They were incubated for 1 hour in 2 x SSC containing 20μg/ml pancreatic ribonuclease. There were 5 mls incubation mixture/filter. After rinsing for 30 minutes with stirring in 4 x SSC, the filters were washed on each side with 20 mls 4 x SSC then dried for 1 hour at 80°C. 27 mM filters were taken through the same washing procedure but were washed with 50 mls 4 x SSC on each side.

The filters were counted in 5 mls toluene based scintillator. Where levels of radioactivity were low, disposable plastic vials were used to reduce and stabilise backgrounds.

Thermal dissociation profiles.

The stability of RNA - DNA hybrids was assayed by washing the filters at increasing temperatures. Filters which had been counted were rinsed in toluene, dried, then rinsed several times in 2 x SSC at room temperature and again dried at room temperature. Hybrids were dissociated by incubating the filters in 0.5 mls 2 x SSC at increasing temperatures. The counts dissociated at each temperature were assayed by adding 0.5 mls water and 10 mls toluene based scintillator/triton X -100 to the eluant.

RESULTS

3. RESULTS

3. RESULTS

3.1 Isolation of 9s RNA from mouse reticulocytes

Preparation of RNA from mouse reticulocytes

The blood from anaemic mice routinely contained about 80% reticulocytes. Two hundred mice gave a yield of 40 ml packed red blood cells after they had been washed 3 times in balanced salts solution. The washing removed the majority of white blood cells. Total RNA was routinely prepared from the post - 18,000 g supernatant fraction of lysed reticulocytes. 30-40 mg total RNA was isolated from 40 mls packed red blood cells.

Figure 1 shows the banding pattern of total RNA when electrophoresed on 2.6% polyacrylamide gels; the major bands correspond to the 28s, 18s and 4s and 5s RNA fractions. The two minor bands between 18s and 5s RNA will be designated "12s" and "9s" RNAs in the results and discussion. In heavily-loaded gels. a band running at approximately 7s is also visible.

The 9s RNA is present at about 2-3 times the amounts of 12s RNA and represents approximately 1.3% of the total RNA. In early experiments, using macaloid as a nuclease inhibitor, RNA breakdown products were sometimes observed between the 18s and 5s RNAs, although with only minor degradation, the 9s RNA band was preferentially degraded. RNA breakdown was more consistently found with RNA isolated from polysomes, probably since this RNA was phenol-extracted at a later stage than the total RNA preparations.

FIGURE 1.

Electrophoresis on 2.6% polyacrylamide gels of RNA isolated from the post-18,300g supernatant fraction of lysed mouse reticulocytes (total reticulocyte RNA).

40μg RNA was applied to the gel. Electrophoresis was for 20 minutes at 3v/cm then for 1 hour at 10v/cm at 4°C.

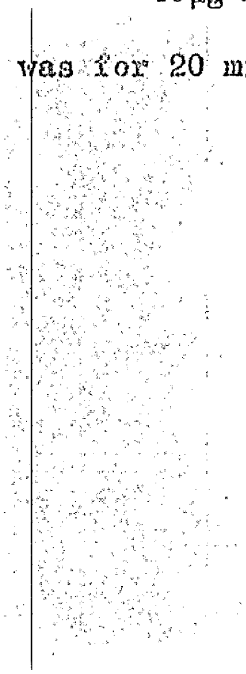
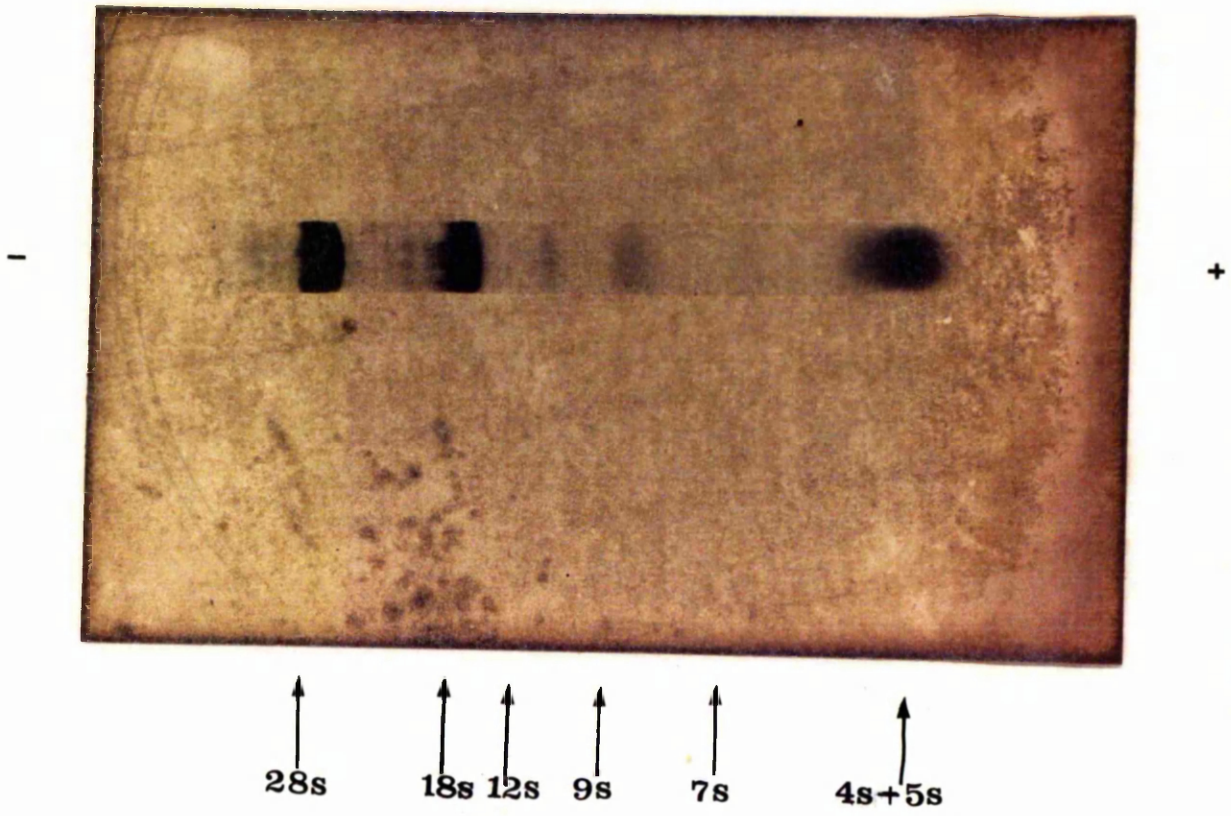


Figure 1.



The introduction of treatment with diethylpyrocarbonate (DEP) to inactivate any ribonuclease in the buffers resulted in consistent RNA preparations with only the 12s, 9s and 7s RNA bands present between the 18s and 5s RNAs.

When 0.1% DEP was added to lysed reticulocytes, most of the RNA associated with ribosomes co-precipitated with the proteins during the phenol extractions and the yield of RNA was reduced by 75%. This co-precipitation did not occur if 0.1% DEP was added to the post -18,300g supernatant or when it was used to sterilise buffers.

The 0.001M $MgCl_2$ used to lyse the reticulocytes leaves most of the white cells intact. The 18,300 g pellet consisted of two layers; a closely-packed pellet under a layer of loosely-packed cell-membranes. The two layers were separated and both were washed with the buffer and centrifuged at 18,300g. This procedure was repeated twice. The polynucleotides isolated from the first 18,300g supernatant fraction and from the two fractions of the precipitate were analysed on 2.6% polyacrylamide gels (Figure 2). The supernatant RNA gave the banding pattern already described. The polynucleotides isolated from the cell membranes contained the same proportions of both 12s RNA and 9s RNA to the rRNA as did the supernatant fraction, although there was some DNA present in the 4s region. The RNA isolated from the cell pellet was heavily

FIGURE 2.

Electrophoresis on 2.6% polyacrylamide gels of polynucleotides from different fractions of lysed mouse reticulocytes, centrifuged at 18,300g for 15 minutes.

a) Supernatant fraction.

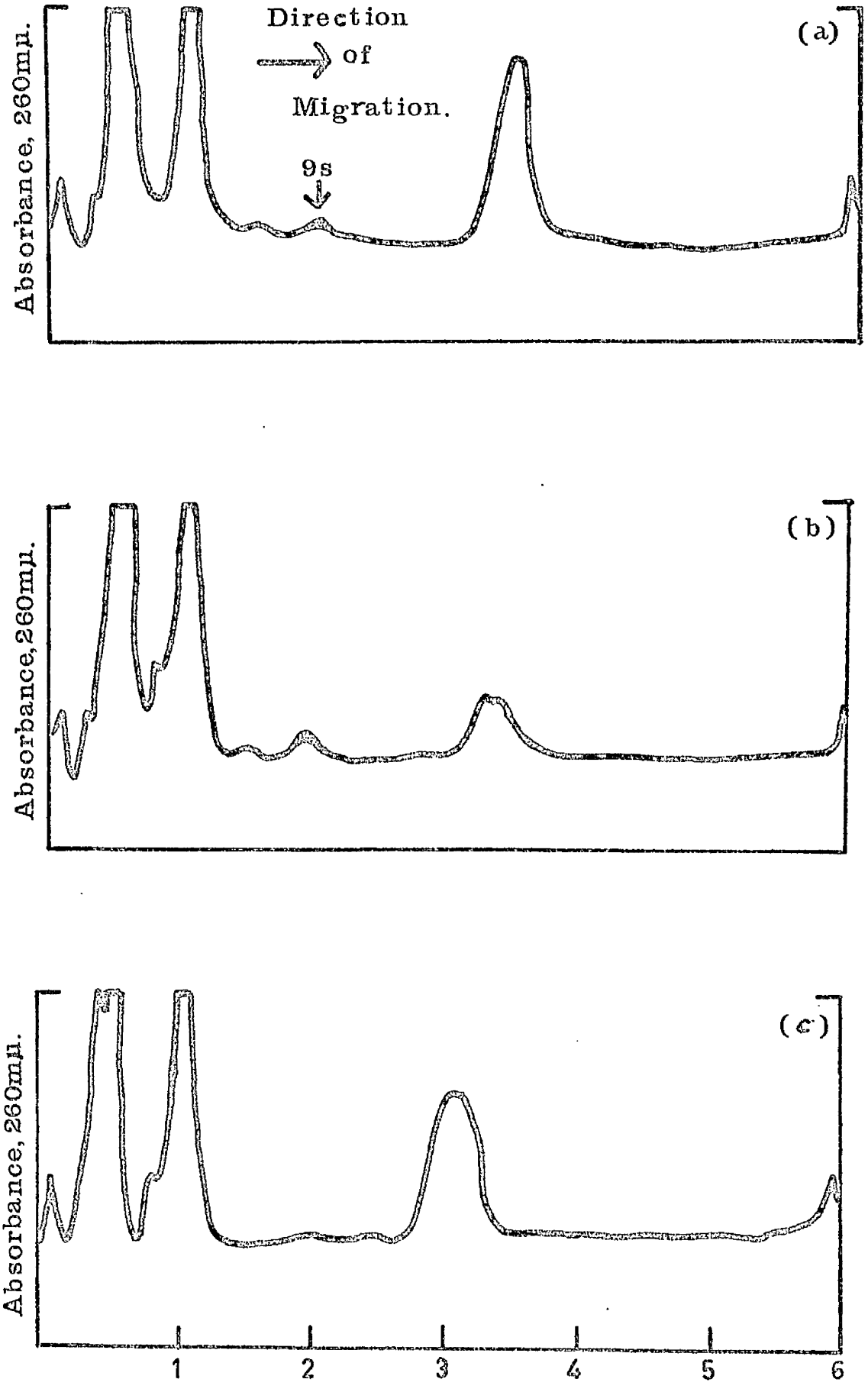
b) loosely-packed pellet.

c) firmly-packed pellet.

In each case, 40µg polynucleotides were applied/gel.

Electrophoresis was for 20 minutes at 3v/cm then for 1 hour at 10v/cm at 4°C.

Figure 2.


Direction
of
Migration.
9s
(a)
Absorbance, 260mμ.
(b)
Absorbance, 260mμ.
(c)
Absorbance, 260mμ.
1
2
3
4
5
6
Distance (cm).

contaminated with DNA and there were no 9sRNA or 12sRNA bands visible.

Gradient recovery and boundary spread in the zonal ultracentrifuge.

A sample of human blood cells was overlaid on a pre-formed gradient in the B-XV zonal rotor. After a short centrifugation, the rotor was unloaded. The effluent was monitored at E_{415} and for percentage sucrose. The results show little gradient diffusion into the underlay or overlay had occurred during this time (Figure 3). The gradient was linear for 1000 ml. It was therefore unaltered by passage through the rotor. The sample width showed little boundary spread although there was some back diffusion into the overlay.

Zonal ultracentrifugation was therefore used in the large-scale routine separation of RNA species. The technique was also used, in preference to centrifugation in swing-out rotors, to separate small amounts of total RNA (< 1 mg). Although the B-XV zonal rotor had a greater capacity, the titanium rotor was latterly used for routine isolations, since its shorter running, loading and unloading times minimised the possibility of RNase action during centrifugation.

The B.D.H. (analar) sucrose used for zonal runs contains a variable amount of RNase activity. Treatment with macaloid did not prevent some RNase activity, but preliminary sterilisation of buffers with DEP completely eliminated any RNase action during centrifugation.

Isolation of 14s mRNP fraction by zonal ultracentrifugation of EDTA-treated reticulocyte polysomes; analysis of fractions.

EDTA treatment of reticulocyte polysomes results in their

FIGURE 3.

Sample of lysed blood cells centrifuged in a 10-20% (w/v) sucrose gradient in the M.S.E. B-XV aluminium zonal rotor.

Sample volume : 40 ml.

Gradient : 1300 ml 10-20% sucrose in buffer (0.05M KCl; 0.001M tris, pH 7.4).

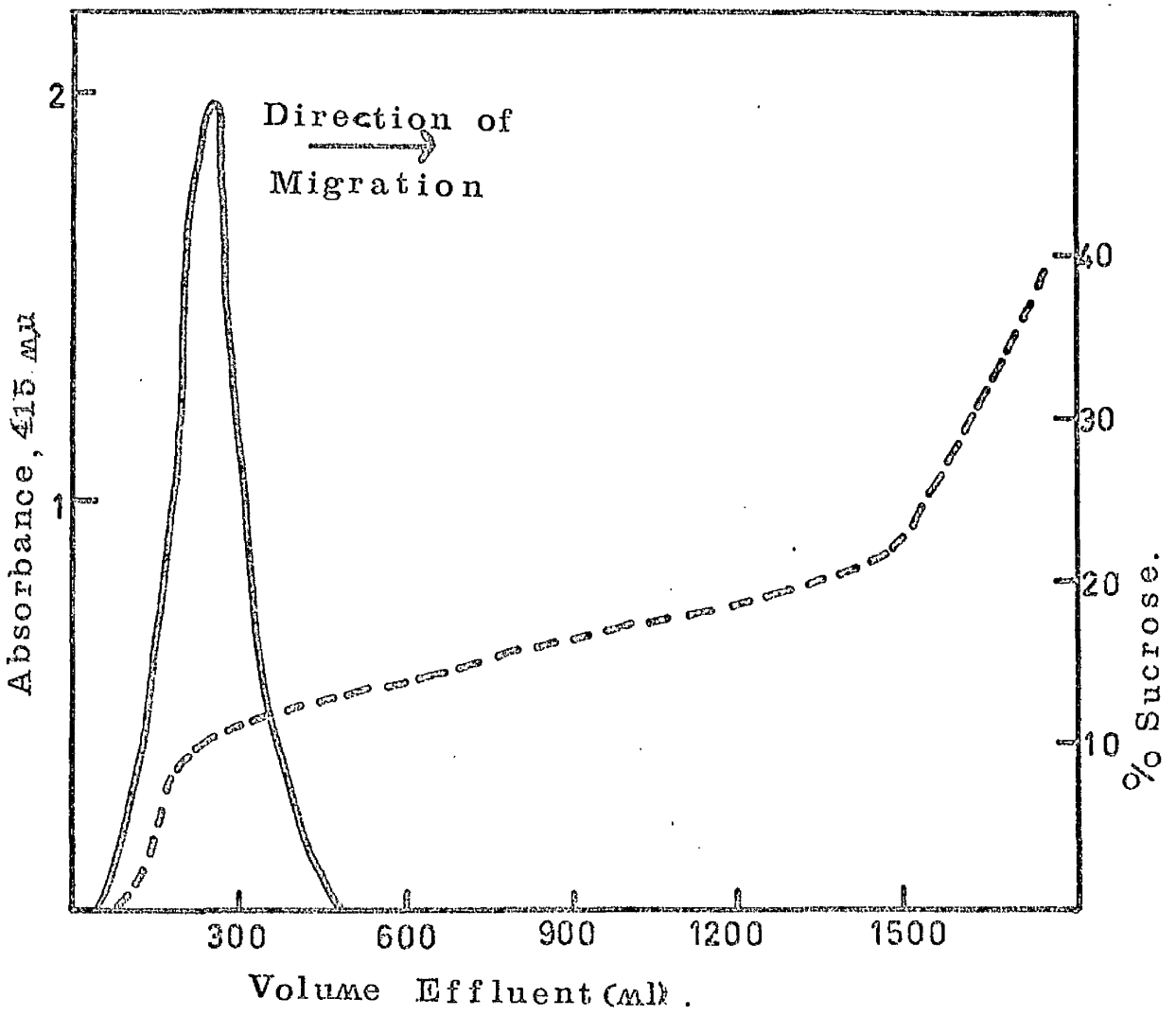
Underlay : 130 mls 40% sucrose in buffer.

Overlay : 200 mls buffer.

Speed : 24,000 rpm.

Time : 40 minutes.

Figure 3.


2
1
Direction of
Migration
Absorbance, 415 mμ
40
30
20
10
% Sucrose.
300
600
900
1200
1500
Volume Effluent (ml).

dissociation into ribosomal subunits with release of a 14s mRNA-protein (mRNP) complex.

The sedimentation profile of EDTA - treated mouse polysomes in the B-XIV zonal rotor is shown in Figure 4 . Three main peaks were resolved. These were globin, the 40s ribosomal subunit and the 60s ribosomal subunit. A small peak, sedimenting at about 14s, was consistently observed between the globin peak and the 40s subunit. The RNA extracted from each peak was analysed on 2.6% polyacrylamide gels (Figure 5). The globin peak contained 4s RNA and some 5sRNA; the 14s peak contained 9s RNA; the 40s subunit contained 18s RNA and a trace of 12s RNA; the 60s subunit contained 28s RNA and some 5s RNA. There was no cross-contamination between fractions.

Recovery was 0.5-1mg 9s RNA from 75-100mg polysomes centrifuged in the B-XV rotor; 0.25 -0.4mg 9s RNA was recovered from 30-40mg polysomes centrifuged on the B-XIV rotor. Increasing the loading of polysomes decreased the resolution of the 14s mRNP complex from the globin and 40s subunit peaks.

Zonal isolation of a fraction enriched in 9s RNA from mouse reticulocyte RNA; analysis of fractions.

The usual starting material for preparation of 9s RNA was the RNA isolated from the reticulocyte post-18,300g supernatant fraction (total RNA). 30-40 mg of total RNA were centrifuged in the B-XIV titanium zonal rotor. A 4 hour run resolved the total RNA into 4 peaks

FIGURE 4.

Resolution of EDTA - treated mouse reticulocyte polysomes after centrifugation in the M.S.E. B-XIV titanium zonal rotor.

Sample : 35 mg EDTA - treated polysomes in 10mls TK buffer.

Gradient : 500 mls 15-30% (w/v) sucrose in TK buffer.

Underlay : 100 mls 40% (w/v) sucrose in TK buffer.

Overlay : 70 mls TK buffer.

Time : 12 hours.

Speed : 45,000 rpm.

Temperature setting : 5.

Figure 4 .

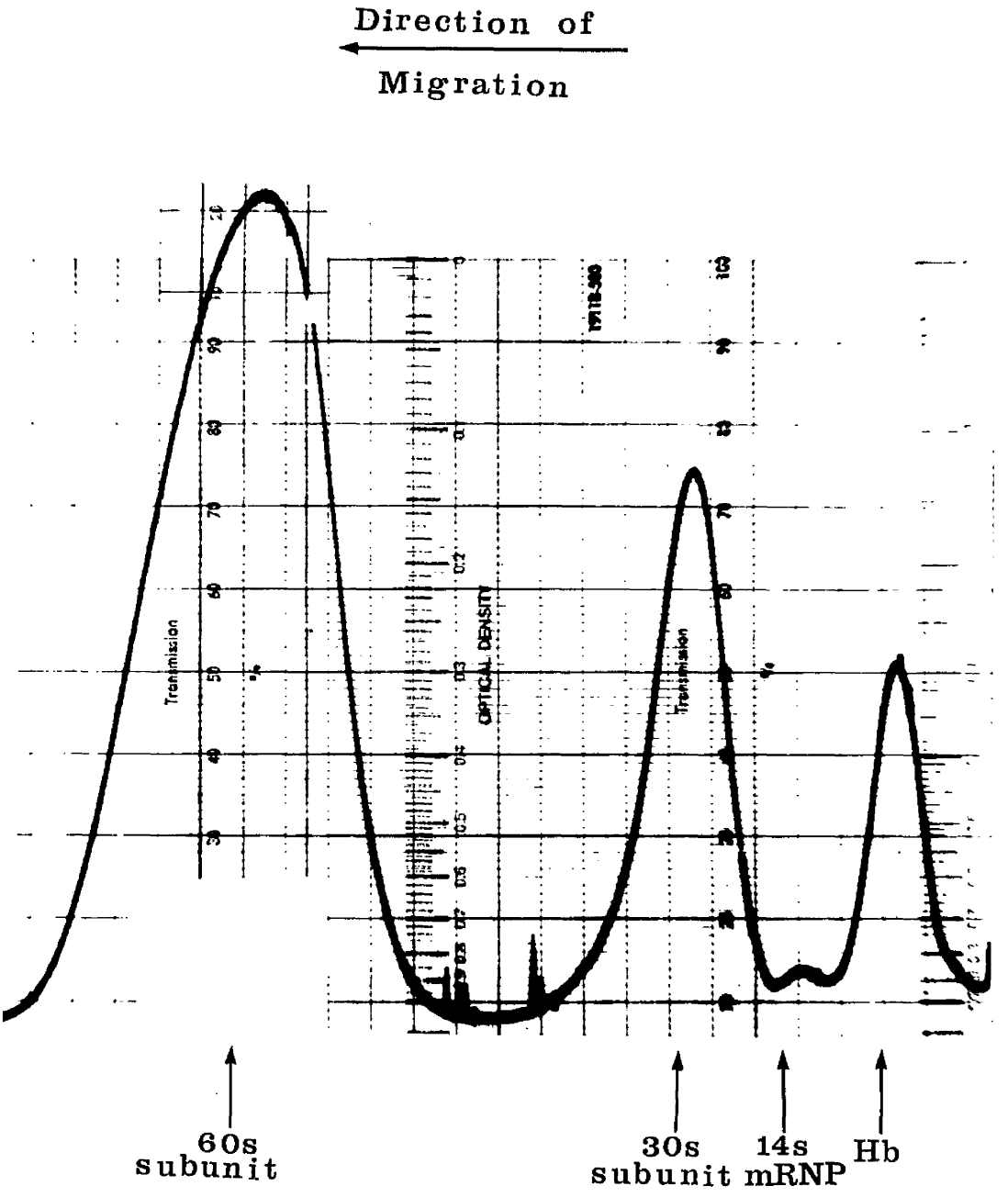

Direction of
Migration
Transmission
OPTICAL DENSITY
Transmission
60s
subunit
30s
subunit
14s
mRNP
Hb

FIGURE 5.

RNA fractions isolated from a zonal centrifugation of EDTA - treated polysomes and analysed on 2.6% polyacrylamide gels. 50µg total RNA and 20µg fractionated RNA were applied/gel.

Electrophoresis was for 20 minutes at 3v/cm then for 90 minutes at 10v/cm. The buffer temperature was 4°C.

a. Total mouse reticulocyte RNA.

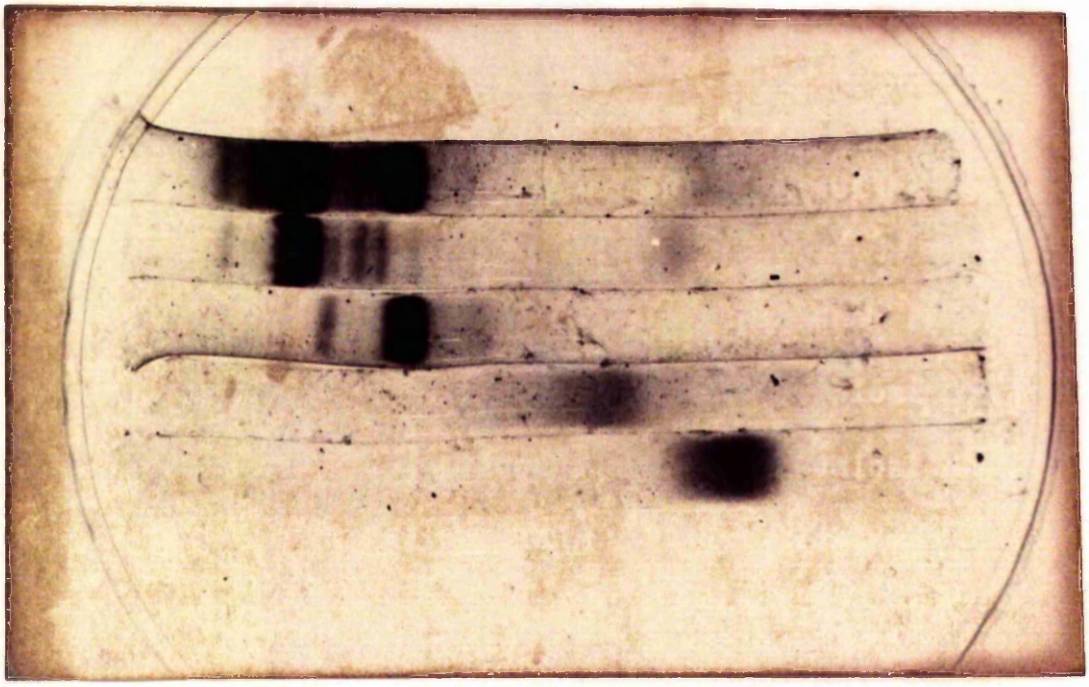
b. RNA isolated from 60s subunit.

c. RNA isolated from 40s subunit.

d. RNA isolated from 14s mRNP particle.

e. RNA isolated from globin peak.

Figure 5.



(Figure 6). The RNA isolated from each peak was analysed on 2.6% polyacrylamide gels (Figure 7). The two heaviest peaks were the 28s and 18s RNAs; the lightest peak contained 4s and 5s RNAs; the peak between 5s RNA and 18s RNA contained 9s RNA, 12s RNA, 7s RNA, 5s RNA and 4s RNA. 0.3-0.4mg 9s-enriched RNA were recovered from one zonal run.

Varying the overlay volumes and the sucrose gradient did not improve theresolution of the 9s and 12s RNAs, but resolution was improved after longer centrifugation times. Figure 8 shows the resolution of RNA species obtained in a 7 hour zonal run. The 9s RNA cuts taken from zonal runs of 4, 7 and 13 hours show increasing purity of the 9s RNA with time of centrifugation (Figure 9). The 9s RNA from the 4 hour zonal cut also contains 12s, 18s and 28s RNAs; the 7 hour cut contains 7s and 28s RNAs; the 13 hour zonal cut contains only 9s RNA. Figure 10 shows three 9s RNA cuts from zonals of 13 hours run on 2.6% polyacrylamide gels. No RNAs of other size classes are seen, even on these heavily-loaded gels. Increasing the running time to over 13 hours compressed the RNAs against the 40% sucrose underlay and therefore gave a 9s RNA fraction more contaminated with other RNA species. Zonal runs of 13 hours were therefore routinely used for isolation of 9s RNA.

Any 9s RNA cuts contaminated with other RNA species were pooled together and re-run on the zonal. If sample volumes of less than 6ml

FIGURE 6.

Resolution of mouse total reticulocyte RNA after centrifugation in the M.S.E. B-XIV titanium zonal rotor.

Sample : 30mg total RNA in 10 mls TKM buffer (1mM tris; 50 mM KCl; 1.5 mM $MgCl_2$, pH 7.4).

Gradient : 500 mls 5 - 20% (w/v) sucrose in TKM buffer.

Underlay : 100 mls 40% (w/v) sucrose in TKM buffer.

Overlay : 70 mls TKM buffer.

Time : 4 hours.

Speed : 45,000 r.p.m.

Temperature setting : 5.

Figure 6.

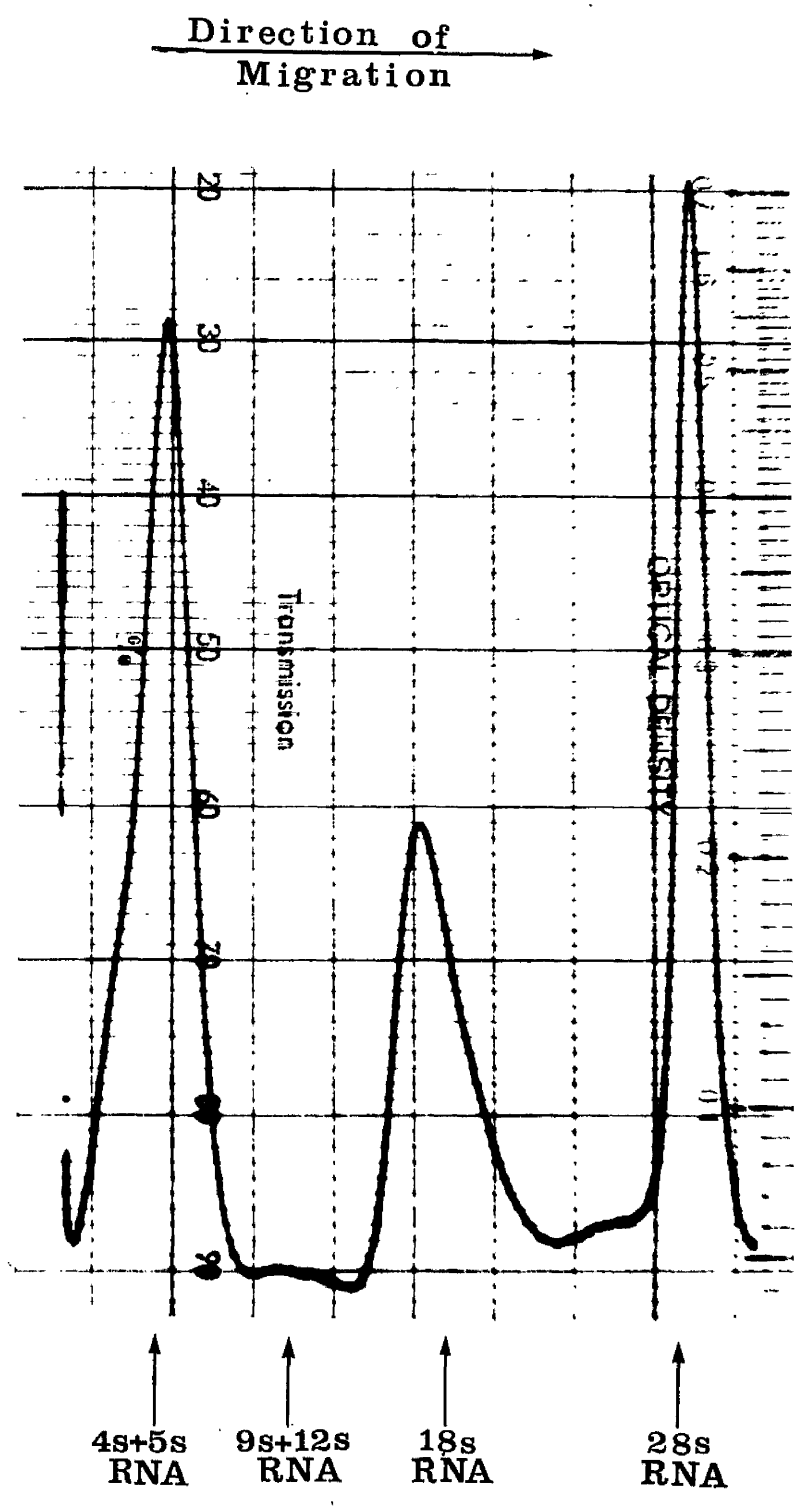

Direction of Migration
20
30
40
50
60
70
80
Transmission
Optical Density
4s+5s RNA
9s+12s RNA
18s RNA
28s RNA

FIGURE 7.

RNA fractions isolated from 4 hour zonal run on total mouse reticulocyte RNA and analysed on 2.6% polyacrylamide gels.

50µg total RNA and 10 µg fractionated RNA were applied/gel.

Electrophoresis was for 20 minutes at 3v/cm then for 1 hour at 10v/cm.

The buffer temperature was 4°C.

a. Total mouse reticulocyte RNA.

b. RNA from 28s RNA peak.

c. RNA from 18s RNA peak.

d. RNA from "9s" RNA peak.

e. RNA from 4s and 5s RNA peak.

Figure 7.

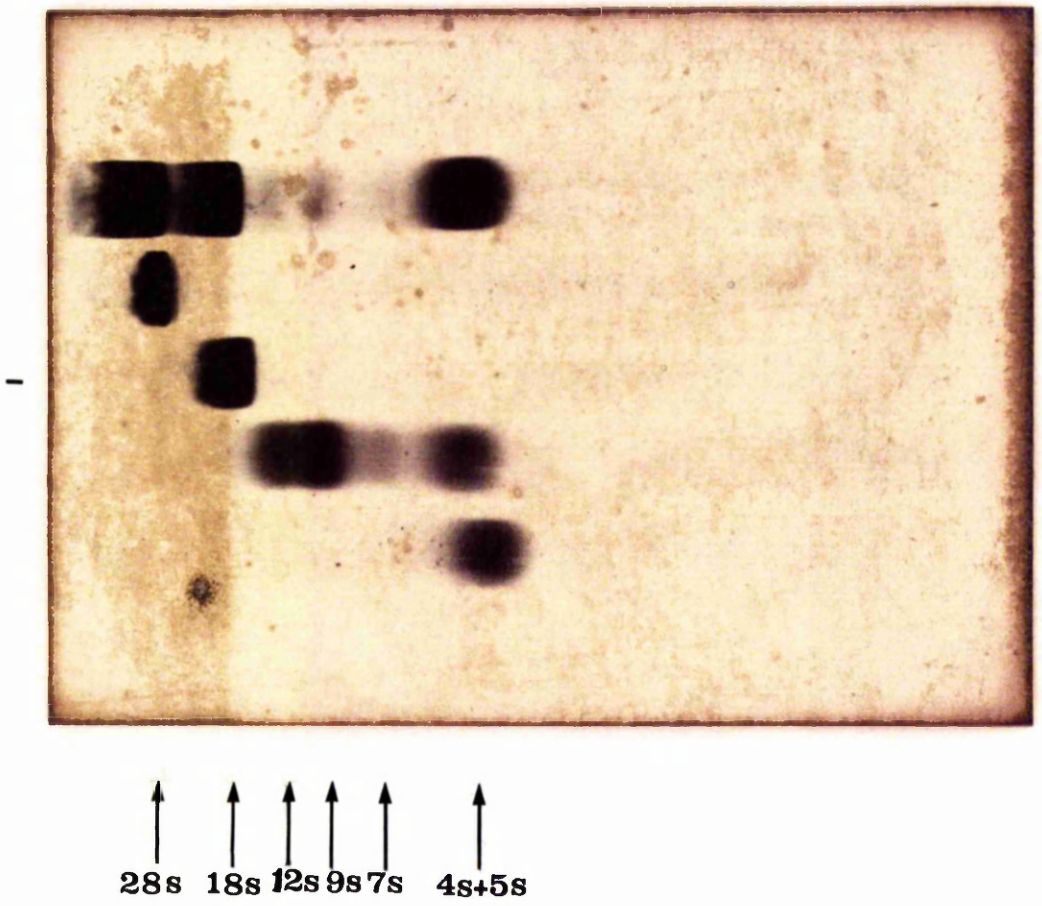


FIGURE 8.

Resolution of total mouse reticulocyte RNA after 7 hours centrifugation in the B-XIV titanium zonal rotor.

Sample : 30 mg total mouse reticulocyte RNA in TKM buffer.

Gradient : 5 - 20% (w/v) sucrose in TKM buffer.

Time : 7 hours.

Speed : 45,000 r.p.m.

Temperature setting : 5.

Figure 8.

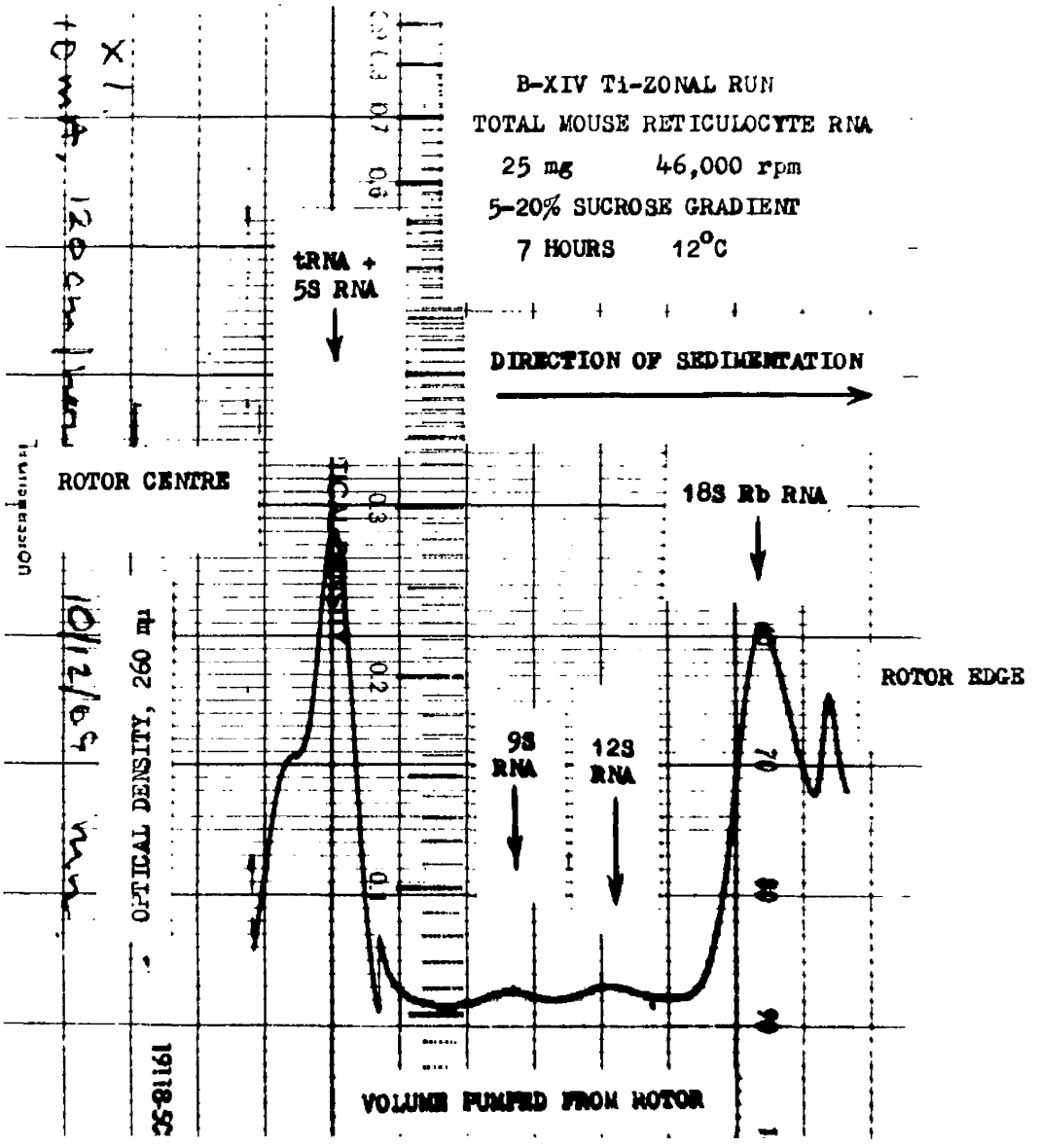

B-XIV T1-ZONAL RUN
TOTAL MOUSE RETICULOCYTE RNA
25 mg 46,000 rpm
5-20% SUCROSE GRADIENT
7 HOURS 12°C
DIRECTION OF SEDIMENTATION
tRNA + 5S RNA
18S Rb RNA
9S RNA
12S RNA
ROTOR CENTRE
ROTOR EDGE
OPTICAL DENSITY, 260 mμ
VOLUME PUMPED FROM ROTOR

FIGURE 9.

Analysis of purity of 9s RNA fractions from several zonal runs by electrophoresis on 6% polyacrylamide gels.

45µg total RNA and 4.5µg 9s RNA were applied/gel.

Electrophoresis was 15 minutes at 3v/cm then for 1 hour at 17v/cm.

The buffer temperature was $4^{o}C$.

a. 9s RNA cut from 7 hour zonal run.

b. 9s RNA cut from 4 hour zonal run.

c. 9s RNA cut from 12 hour zonal run.

d. Total mouse reticulocyte RNA.

FIGURE 10.

Analysis of purity of three 9s RNA preparations from zonals run for 12 hours by electrophoresis on 2.6% polyacrylamide gels.

Electrophoresis was for 20 minutes at 3v/cm then for 50 minutes at 10v/cm.

The buffer temperature was $4^{o}C$.

a - c. 9s RNA cuts (10µg/gel).

d. Total mouse reticulocyte RNA (45ug/gel).

Figure 9.

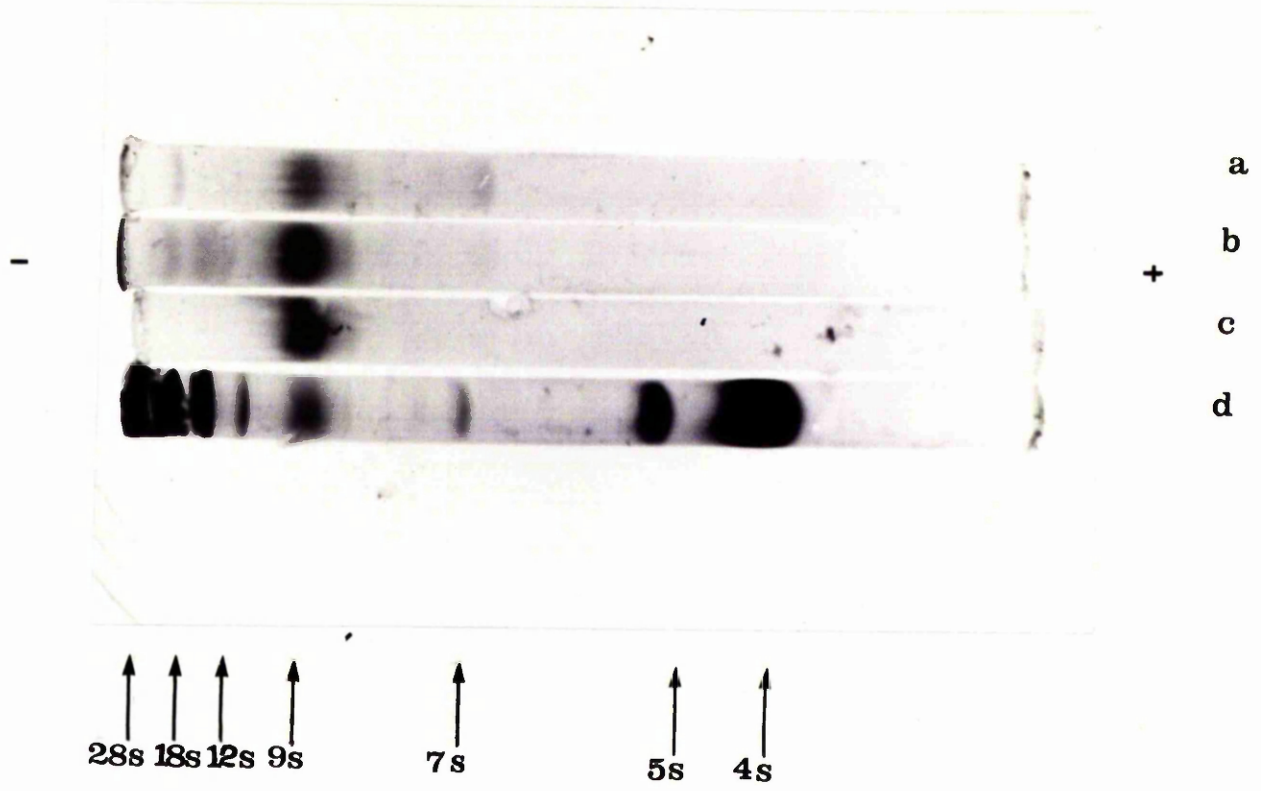
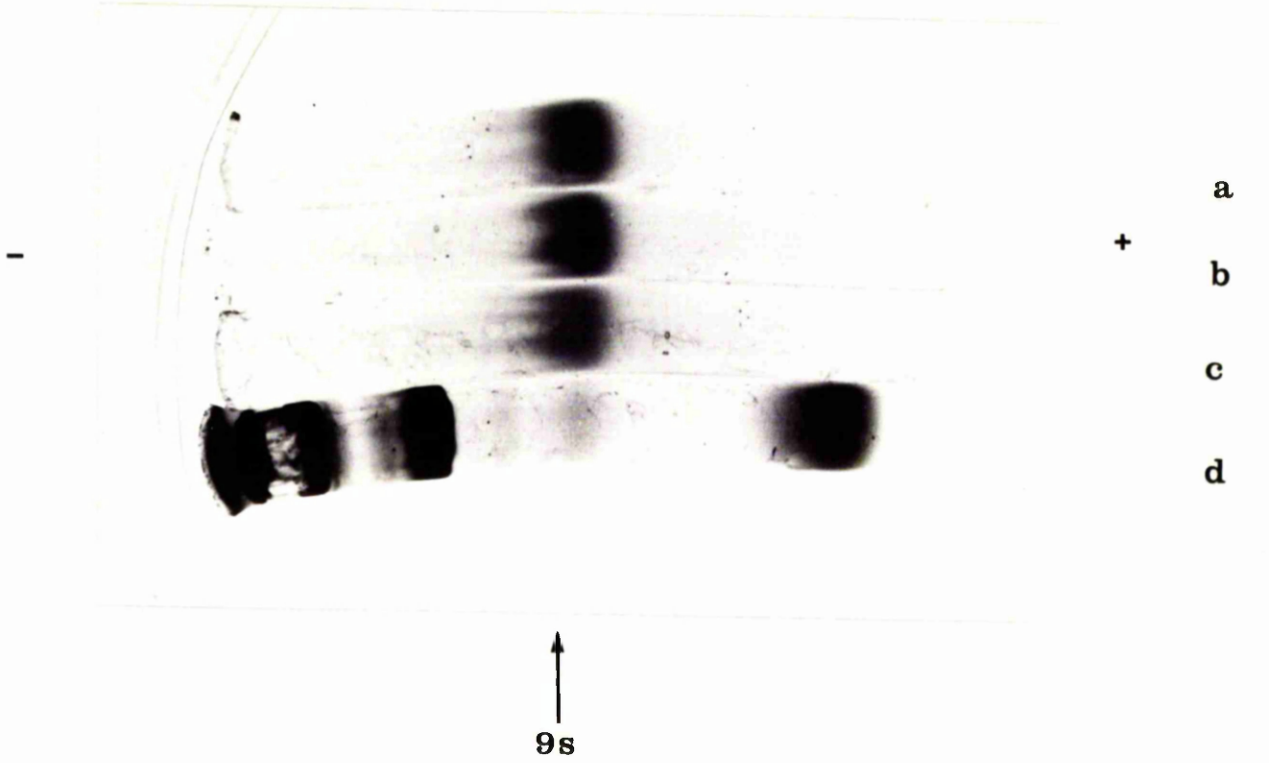


Figure 10.



were used, there was satisfactory resolution of the 9s RNA. Latterly, the efficiency of 9s RNA isolation was improved by increasing the loading of total RNA to 75 mg in the preliminary centrifugation. About 0.75 mg of impure 9s RNA was recovered from one run. The resolution of 2.25 mg impure 9s RNA after a second zonal centrifugation is shown in Figure 11. 2 mg of pure 9s RNA was recovered.

The ratio of 9s RNA to 12s RNA remained unchanged after zonal ultracentrifugation, as did the ratios of both species to 18s RNA. The spectra of both total RNA and isolated 9s RNA had E_{260}/E_{230} ratios greater than 2.36. In contrast, the spectra of several total RNA solutions stored for 1 year at $-20^{\circ}C$ in the presence of DEP showed markedly decreased E_{260}/E_{230} ratios of 1.45 to 1.54.

Preparative polyacrylamide gel electrophoresis of crude 9s RNA zonal cuts.

0.75 - 1 mg of impure 9s RNA, pooled from several zonal runs, was further purified by preparative polyacrylamide gel electrophoresis. Figure 12 shows the separation obtained with a very impure 9s RNA fraction when electrophoresed on a preparative polyacrylamide gel. Each RNA species was recovered pure and undegraded. The impure 9s RNA zonal cut, containing all RNA size classes between 18s and 4s, and the 9s RNA purified on the preparative polyacrylamide gel, are shown in Figure 13. The 9s RNA was free from detectable contamination with RNAs of other size classes. Pooled 9s RNA cuts containing only 12s and 18s contaminants gave the elution pattern shown in Figure 14. Approximately 50% of the RNA was recovered in each run. The use of

FIGURE 11.

Further purification of pooled 9s RNA zonal cuts by centrifugation in the B-XIV titanium zonal rotor.

Sample : 2.25mg impure 9s RNA in TKM buffer.

Gradient : 5 - 20% (w/v) sucrose in TKM buffer.

Time : 13 hours.

Speed : 45,000 r.p.m.

Temperature setting : 5.

Figure 11.

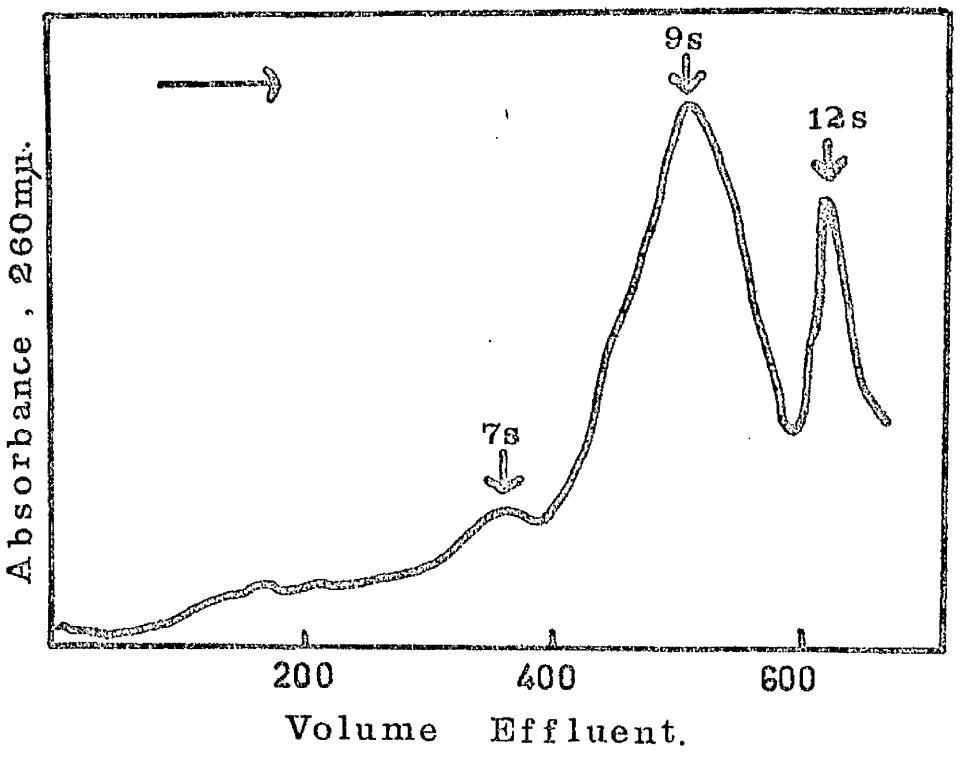


FIGURE 12.

Preparative polyacrylamide gel run on 2.5mg pooled impure 9s RNA cuts isolated from several zonal runs. 75 mls 3% polyacrylamide gel in 0.04 M tris; 0.02M sodium acetate; 0.002 M EDTA, pH 7.8.

Electrophoresis was carried out at 40 volts (50mA) for 1 hour then at 120 volts (200mA). The elution rate was 1ml/minute. 5 minute fractions were collected.

Figure 12.

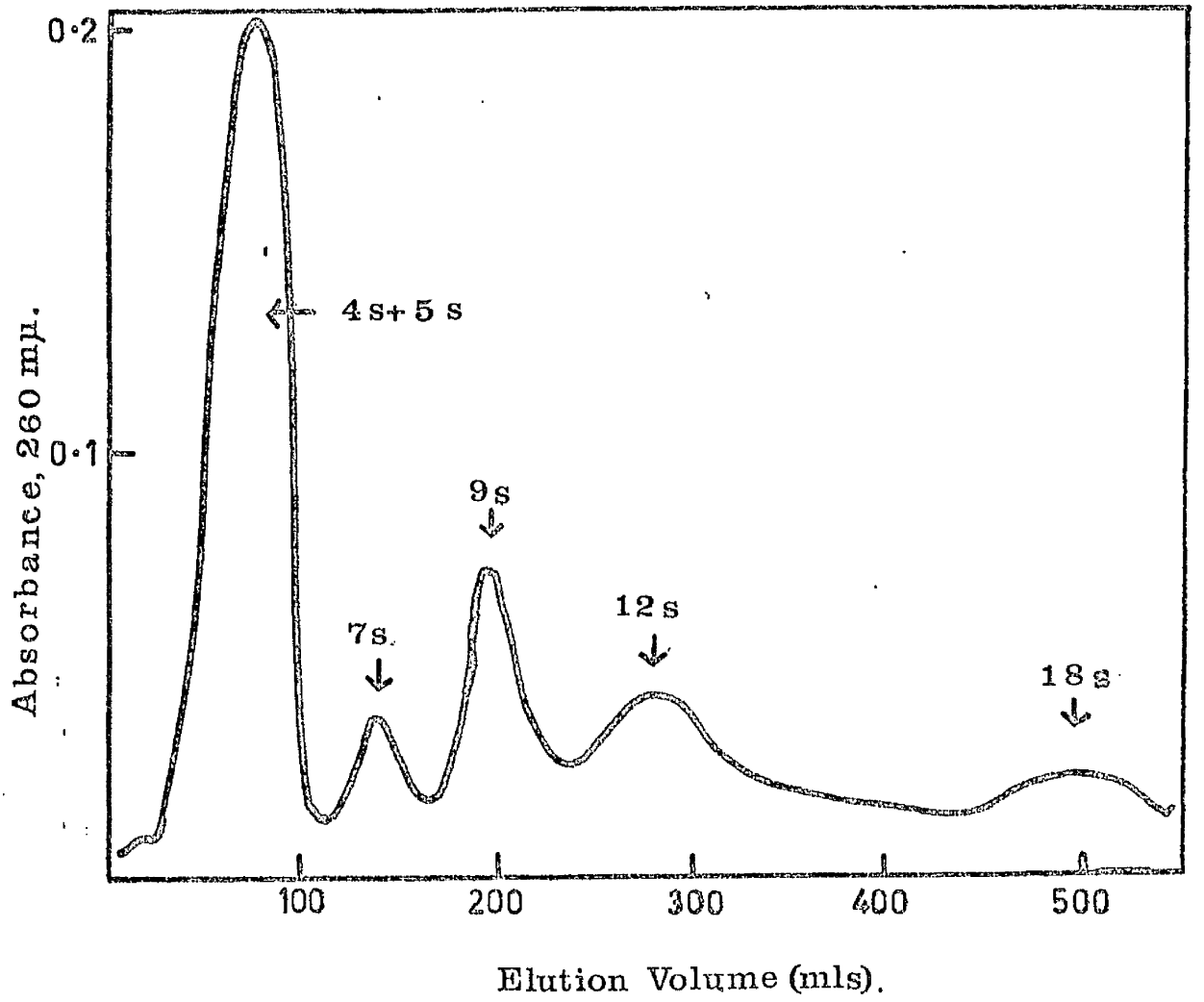

0·2
0·1
Absorbance, 260 mμ.
4s+5s
9s
7s.
12s
18s
100
200
300
400
500
Elution Volume (mls).

FIGURE 13.

2.6% analytical polyacrylamide gel electrophoresis of 9s RNA fractions before and after preparative polyacrylamide gel purification.

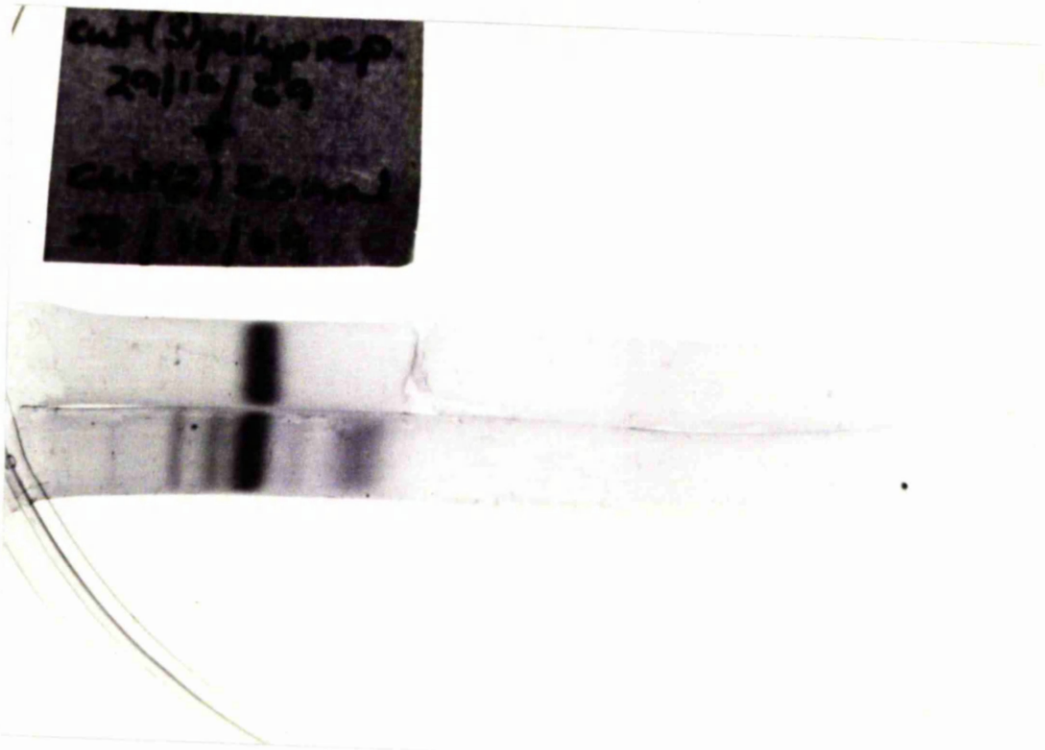
Electrophoresis was for 15 minutes at 3v/cm and then for 30 minutes at 17v/cm.

The buffer temperature was 4^{o}C.

a. 9s RNA fraction from a 75 ml 3% preparative polyacrylamide gel run on the pooled impure 9s RNA zonal cuts.

b. Pooled impure 9s RNA cuts from zonal runs of 4 hours.

Figure 13.



18s 12s 9s 7s 5s

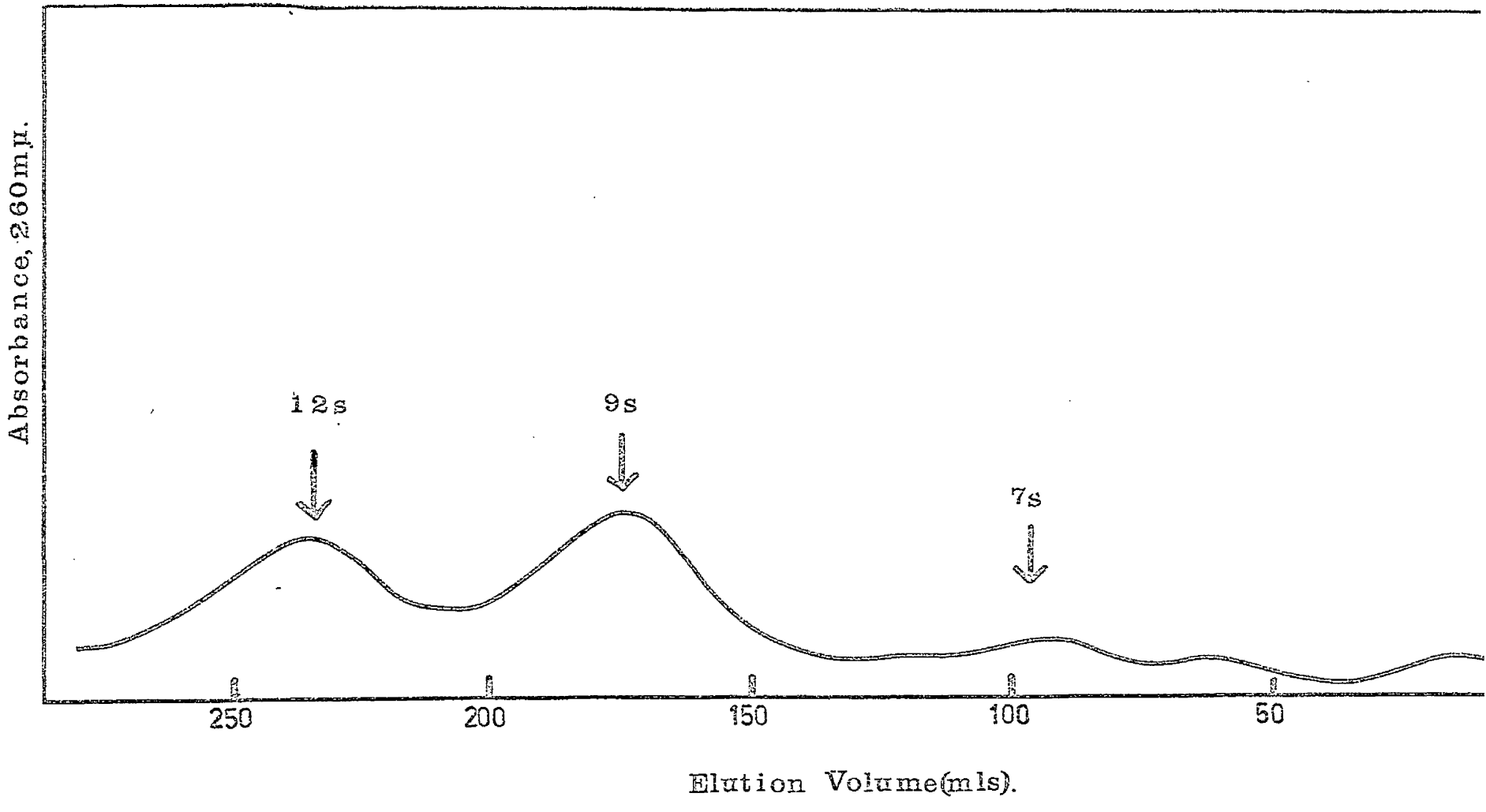
FIGURE 14.

Preparative polyacrylamide gel run on 1.5mg pooled impure 9s RNA cuts from several zonal runs.

100 mls 3% polyacrylamide gel in 0.04 M tris; 0.02 M sodium acetate; 0.002 M EDTA, pH 7.8

Electrophoresis was carried out at 40 volts (50mA) for 1 hour then at 120 volts (200mA). The elution rate was 1ml/minute. 5 minute fractions were collected.

Figure 14.


Absorbance, 260mμ.
12s
9s
7s
250
200
150
100
50
Elution Volume(mls).

high voltages, with faster migration rate of RNA through the gel, gave the highest RNA recoveries.

3.2 Isolation of 9s RNA from cultures of 14 day mouse embryo livers.

Preparation of total RNA

Total nucleic acids were extracted from 14 day mouse embryo livers which had been cultured overnight with ^{3}H- uridine. The yield was an average of 50µg polynucleotides/ liver. Figure 15 shows a chromoscan trace of a total nucleic acid preparation analysed on 2.6% polyacrylamide gels together with a sample pre-treated with 10µg DNase/ml. The "9s" and "12s" peaks in the sample treated with DNase were much reduced, showing heavy DNA contamination in those regions of the total preparation. RNase treatment of a total preparation showed a number of DNA size classes were present.

Similar chromoscan profiles were obtained when total nucleic acids were prepared from cells lysed in the presence and absence of sucrose. DNA contamination of these cytoplasmic preparations was therefore not due to nuclear lysis by the hypertonic 0.001M $MgCl_2$ when the cells were lysed, but was probably due to nuclear lysis or leaking of DNA into the cytoplasm during the culture period.

Isolation of 9s RNA

9s RNA was isolated from DNase - treated cytoplasmic nucleic acids by two methods.

a) 5-10mg total cytoplasmic RNA was run on a 2.4% preparative polyacrylamide gel. This gave good resolution of all molecular

FIGURE 15.

Total polynucleotides isolated from cultured 14 day mouse embryo livers before and after treatment with DNase. 40µg of each sample was electrophoresed on 2.6% analytical polyacrylamide gels.

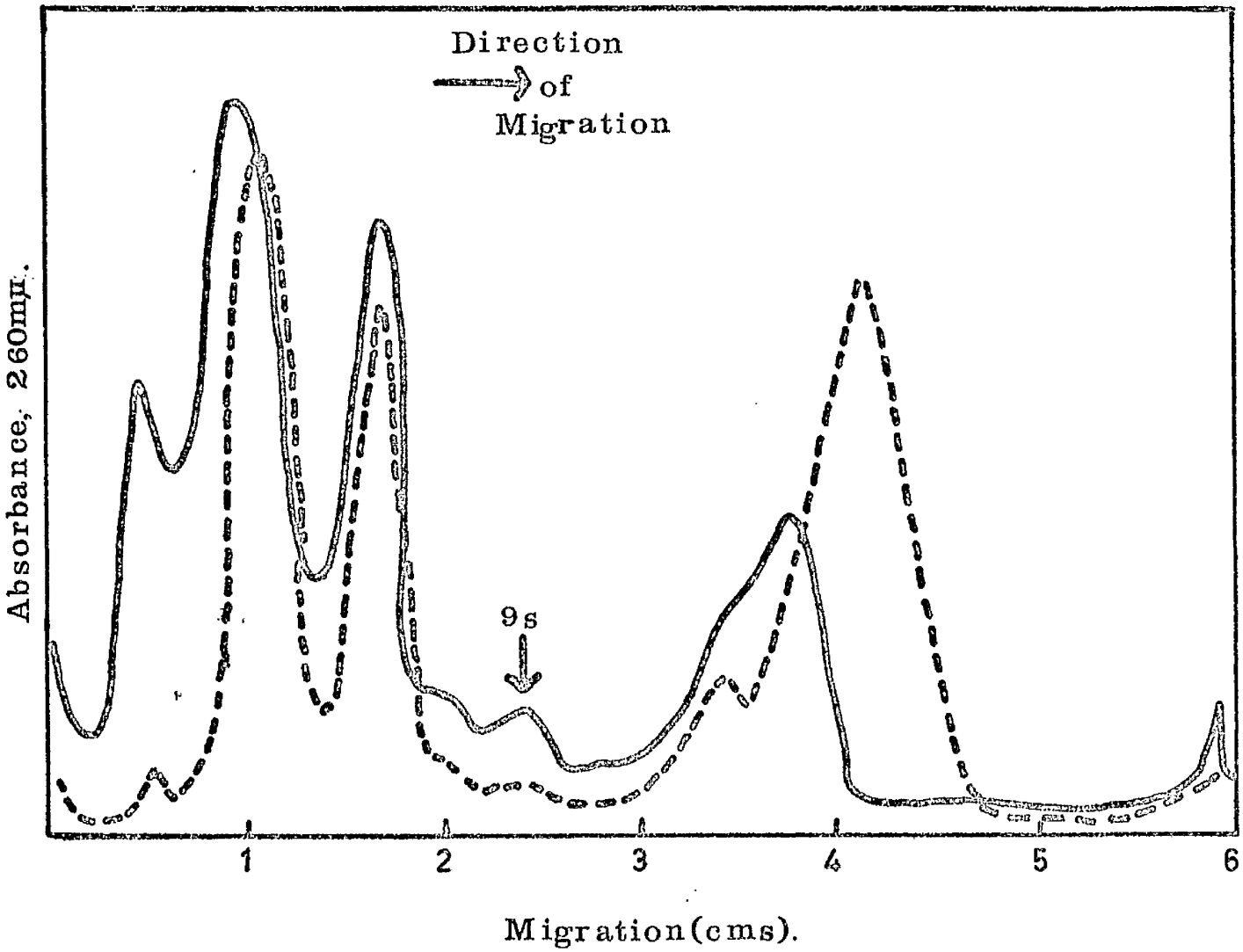
Electrophoresis was for 20 minutes at 3v/cm then for 70 minutes at 10v/cm.

The buffer temperature was 4°C.

_____, polynucleotides before treatment with DNase

– – – –, polynucleotides after treatment with DNase.

Figure 15.


Direction
of
Migration
9s
Absorbance, 260mμ.
1
2
3
4
5
6
Migration (cms).

weight species, but less than 50µg 9s RNA could be recovered from each run.

b) The proportion of low-molecular weight polynucleotides loaded on the preparative polyacrylamide gel was increased by pre-fractionation of the total polynucleotides on a 5-20% sucrose gradient (Figure 16). RNAs of molecular weight less than 18s were pooled and further fractionate on a preparative polyacrylamide gel (Figure 17). 90-140µg of 9s RNA was recovered from each run.

The purity of the 9s RNA was checked on 2.6% analytical polyacrylamide gels. There were no components of other size classes present. The specific activity of the 9s RNA was in the range of 8,000-15,000 cpm/µg. It was always higher than corresponding specific activities of the rRNAs.

Comparison with 9s RNA from mouse reticulocytes

A ^{3}H- 9s RNA isolated from cultured 14 day mouse embryo livers was analysed simultaneously with 9s RNA isolated from mouse reticulocytes on 2.6% polyacrylamide gels. The gels were scanned at E_{260} then sliced and counted as in methods. The ^{3}H counts were located over the reticulocyte 9s RNA (Figure 18). The 9s RNA isolated from the 14 day mouse embryo livers was therefore identical in size to that isolated from reticulocytes.

3.3 Isolation of other RNA species.

a) Landschutz 28s RNA.

Landschutz cells were incubated for 48 hours with 50µc/ml ^{32}P orthophosphate (see methods). RNA extracted from isolated polysomes

FIGURE 16.

Sucrose gradient centrifugation of DNased total polynucleotides from cultured 14 day mouse embyro livers.

20ml 5 - 20% (w/v) sucrose gradients in TKM buffer. 1mg polynucleotides/gradient.

Centrifugation at 4°C, 92,000g for 4 hours.

- - - - - cpm ^{3}H; ——— E_{260}

FIGURE 17.

Preparative polyacrylamide gel electrophoresis of 2mg pooled polynucleotides < 18s from several sucrose gradients.

100 mls 2.6% polyacrylamide gel.

Electrophoresis was carried out at 40 volts (50 mA) for 1 hour then at 120 volts (200mA). The elution rate was 1ml/minute. 5 minute fractions were collected.

- - - - - cpm ^{3}H; ——— E_{260}.

Figure 16.

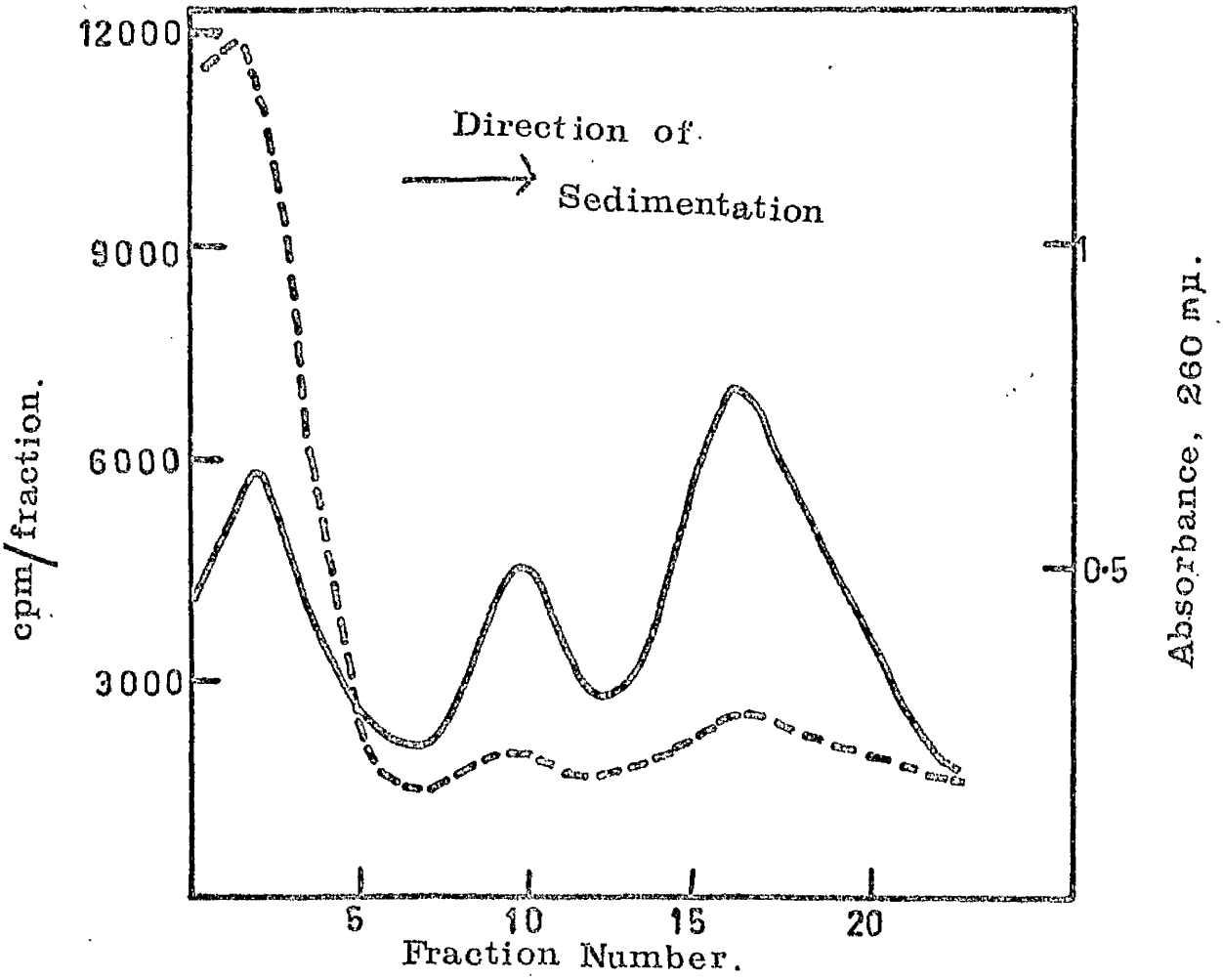


Figure 17.

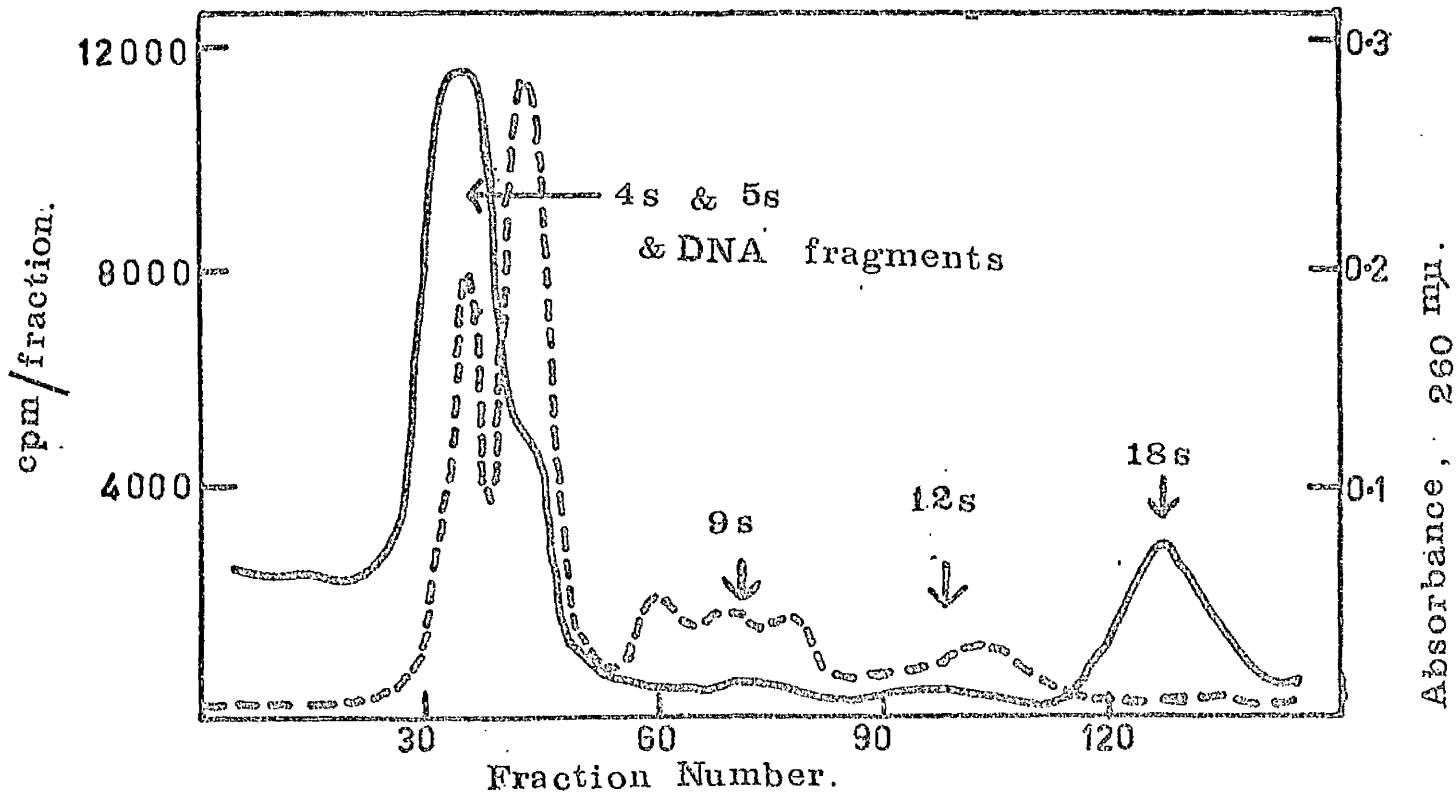


FIGURE 18.

Size comparison of <u>in vitro</u>-labelled ^{3}H - 9s RNA isolated from cultured 14 day mouse embyro livers with a 9s RNA cut isolated from mouse reticulocyte RNA.

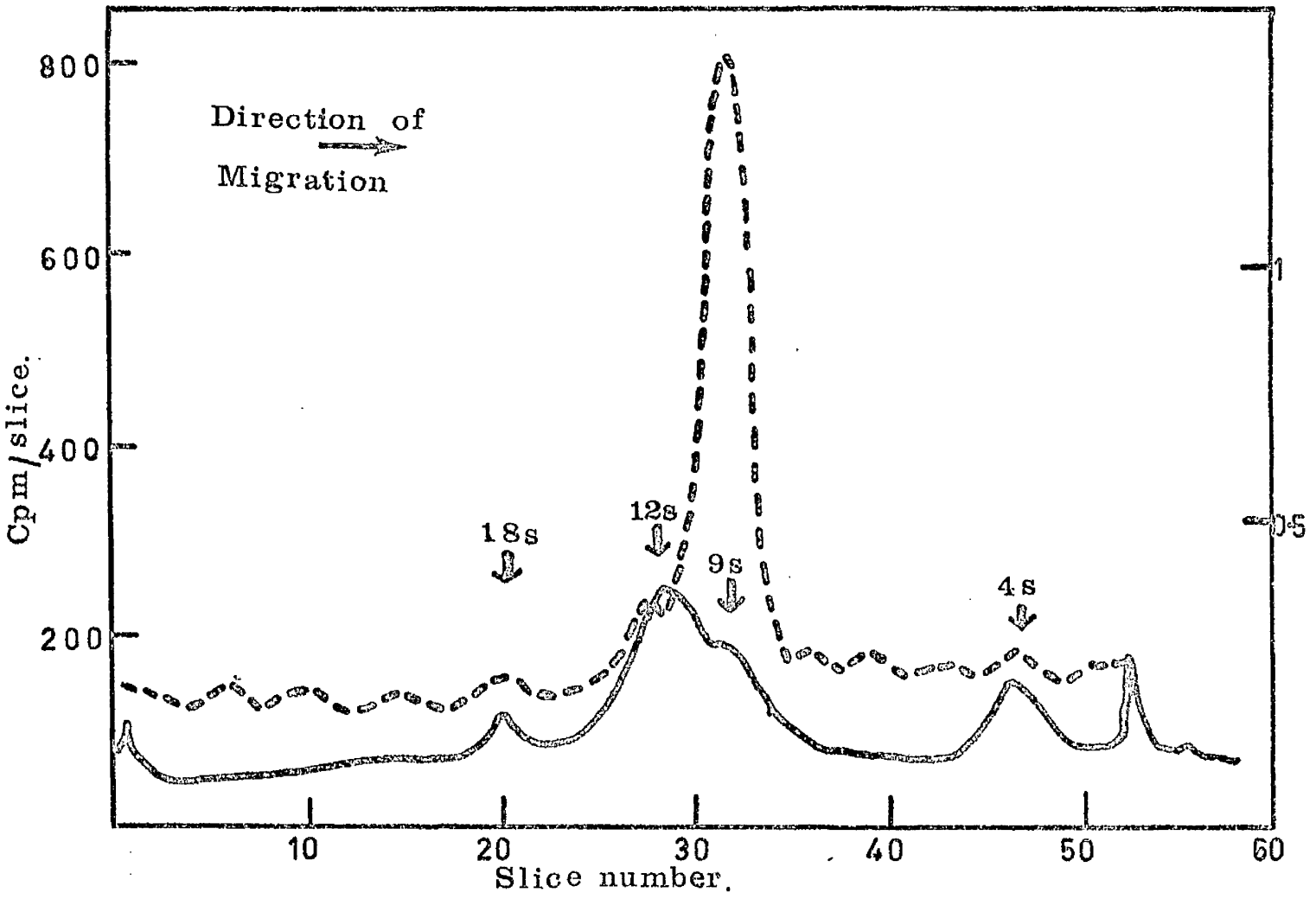
6µg reticulocyte RNA and 1/6µg ^{3}H - 9s RNA (8,000 cpm/µg) were run on a 2.6% polyacrylamide gel.

Electrophoresis was for 90 minutes at 4°C and 12v/cm.

The buffer temperature was 4°C.

- - - -, cpm/slice; ————, E_{260}

Figure 18.


800
600
400
200
Cpm/slice.
Direction of
Migration
18s
12s
9s
4s
1
0·5
10
20
30
40
50
60
Slice number.

was centrifuged in the B-XIV titanium zonal rotor (Figure 19). The ^{32}P RNA from the 28s RNA peak was analysed with cold carrier total mouse reticulocyte RNA on a 2.6% polyacrylamide gel (Figure 20). It was free from RNAs of other size classes.

b) 5s RNA

Unlabelled 5s RNA was isolated from rabbit reticulocyte polysomes. ^{32}P 5s RNA was isolated from polysomes prepared from mouse Landschutz cells cultured with 50μc/ml ^{32}P orthophosphate. After the first separation of RNA on a sephadex G-100 column (Figure 21), the 5s RNA was still contaminated with 4s RNA. The 5s RNA was further purified by a second fractionation on sephadex G-100. The purity of the 5s RNA was analysed by electrophoresis on 6% polyacrylamide gels along with total reticulocyte RNA (Figure 22). The 5s RNA was free from contamination with RNAs of other size classes.

3.4 Further analysis of RNA fractions

Molecular weight determinations

a) Analytical ultracentrifugation

The sedimentation coefficient for untreated 9s RNA, determined by boundary sedimentation, was 8.9 $\pm$ 0.1 (average of 4 runs). There was no degradation after heating the RNA at 63°C in the presence of formaldehyde; the sedimentation coefficient was reduced to 6.2 $\pm$ 0.2 Using the relationship $S_{20,w} = 0.05M^{0.40}$ (Boedtker, 1968), the molecular weight was calculated as 170,000 $\pm$ 8,000 (standard deviation from 7 runs).

FIGURE 19.

1mg Landschutz polysomal RNA centrifuged in a 5-20% (w/v) sucrose gradient in the B-XIV titanium zonal rotor.

Sample : 1mg Landschutz polysomal RNA.

Gradient : 500 ml 10-20% (w/v) sucrose in TKM buffer.

Underlay : 100 mls 40% (w/v) sucrose in TKM buffer.

Overlay : 60 mls TKM buffer.

Speed : 45,000 r.p.m.

Time : 4 hours.

Temperature setting : 5.

FIGURE 20.

2.6% polyacrylamide gel electrophoresis of .01µg ^{32}P - 28s Landschutz RNA isolated by zonal ultracentrifugation and 40µg total mouse reticulocyte RNA.

Electrophoresis was for 20 minutes at 3v/cm then for 1 hour at 10v/cm.

————, E_{260} ; - - - - , cpm/gel slice.

Figure 19.

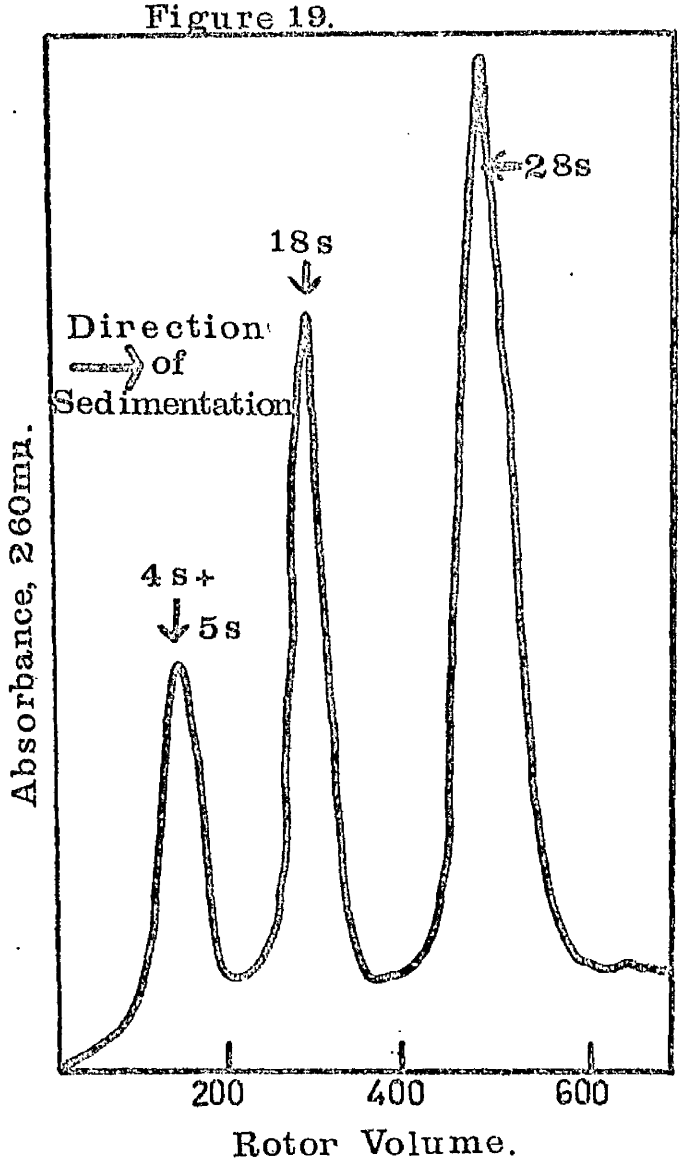


Figure 20.

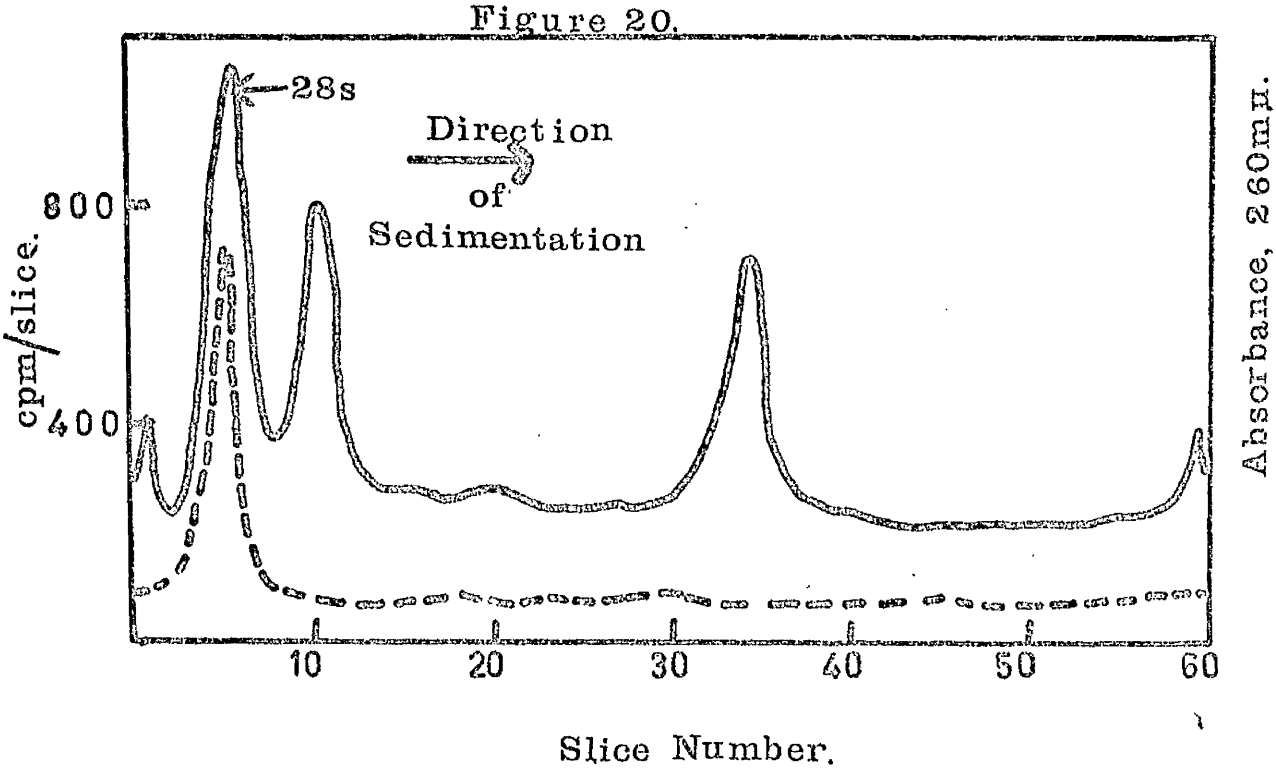


FIGURE 21.

Elution profile of a ^{32}P - 5s RNA - enriched fraction of Landschutz polysomal RNA on a 1.5 x 80 cm G-100 sephadex column. Flow rate : 1ml/10 minutes. Fractions of 2mls collected .

_____, cpm/0.1 ml fraction.

FIGURE 22.

6% polyacrylamide gel electrophoresis of .05µg ^{32}P - 5s Landschutz RNA after two cycles through sephadex G - 100 and 45µg total mouse reticulocyte RNA.

Electrophoresis was for 20 minutes at 3v/cm then for 90 minutes at 10v/cm.

Electrophoresis was at $4^{o}C$.

_____, E_{260} ; _____, cpm/fraction.

Figure 21.

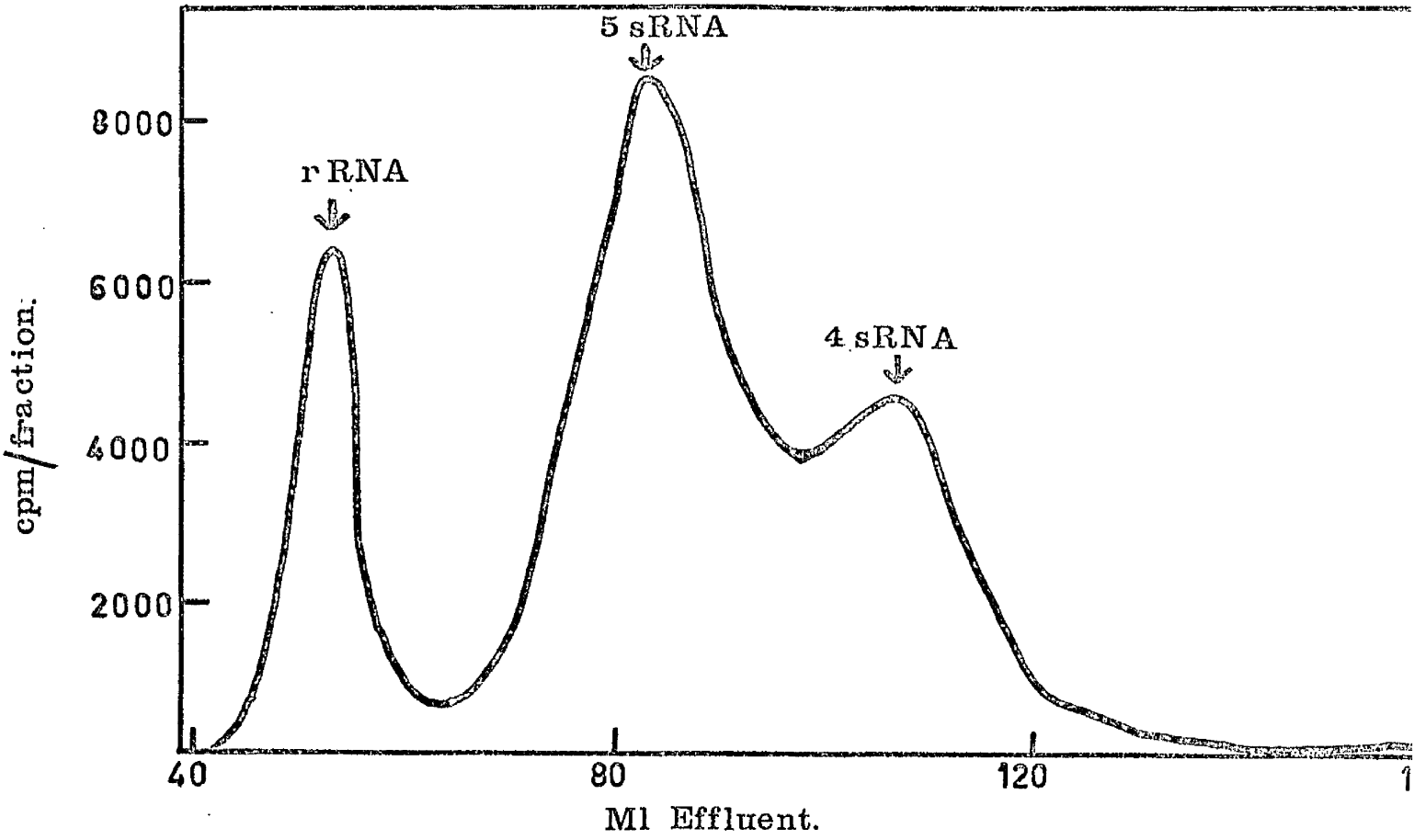
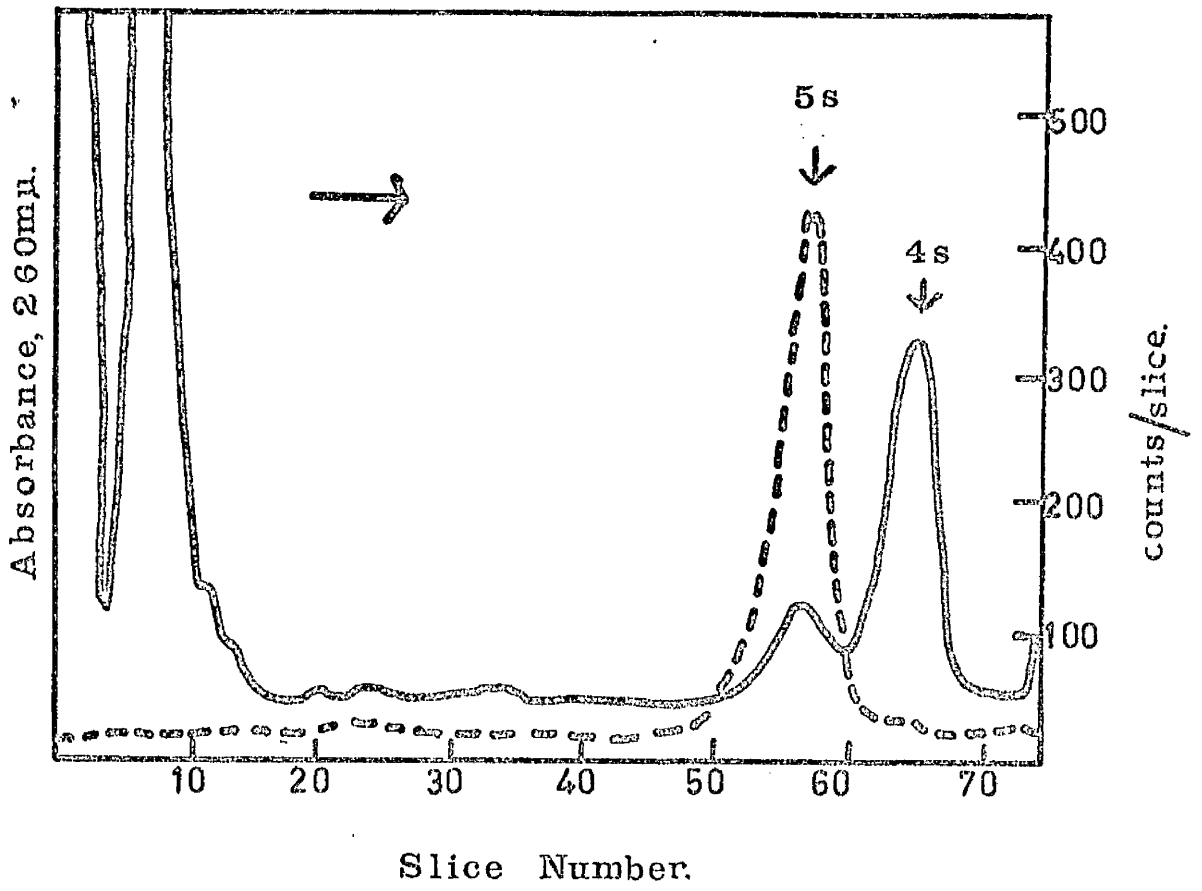


Figure 22..



This analysis of our 9s RNA samples was performed by Dr. R. Eason.

b) Polyacrylamide gel electrophoresis.

The molecular weights of 9s RNA, 12s RNA and 4s RNA were calculated from 10 different total cytoplasmic RNA preparations by their relative migrations on 2.6% polyacrylamide gels. The 28s RNA, 18s RNA and the 5s RNA (where resolved) were used as markers. Molecular weights of 0.65×10^6 and 1.53×10^6 were used for the 18s and 28s RNAs (Hunt, 1970) and 4.0×10^4 for the 5s RNA (Forget and Weissman, 1967). Results showed maximum variation where there was no 5s marker (Table 1). The results calculated with all three ribosomal RNA markers showed good agreement for the three RNA species (Figure 23). The average molecular weight for 9s RNA was $225,700 \pm 13,700$. That for 12s was $373,400 \pm 19,000$. The standard errors were 5% and 6% respectively. These are within the range of the errors in precision of measurement (Lewicki and Sinskey, 1970).

c) Zone ultracentrifugation.

The sedimentation coefficients at different points in the gradient were calculated from the data of a typical zonal run in which RNA species were separated in the B-XIV zonal rotor. The programme developed by Bishop (1966) was used, assuming a density of 1.70 for the RNA. The results (Figure 25) give s values of 10.5s for the 9s RNA and 12.5s for the 12s RNA. These values are only accurate to within 20% due to the inherent inaccuracies in determination of the conditions

TABLE I

Calculation of molecular weights for 9s, 12s and 4s RNA species from their migration on 2.6% polyacrylamide gels relative to the migration of 28s, 18s and 5s RNA species.

Marker RNA species	Estimation of molecular weights		
	4 s	9s	12s
5s, 18s, 28s	25,120	222,100	380,000
5s, 18s, 28s	27,930	222,100	382,100
5s, 18s, 28s	25,120	223,100	363,100
18s, 28s	-	229,100	398,100
18s, 28s	52,480	218,800	380,200
18s, 28s	36,310	213,800	363,100
18s, 28s	38,020	223,900	380,200
18s, 28s	-	239,900	398,100
	28,840	251,200	398,100

Average molecular weight 4s RNA = 34,941 ± 9,448 (27% standard error).

Average molecular weight 9s RNA = 225,770 ± 13,743 (6.09% standard error).

Average molecular weight 12s RNA = 373,400 ± 19,145 (5.13% standard error).

FIGURE 23.

Estimation of the molecular weights of 12s, 9s and 4s RNAs from their relative migration on 2.6% polyacrylamide gels

FIGURE 24.

Estimation of molecular weights of 9s RNA bands from their relative migration on 6% polyacrylamide gels.

Figure 23.

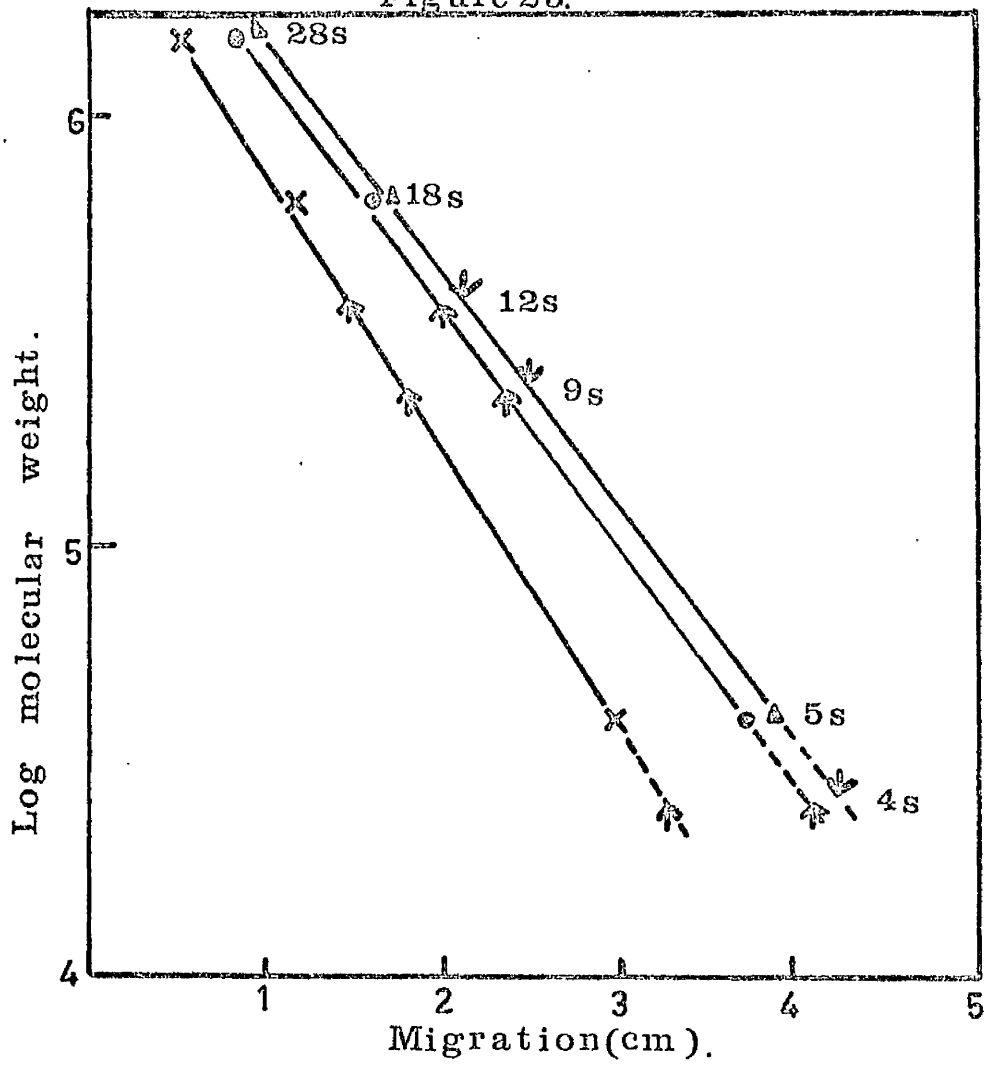


Figure 24.

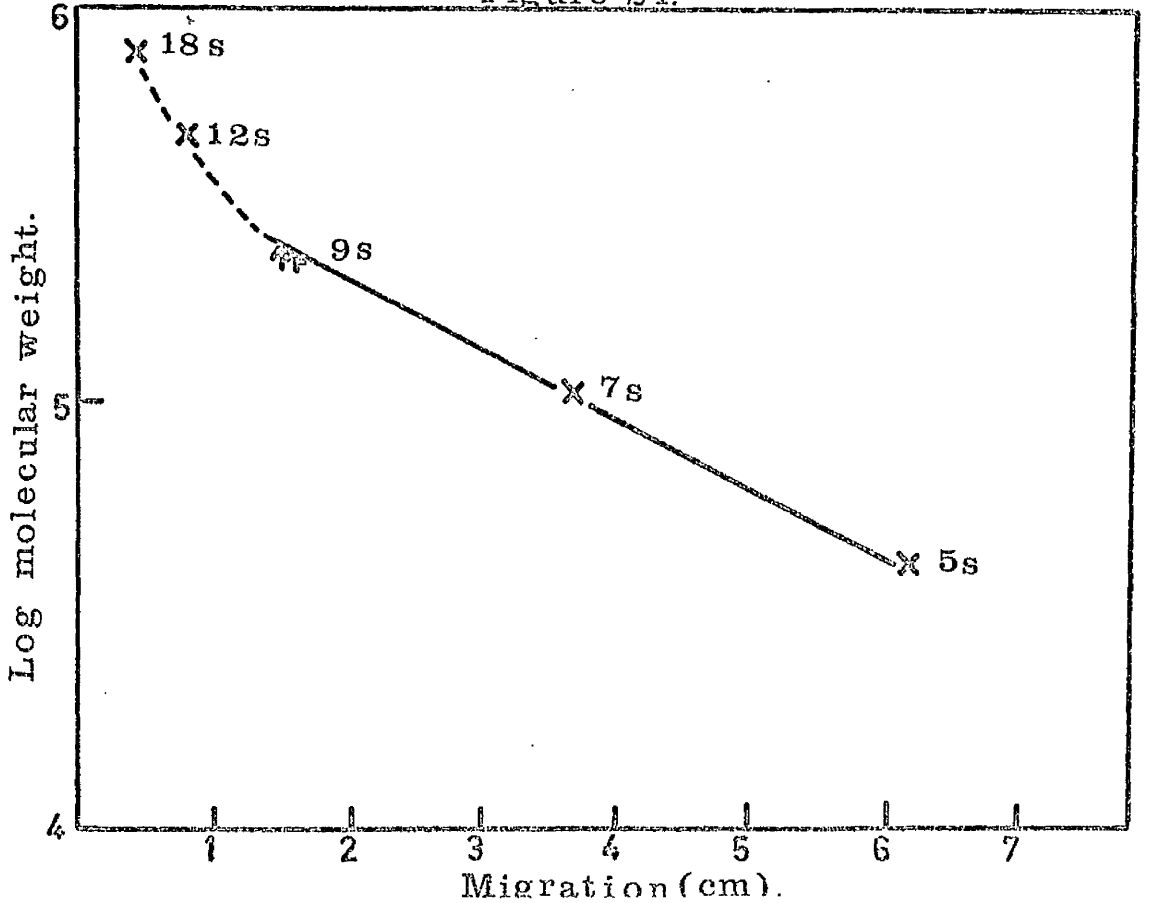


FIGURE 25.

Computer analysis of s values from data of a zone ultracentrifugation in a B-XIV titanium zonal rotor.

Sample : 20mg total mouse reticulocyte RNA in TKM buffer, 2.5% (w/v) sucrose.

Gradient : 500 mls 5-20% sucrose in TKM buffer.

Sample Volume : 10 mls.

Overlay : 90 mls TKM buffer.

Underlay : 70 mls 40% sucrose in TKM buffer

Time : 6 hours

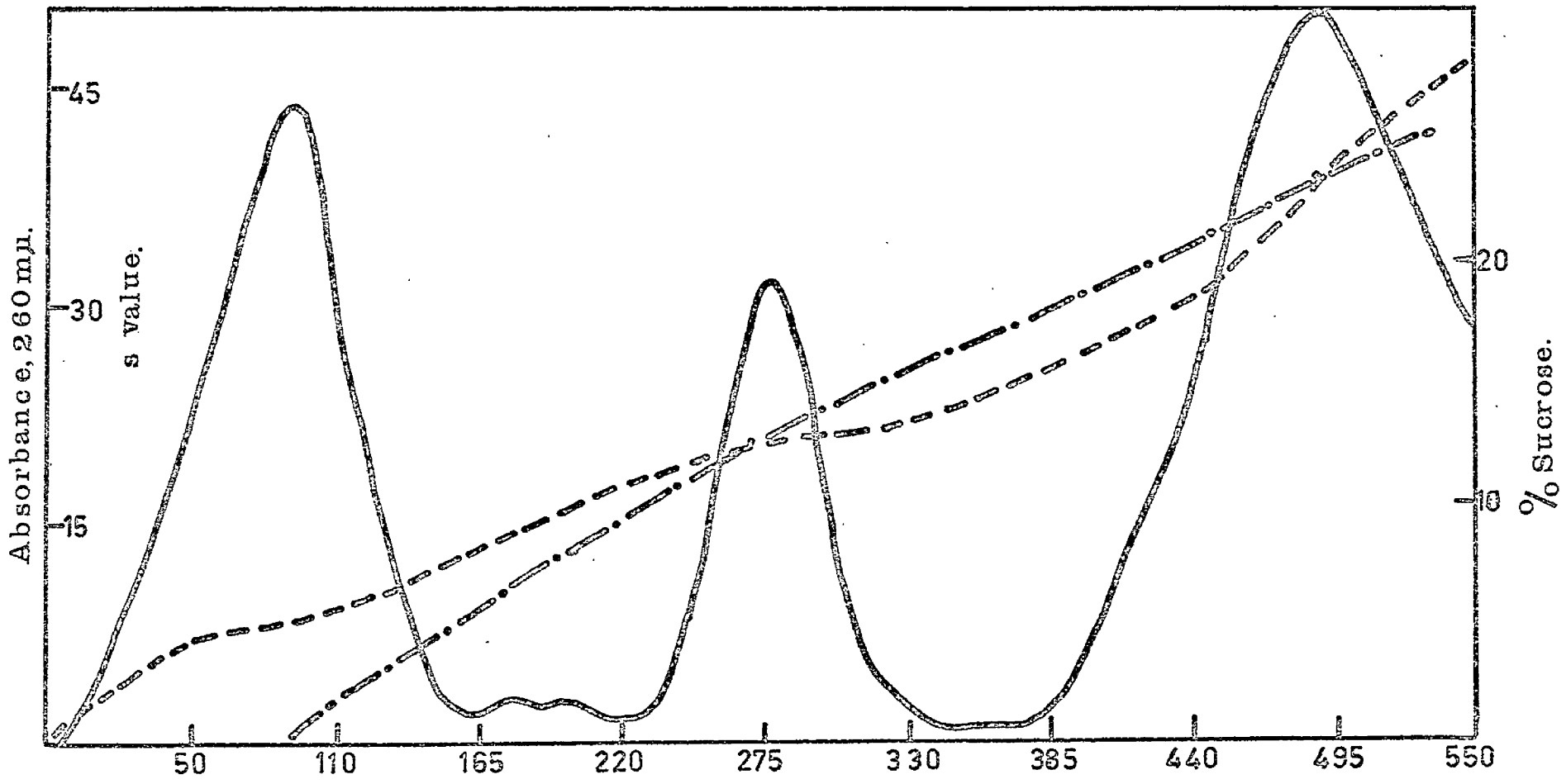
Speed : 39,000 r.p.m.

Temperature setting : 4.25 (6-8°C).

The sucrose concentration of the effluent was monitored at 25 ml intervals.

———, E_{260} ; - - - -, % sucrose; —·—·—, s value.

Figure 25.


45
30
15
s value.
Absorbance, 260 mμ.
20
10
% Sucrose.
50
110
165
220
275
330
385
440
495
550
Rotor Volume.

during the run such as the changing shape of the sucrose gradient. The inaccuracy due to "backwash" of the 4s RNA as the sample and the 5% sucrose diffuse into the overlay during centrifugation causes a decrease in the estimated s value for 4s RNA. Although the s value of the 18s RNA is calculated as 20s, the s value for the 28s RNA is not within the 20% variation. This is probably due to the anomalous shape of the sucrose gradient in that region, since the s value is calculated from the sucrose concentration data.

Data from a zonal run on our total RNA was processed by Mr. J. Leitch.

Analysis of RNA fractions on 6% polyacrylamide gels .

Both the 9s and 12s RNAs migrate as single bands when run on 2.6% polyacrylamide gels, although the 9s RNA band is more diffuse. On 6% polyacrylamide gels, the 9s RNA splits into two major bands present in approximately equal proportions. The 12s RNA is resolved into 2 components; there is approximately 3 times as much of the slower component as there is of the faster (Figure 26).

Several 9s RNA cuts from zonal centrifugations were analysed with a polysomal RNA sample on 6% polyacrylamide gels (Figure 27). At these higher loadings, at least 3 minor bands are seen, running ahead of the major bands. There is also diffuse staining over the 9s RNA region. The relative amounts and migration distances of the two major 9s RNA bands showed little variation in the different 9s RNA samples isolated from zonal centrifugations of total reticulocyte RNA. There was more variation in the relative proportions of the

FIGURE 26.

6% polyacrylamide gel electrophoresis run on mouse reticulocyte polysomal RNA (400μg).

Gels were 1 cm diameter and 5 cm long.

Electrophoresis was for 30 minutes at 10v/cm then for 200 minutes at 20v/cm, 13 mA/gel.

The buffer temperature was 6°C.

FIGURE 27.

6% polyacrylamide gel electrophoresis of mouse reticulocyte mRNA fractions isolated from zonal ultra-centrifuge runs on total mouse reticulocyte RNA.

Gel ∝ was loaded with total mouse reticulocyte polysomal RNA.

Gels were 1 cm diameter and 5 cm long.

Electrophoresis was for 30 minutes at 10v/cm then for 200 minutes at 20v/cm, 13 mA/gel.

The buffer temperature was 6°C.

Figure 26.

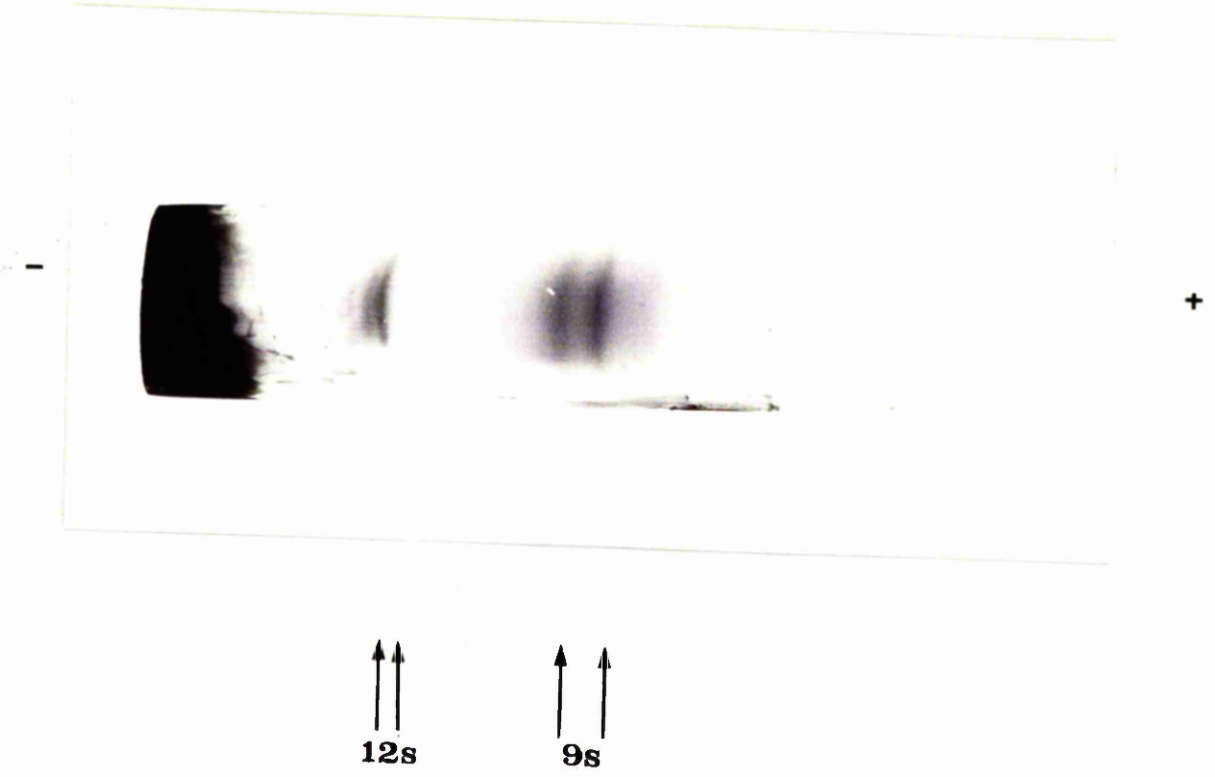
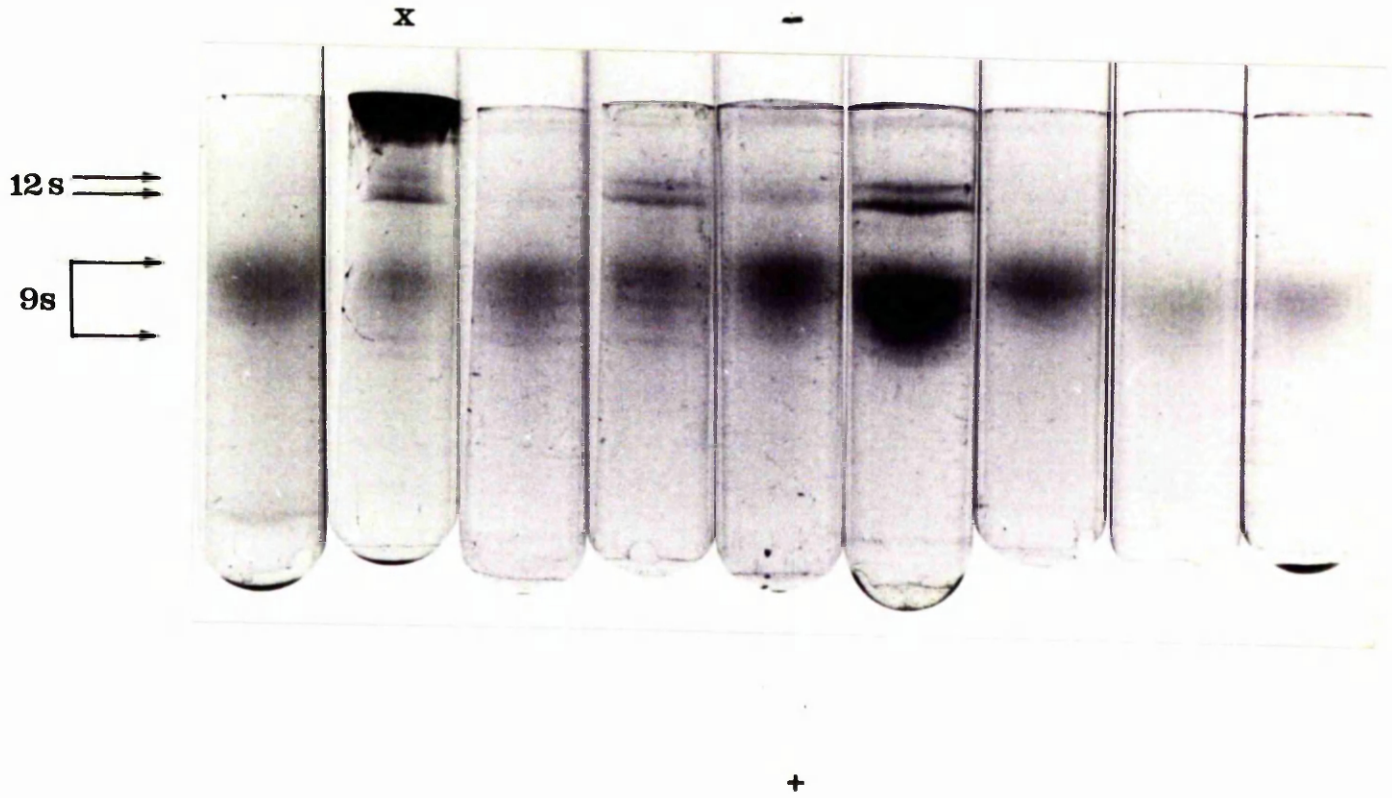


Figure 27.



minor bands and the degree of background staining. The 9s RNA in total polysomal RNA showed a more distinct banding pattern. Further electrophoretic runs showed that the banding patterns in the 9s RNA region were similar to those in total reticulocyte RNA and in 9s RNA isolated from the 14s mRNP particle from EDTA-treated mouse reticulocyte polysomes.

The major 9s RNA components differ by approximately 5,000 in molecular weight (Figure 24). The molecular weight of 7s RNA was calculated as 87,100 from its migration on a 2.6% polyacrylamide gel. The data for the 9s RNA were obtained by extrapolation from the positions of the 5s and 7s RNA species on 6% gels.on the assumption that the migration of RNA species on 6% gels is linear up to s values of approximately 10 (McPhie, 1966). The measurements are therefore less precise than the average molecular weight calculated for 9s RNA on the 2.6% polyacrylamide gels. The lighter 9s band had a molecular weight of 218,800 and the heavier a molecular weight of 223,900.

2.5 In vitro methylation of RNA.

Vigorous shaking of the biphasic methylating mixture was necessary for methylation of the RNA. Specific activities of less than 1,000 cpm/μg RNA were obtained when methylation was carried out in a shaking water bath. Specific activities were increased by more vigorous shaking using a tube-shaker. Sealing of the tubes used for methylating with rubber solution and tape was necessary to prevent

leakage of the ether/benzene phase.

0.5 - 1.5 mg RNA were methylated at one time and specific activities obtained ranged from 2,500 to 5,000 cpm/µg RNA. These figures correspond to between 0.6 and 1.2% of the bases in RNA being methylated. The highest specific activities were obtained with the higher concentrations of RNA. The methylated RNA was free from any residual dimethyl sulphate after 3 ethanol precipitations and passage through a G-25 sephadex column since the supernatant fraction, obtained after precipitation of the column-purified RNA, contained no radioactivity. The RNA, dissolved in hybridisation medium, was dialysed overnight against the medium at 4^{0}C. No counts were dialysed out at this stage.

The methylated RNA was undegraded and ran identically to unmethylated 9s RNA on 2.6% analytical polyacrylamide gels (Figure 28).

3.6 Preparation of DNA

Purification of DNA

RNase-treated DNA was still contaminated with RNase after pronase treatment and several chloroform- isooctanol extractions. After the DNA was treated with iodoacetate, it contained no RNase, as determined by the RNase filter assay. However, when this DNA was incubated with total RNA for 1 hour at room temperature, the total RNA was more degraded than a control RNA, incubated without DNA, when the two RNA preparations were analysed on 2.6% polyacrylamide gels. Banding of partially-purified DNA, not treated with RNase, in caesium

FIGURE 28.

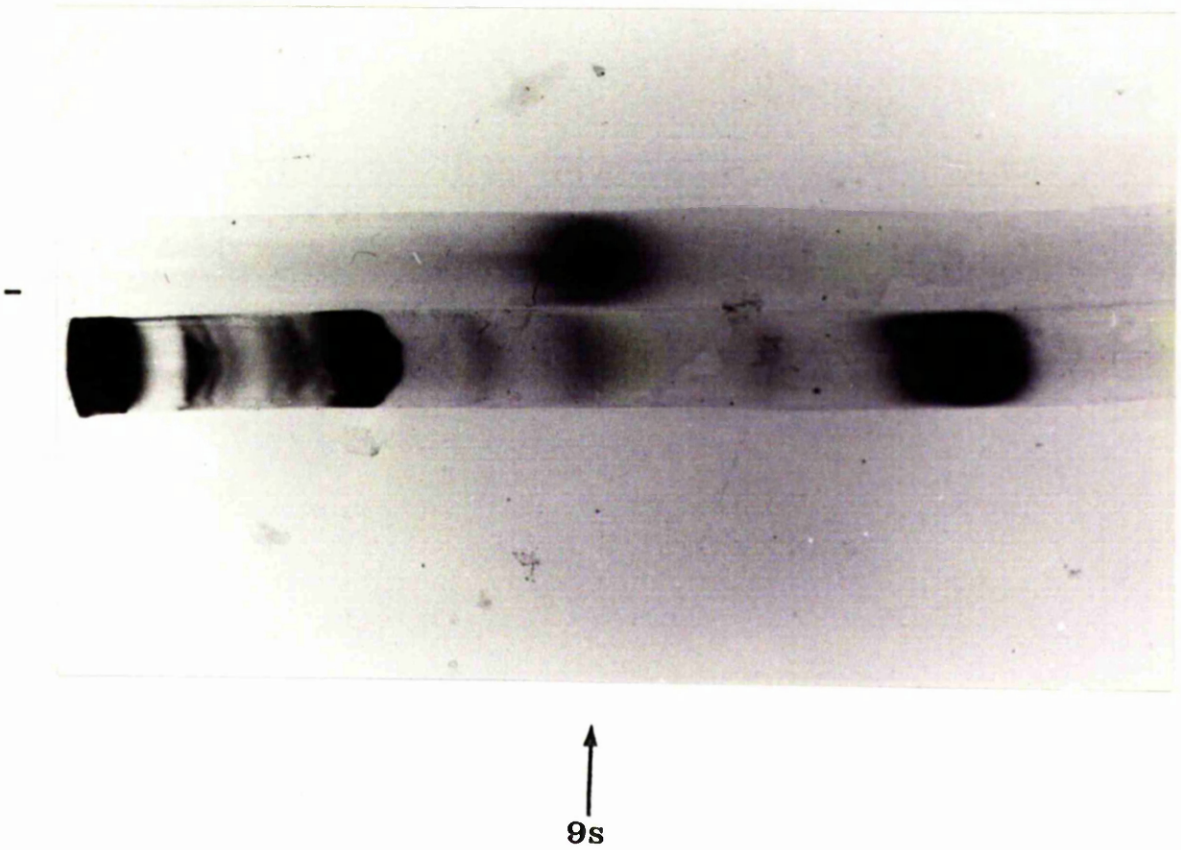
^{3}H - methylated 9s RNA and unmethylated total mouse reticulocyte RNA analysed on 2.6% polyacrylamide gels. Electrophoresis was for 15 minutes at 3v/cm then for 40 minutes at 17v/cm.

The buffer temperature was $4^{o}C$.

a. ^{3}H - methylated 9s RNA (10µg).

b. Total mouse reticulocyte RNA (50µg).

Figure 28.



chloride was therefore used to purify the DNA from RNA and protein contaminants.

Figures 29 and 30 show the isopycnic caesium chloride banding of DNA in the B-XIV titanium zonal rotor and the titanium 8 x 25 fixed angle rotor respectively. Both methods give good resolution of the DNA band from protein and RNA. Although some contamination of the DNA band with pelletted RNA is likely when fractions are drop-collected from the 8 x 25 tubes, this was the method routinely used for DNA purification. Up to 5mg DNA (66% recovery) was purified in a fixed angle head run and up to 10 mg (50% recovery) was purified from a zonal run.

Characterisation of DNA

Size

Samples of caesium chloride-purified E.coli DNA and total mouse embryo DNA were centrifuged through a 5-25% sucrose gradient in a 3 x 23 swing-out head for 13.25 hours at a speed of 23,000 rp.m. The s values were determined by the relationship $S = \frac{\Delta I \times 10^{13}}{w^2 t}$ where

S = sedimentation constant

I = time integral (Tables in McEwan, 1967).

w = speed in radians/sec.

t = time (sec).

$w^2t = \left(\frac{23,000 \times 2\pi}{60}\right)^2 \times 13.25 \times 60 \times 60 = 2.77 \times 10^{11}$.

The slope of the sucrose gradient 3_o

$= \frac{Z_1 r_2 - Z_2 r_1}{r_2 - r_1}$ where

FIGURE 29.

Isopycnic banding of total mouse embryo DNA in caesium chloride in the E-XIV titanium zonal rotor.

20mg total mouse embyro DNA in caesium chloride (1.72 gm/ml; 400mls).

Overlay : 275 mls air

Time : 72 hours

Speed : 45,000 r.p.m.

Temperature : 20°C.

———, E_{260} ; — — —, Buoyant density CsCl.

FIGURE 30.

Isopycnic banding of total mouse embyro DNA in caesium chloride.

M.S.E. 8 x 25 titanium rotor.

1mg DNA in 15 mls caesium chloride (1.72gm/ml) overlaid with 5 ml paraffin.

Time : 48 hours.

Speed : 190,000g

Temperature : 20°C.

———, E_{260}; — — —, Refractive index CsCl.

Figure 29.

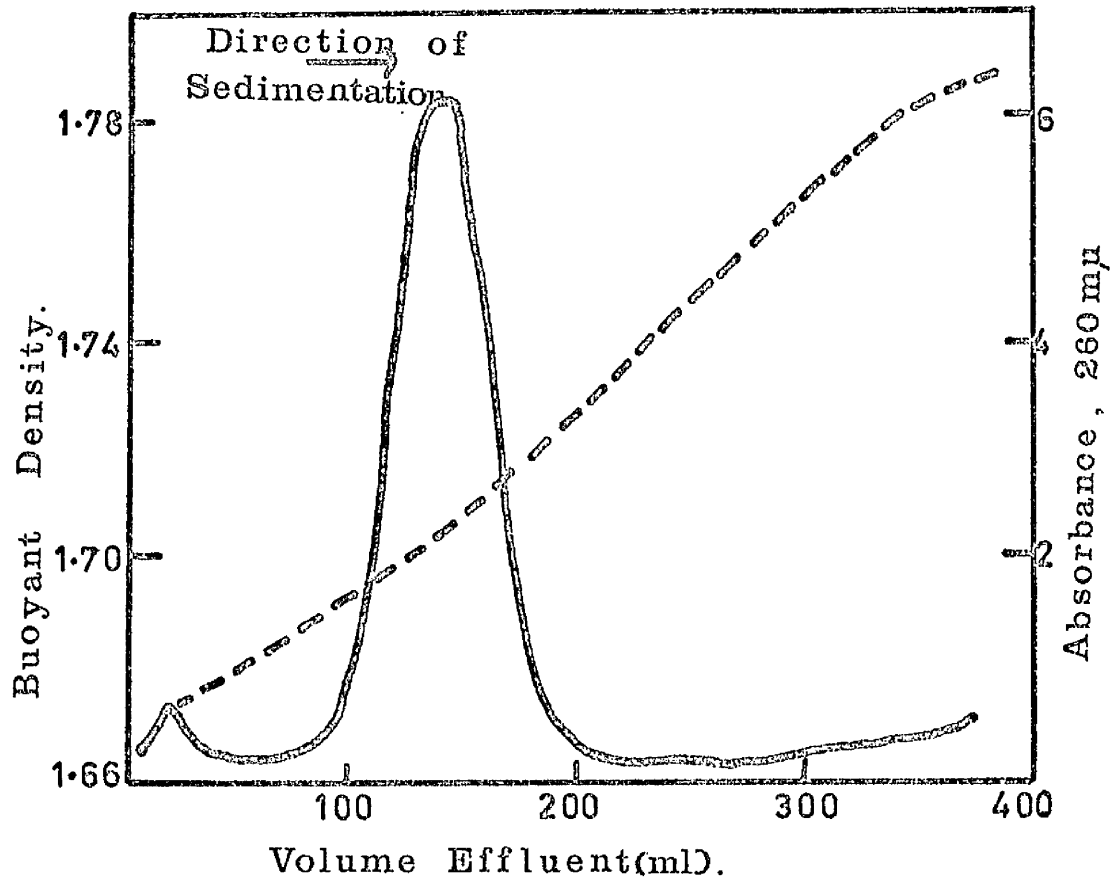
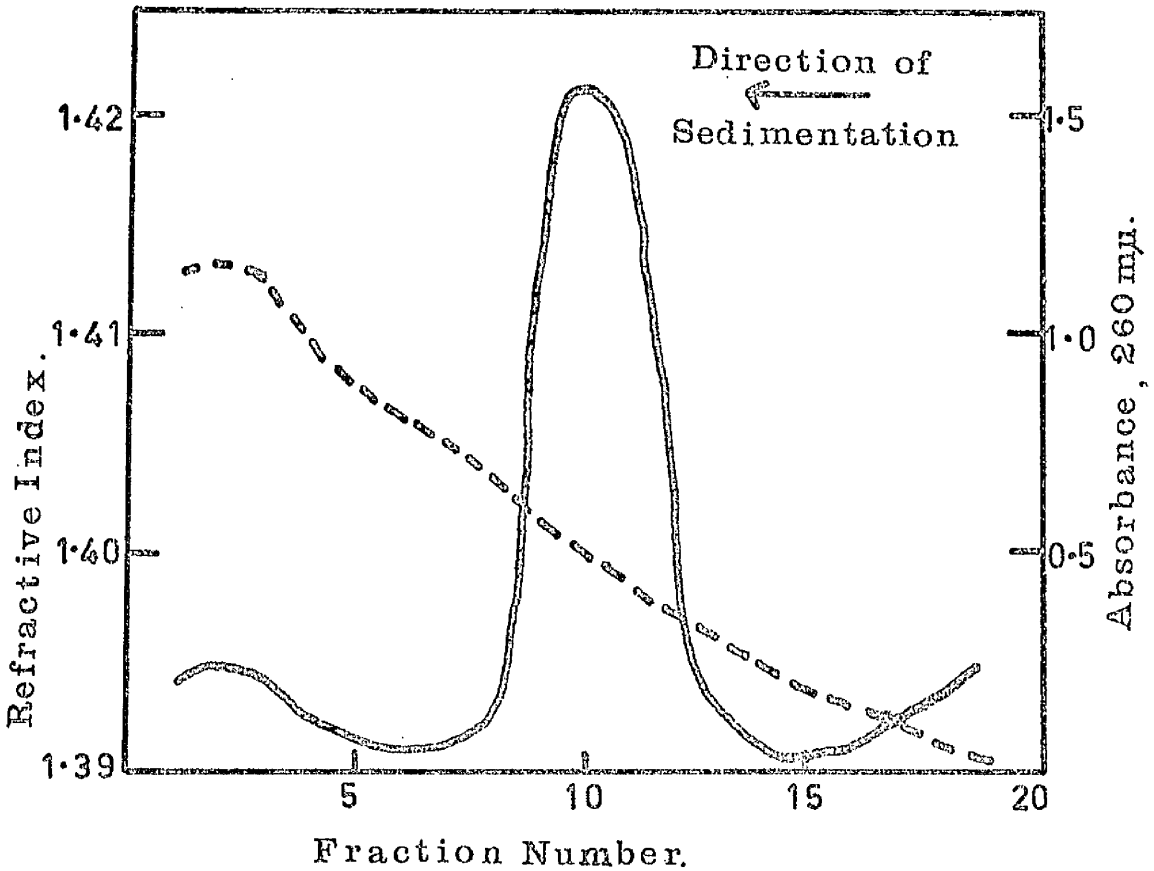


Figure 30.



Z_1 = sucrose concentration at r_1 (distance in cm in direction of centrifugal force field).

Z_2 = sucrose concentration at r_2.

This was determined for r_1 = 8.5 cm at a 10% sucrose concentration and r_2 = 10.9 cm at a 20% sucrose concentration.

$\therefore Z_o = -25.4$. The values for $\triangle I$ were determined from the slope of the sucrose gradient. Using the formula, the s values at different points in the gradient were calculated and the s values at peak heights determined for the two DNAs. S value

ΔI_{24} =	1.17 - 0.16	=	1.01	36.5
ΔI_{20} =	0.90 - "	=	0.74	26.8
ΔI_{16} =	0.69 - "	=	0.53	19.2
ΔI_{12} =	0.49 - "	=	0.33	11.9
ΔI_{8} =	0.32 - "	=	0.16	5.8
ΔI_{6} =	0.24 - "	=	0.08	2.9

The molecular weights were calculated using the relationship $S = 0.0528M^{0.4}$ (Where M = molecular weight in 0.9M NaCl/0.1M NaOH; Studier, 1965).

Single stranded molecular weight at peak height E.coli DNA = 300,000.

Single stranded molecular weight at peak height total mouse embryo DNA = 400,000.

This experiment was performed in collaboration with Dr. A. Hell.

Protein content

The protein contents of DNA samples before and after purification by caesium chloride centrifugation were less than 0.5%. This was the limit of determination for the amounts of DNA used in the estimations (0.5 mg). The effect of caesium chloride purification on the protein content of DNA could not therefore be assessed.

RNA content

The RNA content of DNA preparations was determined. Crude mouse embryo DNA had a 20% RNA contamination. This was reduced to between 2.1 - 5.0% after the DNA was banded in caesium chloride in the 8 x 25 titanium angle head.

3.7 Hybridisation procedures.

Filter retention of DNA

a) DNA retention during standard hybridisation procedures.

^{3}H-DNA (4530 cpm/µg) was prepared from 14 day mouse embryo livers cultured with ^{3}H - uridine for 20 hours. The crude DNA was purified by isopycnic centrifugation in caesium chloride.

Membrane filters were washed and 10-20µg ^{3}H-DNA was immobilised on each filter. The filters were either washed on each side, dried and counted, or subjected to each step in the normal hybridisation procedure. Blank filters were prepared as usual and were incubated with the DNA - containing filters. Filters to be processed were pre-incubated in Denhardt's medium either in water or in 4 x SSC.

They were then incubated either in 2 x SSC/0.1% SDS at 67°C or in 4 x SSC/50% formamide at 37°C and washed and RNased as usual.

The results are summarised in Table 2. 1-2% of the counts were lost on the initial loading of the filters. This was probably due to residual contamination of the DNA with ^{3}H-RNA. Pre-incubation of the filters with Denhardt's medium in water resulted in 46-48% DNA loss from all hybridised filters. When filters were pre-incubated with Denhardt's medium in 4 x SSC, filter retention of DNA was 80% after incubation at 67°C and 90% after incubation in formamide at 37°C. Transfer of DNA onto blank filters was 3% in the 67°C incubation and 0.45% in the 37°C incubation.

Although hybridisation at 37°C in 4 x SSC/50% formamide will therefore give better retention of DNA on filters than will hybridisation at 67°C in 2 x SSC/0.1% SDS, about 12% of the DNA is lost under the most favourable hybridisation conditions. The amount of DNA lost did not differ significantly with duplicate filters so retention of unlabelled DNA to filters was routinely checked by monitoring the total filtrate for absorbance at E_{260} after loading the filters with DNA. The saturation curves and double reciprocal plots in the hybridisation results have not been corrected for DNA loss. This correction is made in the final calculation of DNA hybridised.

b) <u>DNA retention at different formamide concentrations</u>

^{3}H-DNA (4530 cpm/μg) retention on filters was monitored after incubation at different formamide concentrations in 4 x SSC

TABLE 2.

Retention of ^{3}H - DNA on filters

Pre-incubation in Denhardt's medium	Incubation conditions	cpm/filter + DNA	cpm/filter blanks
None	None	68062, 63140,	-
None	None	61901	-
in water	67°C/2 x SSC	33634, 33741	1431, 1953
in water	37°C/50%f.* /4 x SSC	34036, 33086	486, 761
in 4 x SSC	67°C/2 x SSC	51318, 41721	2107, 1779
in 4 x SSC	37°C/50%f.*/4 x SCC	59362, 56606	323, 264

f.* = formamide

15µg ^{3}H - 14 day mouse embryo liver DNA was loaded on each filter. Control DNA filters were washed on each side with 20 mls 4 x SSC, dried and counted.

^{3}H - DNA and blank filters were pre-incubated in Denhardt's medium, either in water or in 4 x SSC. 2 blank and 2 DNA filters were then incubated for 16 hours in 0.2 mls of either 2 x SSC (67°C) or 4 x SSC/50% formamide (37°C). The filters were washed in the usual way, dried and counted.

at 36.5C. When the incubation medium was not adjusted to pH7, the DNA loss rose from 10% at pH 7.3 to 17% at pH 8.3 (62% formamide/4 x SSC). Transfer of DNA to blank filters rose from 0.3% to 1.4%. When the formamide solutions were adjusted to pH 7, there was a DNA loss of 7-10% (Table 3). Transfer of DNA to blank filters increased from 0.4% to 0.7% at the higher formamide inputs.

The variations in the levels of DNA retentions to filters in this and the previous experiment may be partially due to pipetting errors in dispensing 4 mls DNA solution onto the filters (±4%). Most of the DNA was lost during the incubation periods and not during the washings. It is probable that the loss of DNA was due to its low molecular weight (4×10^5).

c) DNA retention at different temperatures

Filters loaded with 20µg ^{3}H - DNA (4530 cpm/µg) were incubated under hybridisation conditions, washed, dried and counted. The filters were heated at increasing temperatures in aliquots of 2 x SSC or 1 x SSC as in the determination of T_ms. The counts lost at each temperature were determined (Figure 31). 40% DNA was lost when filters were heated to 90°C in 1x SSC; 15% DNA was lost from filters incubated in 2 x SSC. The T_m determinations from RNA - DNA hybrids were therefore carried out in 2 x SSC.

Reduction of non-specific binding of RNA to blank filters

a) Incubation in 0.1% SDS

TABLE 3.

Retention of ^{3}H - DNA on filters after incubation in varying formamide concentrations.

% formamide in incubation medium	cpm transferred to 0.2 mls incubation medium	cpm/filter + DNA	cpm/filter blanks
38	1080	11444, 10702	45,55
44	700	11942, 11370	57,63
50	1040	10101, 11747	62,78
56	1100	11708, 10840	76,86
62	805	12201, 11451	81,97

3µg ^{3}H - DNA (4530 cpm/µg) was loaded onto each filter. ^{3}H - DNA and blank filters were pre-incubated in Denhardt's medium in 4 x SSC. 2 ^{3}H - DNA and 2 blank filters were incubated in 0.2 mls 4 x SSC at different formamide concentrations. Incubation was for 16 hours at 36.5°C. The filters were washed, dried and counted in the usual way. An aliquot of the incubation medium was counted in 1 ml aqueous phase in 10 mls toluene-based scintillator/triton X - 100.

FIGURE 31.

Loss of ^{3}H DNA from filters when heated at different temperatures.

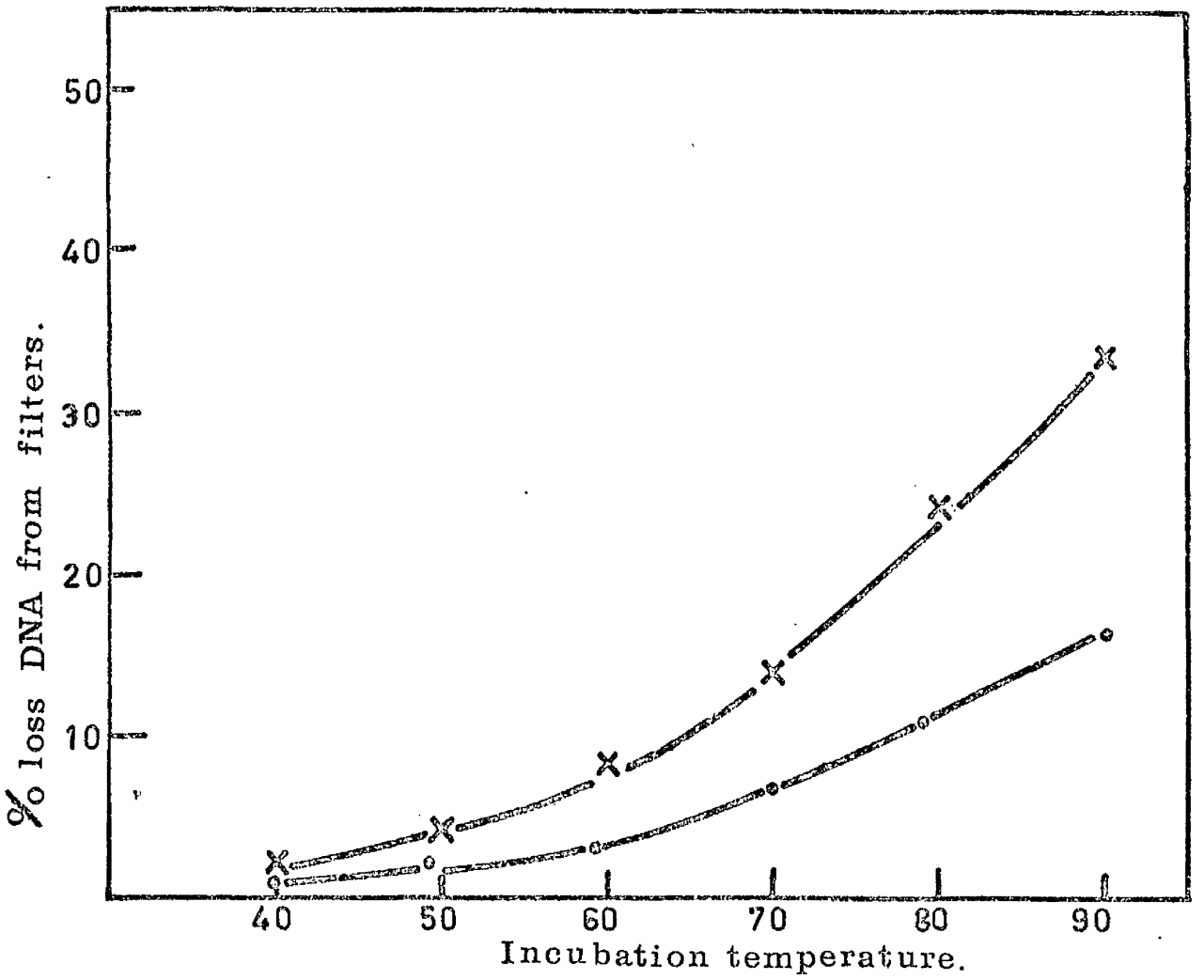
Duplicate filters were loaded with 20µg ^{3}H-DNA (4530 cpm/µg) from 14 day cultured mouse embyro livers.

The filters were incubated under hybridisation conditions in 4 x SSC/50% formamide at 36.5°C, washed, dried and counted. They were washed in 2 x SSC at room temperature, dried, then were heated for 10 minutes at increasing temperatures in a series of vials containing either 1 ml 1 x SSC or 1 ml 2 x SSC. The eluants were counted in 10 mls toluene based scintillator/triton X - 100.

x ——— x, loss in 1 x SSC;

o ——— o, loss in 2 x SSC.

Figure 31.


50
40
30
20
10
% loss DNA from filters.
40
50
60
70
80
90
Incubation temperature.

Incubation of ^{32}P-labelled 28s RNA in 2 x SSC/0.1% SDS at 67°C lowered the counts on blank filters from 0.15% (incubation in the absence of SDS) to 0.075% of the input RNA. Duplicates were also more reproducible. Hybridisations at 67°C were therefore carried out in the presence of 0.1% SDS.

b) Washing outer rim of filters.

^{32}P - 28s RNA was hybridised to DNA filters under the usual conditions at 67°C. After incubation, the DNA filters and the blanks were washed only on the filtration apparatus. The filters were dried and counted. They were then cut into an inner circle, containing all the DNA, and an outer ring, using the no.12 cork borer. The parts were counted separately (Table 4). There was more non-specific binding of RNA to the outer ring of the filters than to the washed centre portion. A 30 minutes incubation in 4 x SSC, with stirring, after the RNase incubation, reduced the non-specific binding to the unwashed outer portion of the filters. The wash in 4 x SSC was therefore included in the routine washing procedure.

c) Non-specific binding of methylated RNA.

Methylation of RNA resulted in an increased non-specific retention of RNA on blank filters. This non-specific binding could be reduced by a combination of several procedures (Table 5).

Initial experiments with ^{3}H-dimethyl sulphate-labelled 18s RNA gave 6% retention of input RNA on blank filters after incubation in 2 x SSC/0.1% SDS at 67°C. Sonication of the RNA reduced the

TABLE 4.

Non-specific RNA retention on outer rim of filters.

Filters ± DNA	cpm inner circle	cpm outer ring	% total counts on outer ring
-	74	138	65
-	32	83	72
-	65	160	71
+	168	153	48

Blank filters and a filter loaded with 20µg DNA were incubated with an aliquot of ^{32}P - 28s RNA in 2 x SSC/0.1% SDS. Incubation was for 18 hours at 67°C. The filters were processed (see Results section) and the counts on the outer and inner parts of the filters were determined.

TABLE 5.

Non-specific binding of methylated RNAs

Treatment	% retention RNA to blank filters
1. ^{3}H - 18s RNA, 2 x SSC/0.1% SLS/67°C	6%
2. Sonication ^{3}H - 18s RNA	4%
3. Pre-treatment filters in Denhardt's medium	0.3 - 0.4%
4. ^{3}H - 5s RNA, 4 x SSC/50% f.+ + Deinhardt pre-treatment	0.095%
5. (4) + 0.1% SDS in incubation mixture	0.095%
6. Passing ^{3}H - 9s RNA through blank filter	0.095%
7. Further phenol extraction of ^{3}H - 9s RNA + (4)	0.004%

+ f. = formamide

Different preparations of RNA were incubated under the specified conditions. Hybridisations were in 0.2 mls hybridisation medium containing 2 DNA and 2 blank filters. All filters were washed as usual. The ^{3}H-RNA sticking to the filters was expressed as a percentage of the total ^{3}H-RNA input.

non-specific binding to 4%. When both blank and DNA filters were pre-incubated in Denhardt's medium, the non-specific binding of ^{3}H-methylated 5s RNA to blank filters was reduced to 0.3-0.4% of the input counts. Although this had reduced the non-specific binding by a factor of 10, the background was still too high for the detection of low levels of hybridisation. When filters, pre-treated with Denhardt's medium, were incubated with the 5s RNA in 50% formamide/4 x SSC at 36.5°C, the background was reduced to 0.095% of input counts. Addition of 0.1% SDS to the incubation medium had no effect on the RNA binding to either blank or DNA filters.

Passing ^{3}H-methylated RNA through a blank filter gave the same level of binding as did the incubation of a filter with the same amount of RNA during hybridisation. This background was also similar whether the incubation medium contained DNA filters or only blank filters. It was therefore unlikely that the counts on blank filters were caused by uptake of DNA by the blank filters and a consequent hybridisation with the methylated RNA. In later experiments, a further phenol extraction of "sticky" methylated RNAs reduced backgrounds in later experiments to .004%. Residual protein contamination of the RNA was therefore responsible for some of the non-specific binding to blank filters. This protein contaminant could have increased the counts sticking to blank filters in two ways; either by increasing the retention of labelled RNA to the blank filters or by the retention of the methylated protein

itself.

Comparisons of hybridisations in formamide and in SSC.

a) Saturation curves.

Landschutz ^{32}P-28s RNA was hybridised to total mouse embryo DNA at 67°C in 2 x SSC/0.1% SDS and at 36.5°C in 2 x SSC/50% formamide. The saturation curves and double-reciprocal plots are shown in Figures 32 and 33. The approach to saturation is similar for both curves. When the corrections are made for the loss of DNA from the filters (12% loss for the incubation at 36.5°C and 22% loss for the incubation at 67°C), the saturation level is higher for the hybrid formed at 67°C. The corrected double reciprocal plots give saturation values of 0.0128% (36.5°C incubation) and 0.0136% (67°C incubation) at infinite RNA inputs. These values correspond to 62 (36.5°C) and 66 (67°C) 28s DNA cistrons/haploid genome, assuming a molecular weight of 1.53×10^6 for 28s RNA (Hunt, 1970) and a DNA content of 3×10^{12} daltons for a diploid mouse cell (Vendrely and Vendrely, 1949).

b) Time course.

0.9µg ^{32}P-28s RNA was hybridised to total mouse embryo DNA in incubations of 2 x SSC/50% formamide at 36.5°C or 2 x SSC/0.1% SDS at 67°C for the various times indicated (Figure 34). The 67°C hybridisation is complete after 1 hour whereas the hybridisation in formamide at 36.5°C is only complete after 4-8 hours.

FIGURE 32.

Partial saturation curve for ^{32}P - 28s Landschutz RNA hybridised to total mouse embryo DNA. The DNA had been purified by isopycnic banding in caesium chloride or by the method of Marmur. Filters were loaded with 20µg DNA. 2 DNA and 2 blank filters were incubated in 0.2 mls hybridisation medium at different RNA inputs for the 36.5°C incubations. Duplicates containing 1 DNA and 1 blank filter in 0.1 mls hybridisation medium were incubated at different RNA inputs for the 67°C incubations.

Hybridisation was for 16 hours.

Corrections were made for DNA loss from filters during hybridisation.

△ ——— △ , Hybridisation to CsCl-purified DNA in 2 x SSC/0.1% SDS at 67°C.

○ ——— ○ , Hybridisation to CsCl-purified DNA in 50% formamide/2 x SSC at 36.5°C.

x ——— x , Hybridisation to Marmur-purified DNA in 50% formamide/2 x SSC at 36.5°C

FIGURE 33.

Double reciprocal plots for the data shown in Figure 32. At infinite concentrations of input RNA, the saturation values for DNA are calculated as

△ ——— △ , .0136%

○ ——— ○ , .0128%

x ——— x , .0140%

Figure 32.

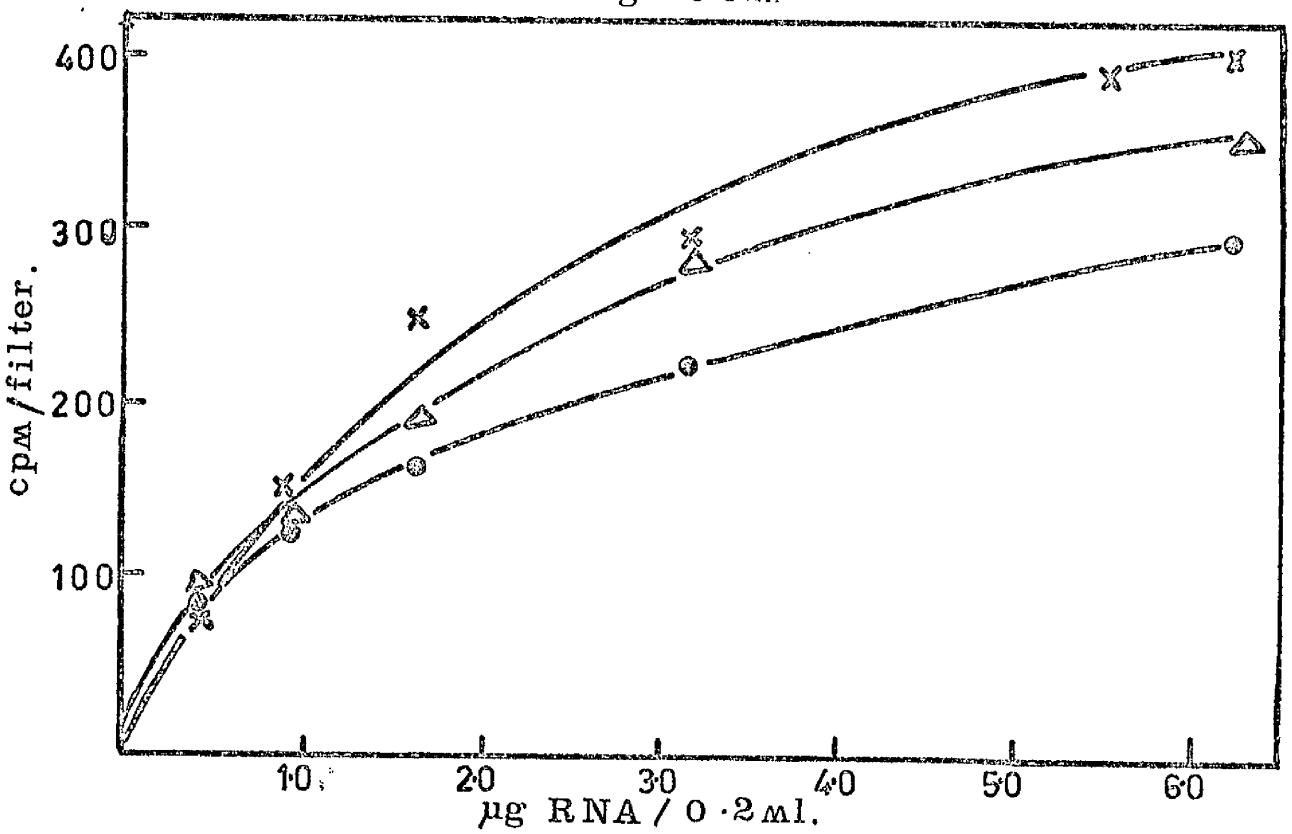


Figure 33.

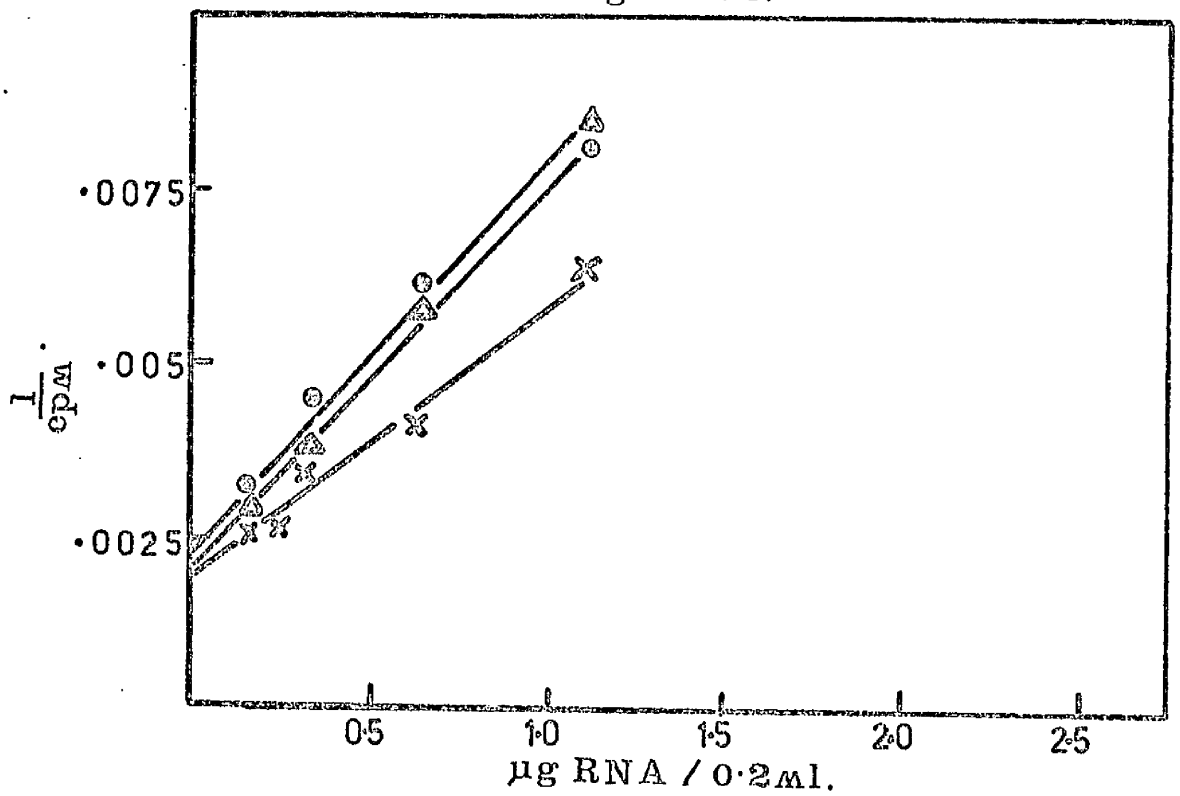


FIGURE 34.

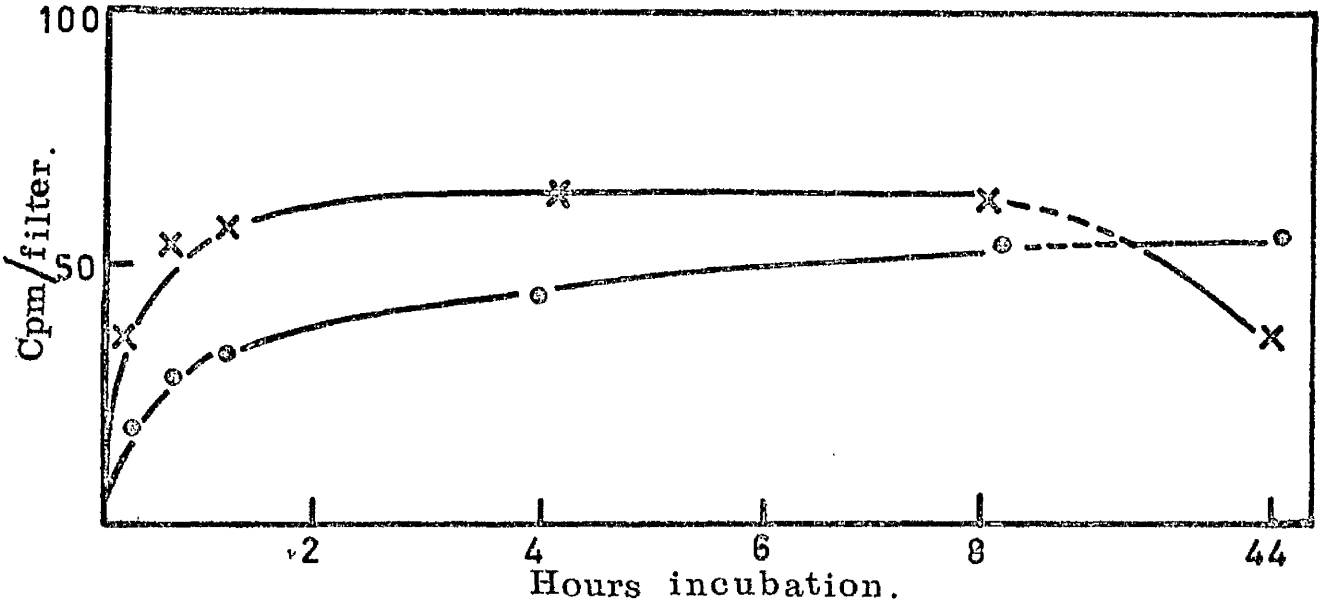
Time course of hybridisation of ^{32}P - 28s RNA with total mouse embyro DNA.

x——————x, 1 DNA and 1 blank filter were incubated in duplicate vials with 0.9µg ^{32}P - 28s RNA in 0.1 ml 2 x SSC/0.1% SDS.

o——————o, 2 DNA and 2 blank filters were incubated in a vial with 1.8µg ^{32}P - 28s RNA in 0.2 mls 50% formamide/2 x SSC.

All components of the reaction mixtures were pre-equilibrated separately to the temperature of incubation. Incubations were for the times indicated.

Figure 34.



c) Thermal dissociation profiles.

The melting profiles of ^{32}P-28s RNA - DNA hybrids, formed at different RNA inputs at both 67°C and 36.5°C, are shown in Figure 35. Hybrids formed at 67°C had the same T_m(50°C) at both RNA inputs. Hybrids formed at 36.5°C had T_ms of 54°C (2.7µg RNA and 62°C (0.9µg RNA). The T_ms of hybrids formed at 36.5°C were therefore higher than those formed at 67°C, at equivalent RNA inputs.

A similar result was obtained with an impure ^{32}P-5s RNA sample hybridised both at 67°C and at 36.5°C at equivalent RNA inputs (Figure 36). The T_m of the hybrid formed at 36.5°C in formamide was 8°C higher than that formed at 67°C.

Comparison of ^{32}P - 28s RNA hybridisation to DNAs of different size.

a) Saturation curve.

^{32}P - 28s RNA was hybridised to DNA, purified by the method of Marmur (see methods), in 50% formamide/2 x SSC at 36.5°C. The saturation curve and double reciprocal plot were compared with those using caesium chloride-purified DNA (Figures 32 and 33). The DNA purified by the method of Marmur saturated at a higher level than did the caesium chloride-purified DNA. At infinite RNA inputs, there was 0.014% hybridisation to the Marmur-prepared DNA and 0.0128% hybridisation to the caesium chloride- purified DNA. These values correspond to 68 and 62 28s cistron copies/

FIGURE 35.

Melting profiles of ^{32}P - 28s RNA - DNA hybrids formed under different conditions and at varying RNA inputs.

1. Hybridisation in 0.1ml 2 x SSC/0.1% SDS at 67°C for 16 hours.

o ——— o, 0.45µg input ^{32}P - 28s RNA.

△ ——— △ 1.35µg input ^{32}P - 28s RNA.

2. Hybridisation in 0.2 mls 50% formamide/2 x SSC at 36.5°C for 16 hours.

x ——— x, 0.9µg input ^{32}P - 28s RNA.

o ——— o, 2.7ug input ^{32}P - 28s RNA.

FIGURE 36.

Melting profiles of ^{32}P - 5s RNA - DNA hybrids formed at 67°C and at 36.5°C.

o ——— o, Hybridisation of 0.3µg ^{32}P - 5s RNA in 0.1 ml 2 x SSC/0.1% SDS at 67°C for 16 hours.

x ——— x, Hybridisation of 0.6µg ^{32}P - 5s RNA in 0.2ml 2 x SSC/50% formamide at 36.5°C for 16 hours.

Figure 35.

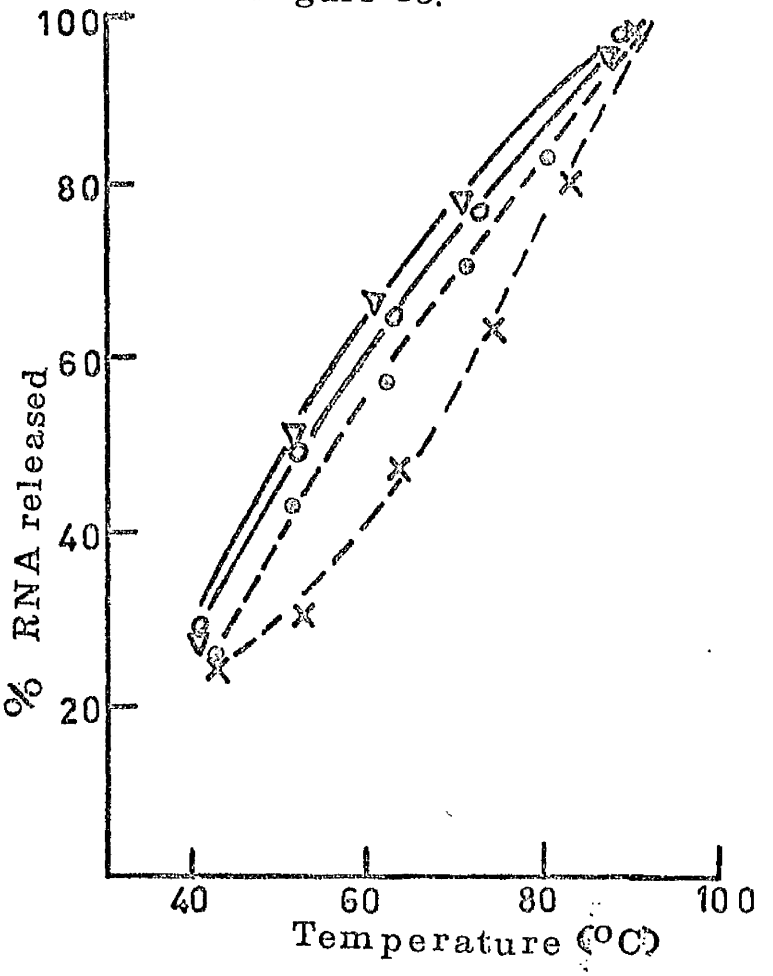
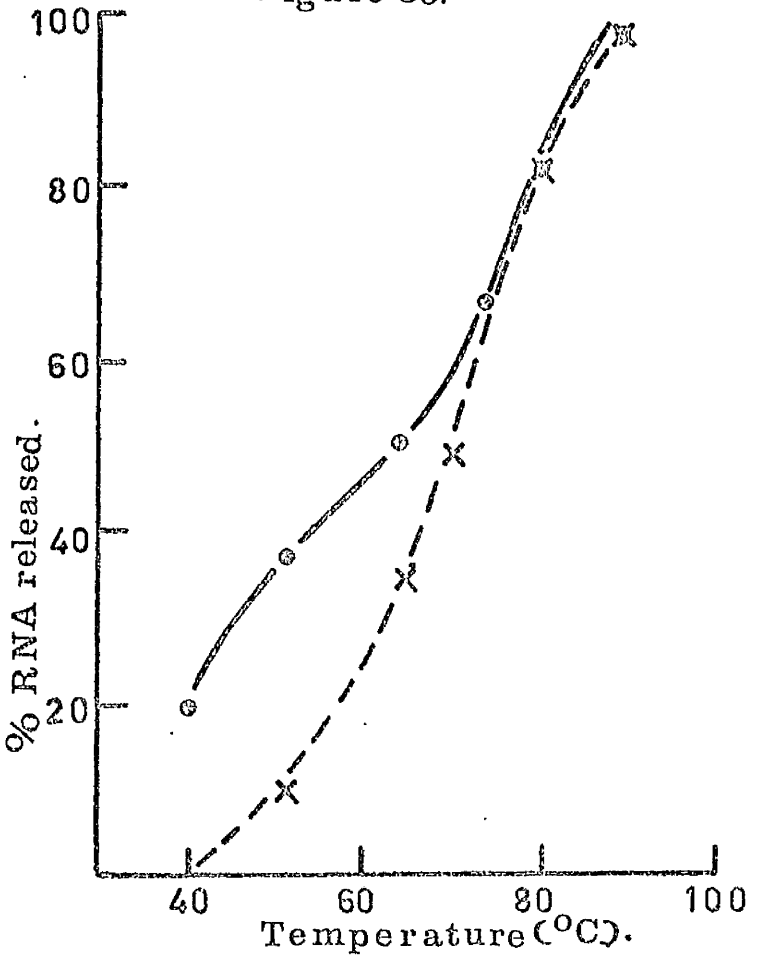


Figure 36.



haploid genome respectively. The Marmur-prepared DNA had 96-100% retention to filters throughout the hybridisation procedure (Dr. G. Threlfall, personal communication). The differences in saturation values could therefore be caused by the 10 - 15% loss of caesium-chloride purified DNA during the hybridisation procedure.

b) Thermal dissociation profiles

The melting profiles of ^{32}P-28s RNA hybrids formed with caesium chloride-purified DNA at different RNA inputs are shown in Figure 37. Similar melting profiles for Marmur-prepared DNA are shown in Figure 38. Hybrids formed with caesium chloride-purified DNA had T_ms of 54°C at an RNA input of 2.7µg and T_ms of 62°C at an input of 0.9µg. The hybrids formed with Marmur-purified DNA had T_ms of 62°C at an RNA input of 5.4µg and of 67°C at an RNA input of 2.7µg. The T_ms of hybrids formed with Marmur-prepared DNA were therefore 5° - 17°C higher than those formed with caesium chloride-purified DNA. Since the Marmur-prepared DNA was probably of a higher molecular weight than the caesium chloride-purified DNA, the difference in hybrid stability may be due to the size difference between the two DNAs when hybridised to an RNA of molecular weight 1.53×10^6.

There was again a lowering of hybrid stability, as indicated by a lowering of the T_ms, at higher RNA inputs. It is therefore probable that more mismatched hybrids are being formed at higher RNA inputs.

FIGURE 37.

Melting profiles of ^{32}P - 28s RNA - DNA hybrids formed in 0.2 mls 50% formamide/2 x SSC at 36.5°C for 16 hours. The DNA was purified by isopycnic caesium chloride centrifugation.

•———— • 0.9µg input ^{32}P - 28s RNA.

x———— x 2.7µg input ^{32}P - 28s RNA

FIGURE 38.

Melting profiles of ^{32}P - 28s RNA - DNA hybrids formed in 0.2 mls 50% formamide/2 x SSC at 36.5°C for 16 hours. The DNA was purified by the method of Marmur.

•———— • 2.7µg input ^{32}P - 28s RNA.

x———— x 5.4µg input ^{32}P - 28s RNA.

Figure 37.

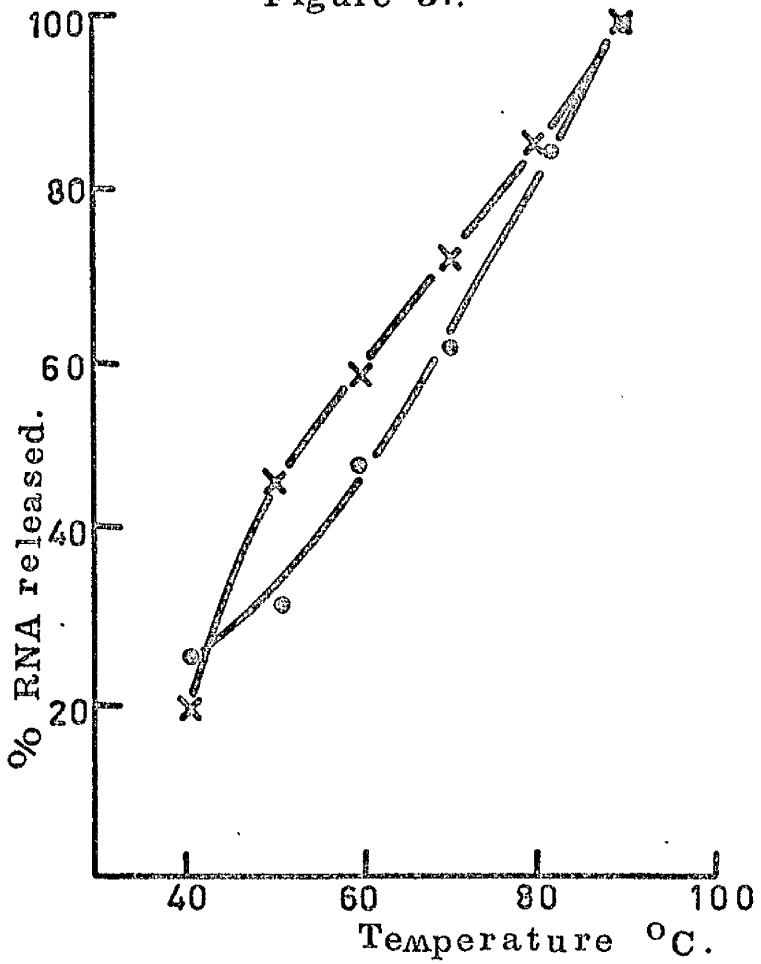
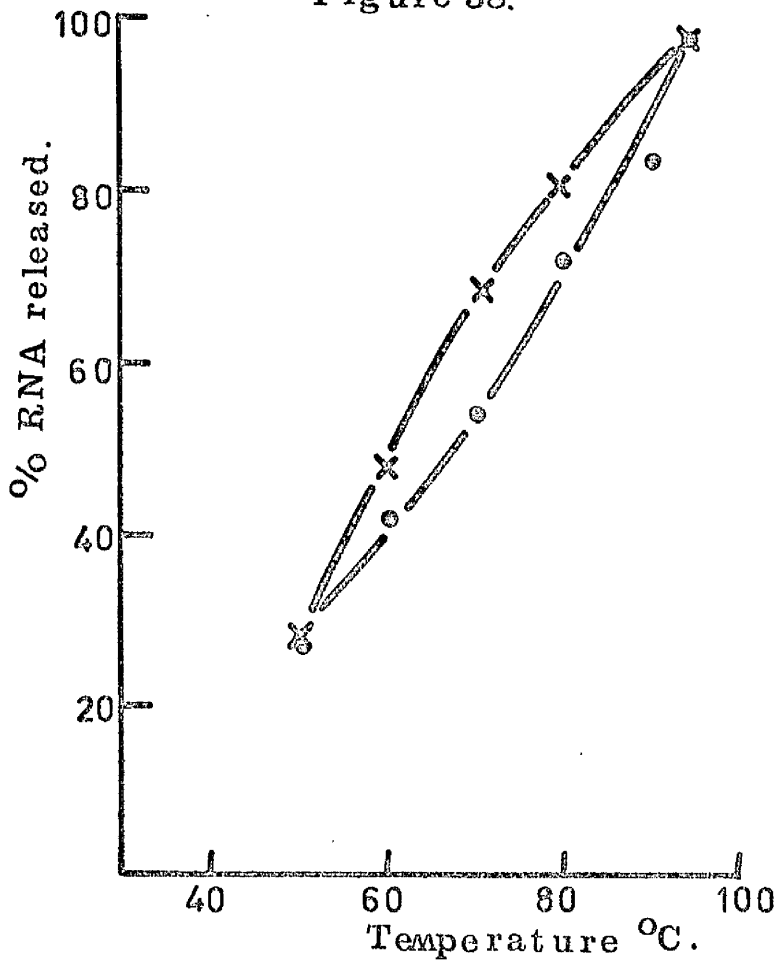


Figure 38.



Hybridisation of methylated and non-methylated 5s RNAs.

^{32}P - 5s RNA was prepared from Landschutz cells cultured with ^{32}P - orthophosphate. Part of the pure ^{32}P - 5s RNA was methylated *in vitro*.

a) Saturation curves

Varying amounts of the 5s RNA samples were hybridised to total mouse embryo DNA. The partial saturation curves for the methylated ^{32}P - 5s RNA and the ^{32}P - 5s RNA are shown in Figure 3 The approach to saturation was rapid for the ^{32}P - 5s RNA since at RNA/DNA inputs of 0.015, 75% of the potential hybrid was formed. The methylated ^{32}P - 5s RNA showed a more gradual approach to saturation. The double reciprocal plots for the methylated and non-methylated ^{32}P - 5s RNAs (Figure 40) give saturation value of 0.01125% DNA and 0.0108% DNA respectively. These values correspond to 2120 and 2080 DNA cistrons coding for 5s RNA in a haploid genome, assuming a molecular weight of 4×10^4 for 5s RNA (Forget and Weissman, 1967) and a DNA content of 3×10^{12} daltons for a diploid mouse cell (Vendrely and Vendrely, 1949).

b) Thermal dissociation profiles

The thermal melting profiles of the methylated and unmethylated ^{32}P - 5s RNA hybrids were compared at equivalent RNA inputs (Figure 41). The T_m of the methylated ^{32}P - 5s RNA hybrid was 67°C, 7°C lower than the T_m of the ^{32}P - 5s RNA hybrid (74°C).

A comparison was also made between hybrids formed at one RNA input from a methylated rabbit 5s RNA preparation and an

FIGURE 39.

Partial saturation curves for ^{32}P - 5s RNA and methylated ^{32}P - 5s RNA hybridised to total mouse embryo DNA purified by isopycnic centrifugation in caesium chloride.

Hybridisation was in 50% formamide/2 x SSC at different inputs of RNA for 16 hours at 36.5°C.

o———o ^{32}P - 5s RNA.

x———x methylated ^{32}P - 5s RNA.

FIGURE 40.

Double reciprocal plots for the data shown in Figure 39. At infinite concentrations of RNA, the ^{32}P - 5s RNA saturated at a value of 0.0112% DNA and the methylated ^{32}P - 5s RNA saturated at a value of 0.0108% DNA.

Figure 39.

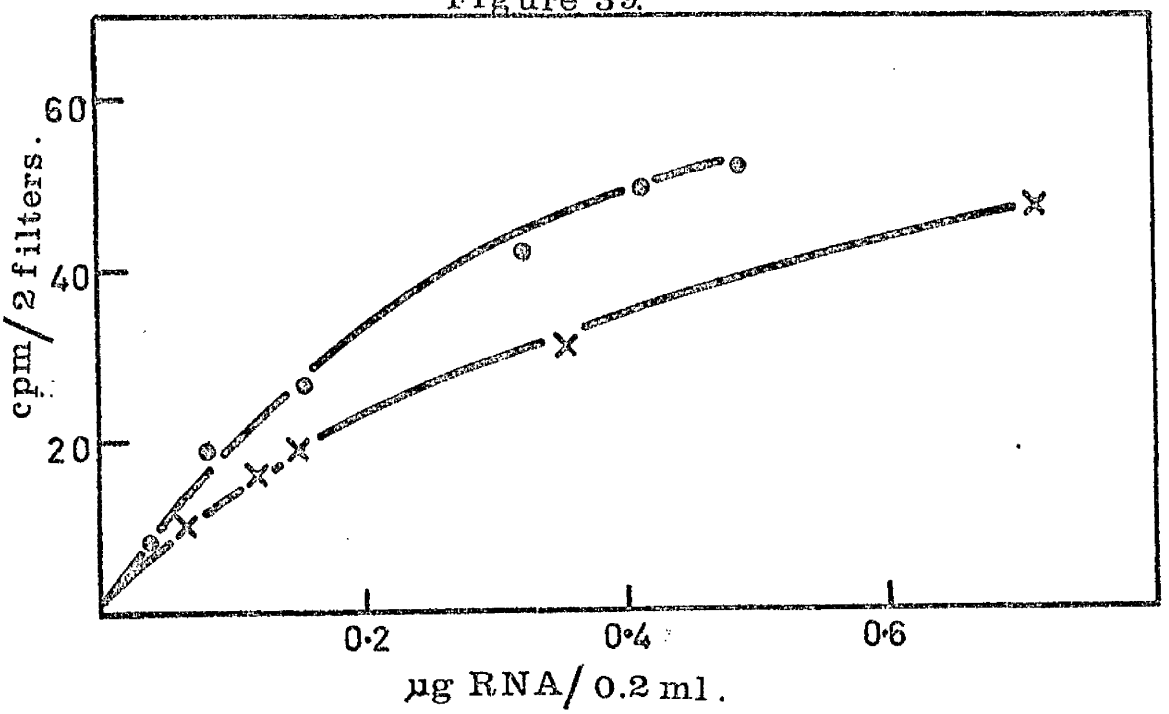


Figure 40.

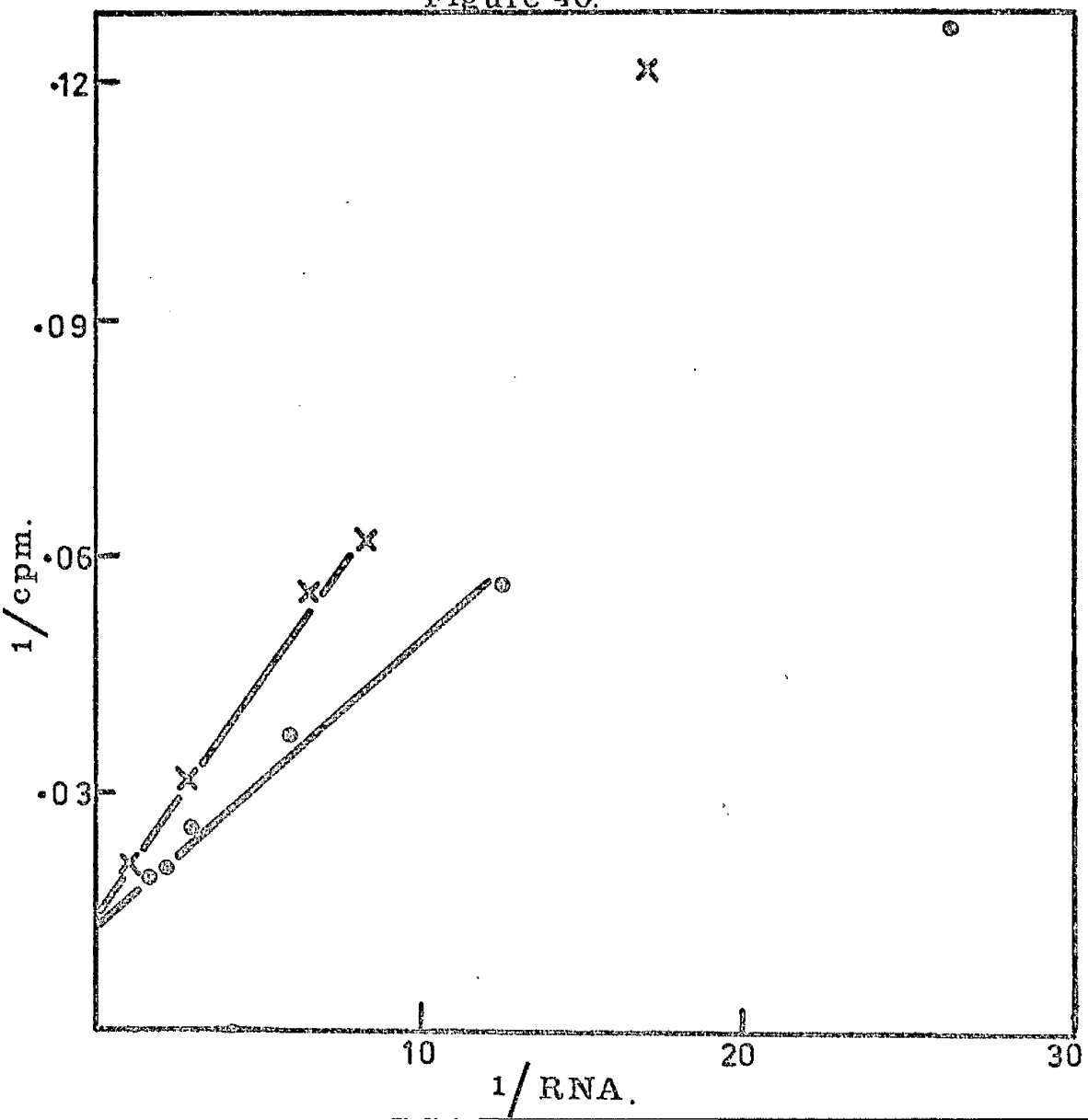


FIGURE 41

Melting profiles of ^{32}P - 5s RNA and ^{3}H - methylated ^{32}P - 5s RNA - DNA hybrids (Figure 35). Several filters at similar RNA inputs were pooled and melting profiles were performed as in methods.

o ---------- o ^{32}P - 5s RNA

x ---------- x ^{3}H - methylated ^{32}P - 5s RNA.

FIGURE 42.

Melting profiles of impure ^{32}P - 5s RNA - DNA hybrids and ^{3}H - methylated 5s RNA - DNA hybrids at inputs of 0.6µg RNA. Melting profiles were performed as in methods.

o ---------- o Impure ^{32}P - 5s RNA.

x ---------- x ^{3}H - methylated 5s RNA.

Figure 41.

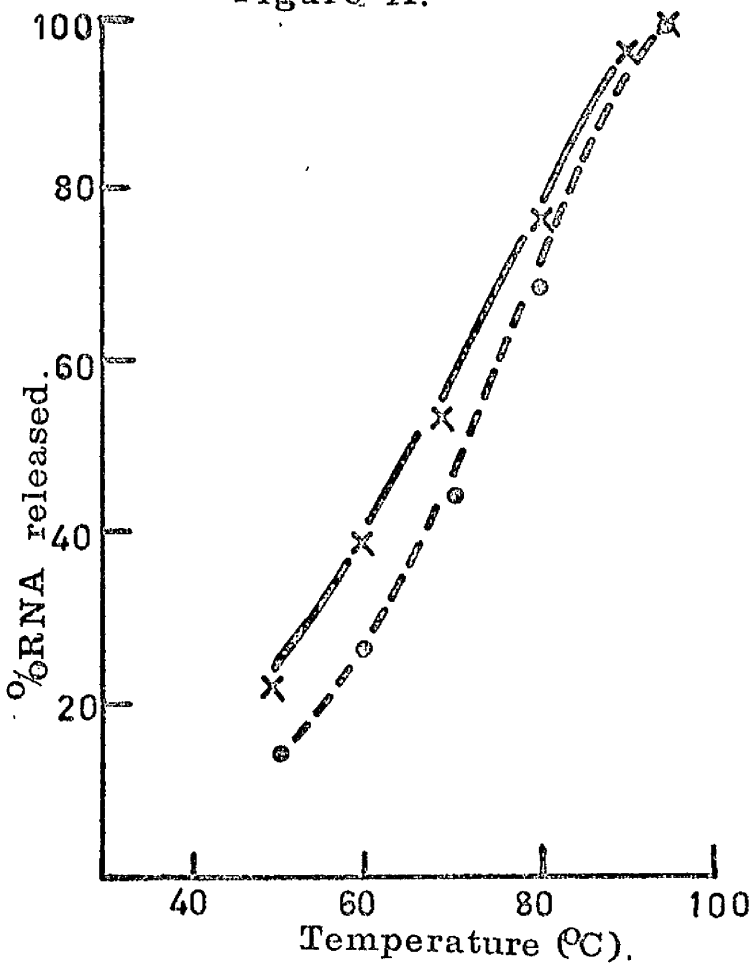
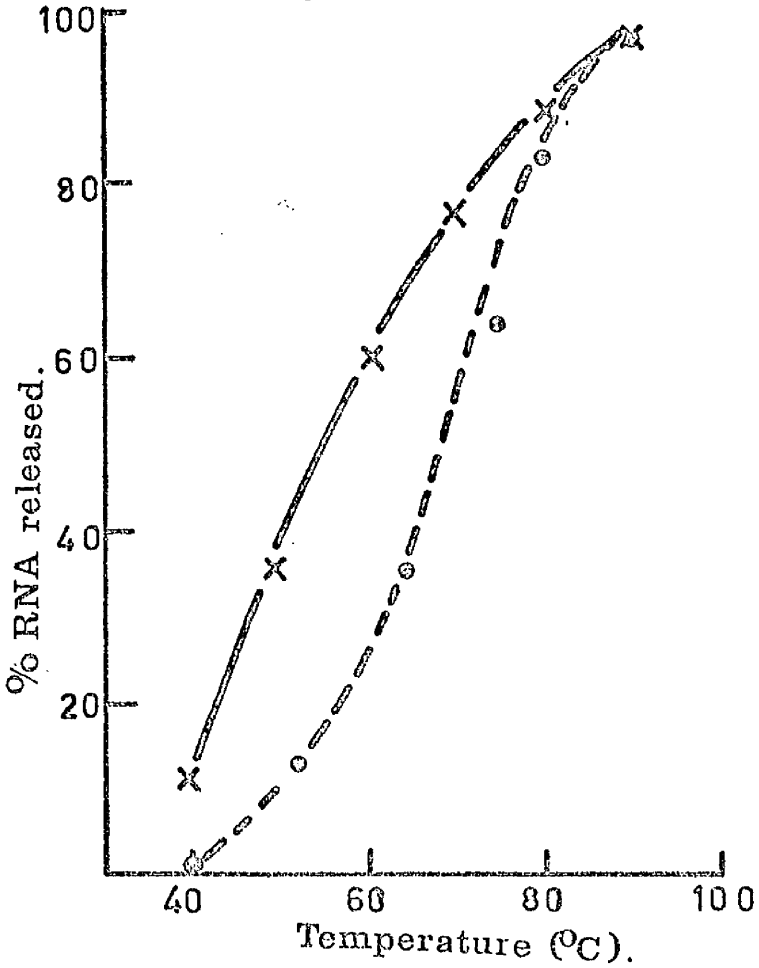


Figure 42.



impure mouse ^{32}P - 5s RNA preparation (20% contamination with 4s RNA). The pure methylated rabbit 5s RNA hybrid had a T_m of 56°C, whereas the impure mouse ^{32}P - 5s RNA had a T_m of 70°C (Figure 42).

3.8 Hybridisation of 9s RNA.

Hybridisation of ^{3}H - 9s RNA from cultured 14 day mouse embryo livers to total mouse embryo DNA.

^{3}H - 9s RNA was isolated from 14 day mouse embryo livers cultured in the presence of ^{3}H - uridine. The 9s RNA was not contaminated with RNA of other size classes. A partial saturation curve of the ^{3}H - 9s RNA to total mouse embryo DNA is shown in Figure 43. The double reciprocal plot gives a saturation value of 0.28% DNA at infinite RNA inputs (Figure 44).

Hybridisation of ^{3}H - methylated 9s RNA to total mouse embryo DNA and E.coli DNA.

Varying amounts of ^{3}H - methylated 9s RNA were incubated with filters loaded with total mouse embryo DNA. The partial saturation curves and double reciprocal plots for 4 different methylated 9s RNA preparations are shown in Figures 45 and 46. Both the approach to saturation and the saturation levels vary with different preparations of 9s RNA. With the 12% correction for loss of DNA from filters during hybridsation, the saturation values at infinite RNA input range from 0.084% to 0.57%. These saturation values would correspond to between 3715 and 25,150 DNA cistrons in a haploid mouse genome, if the cistrons coded for an RNA

FIGURE 43.

Partial saturation curve for ^{3}H - 9s RNA (6770 cpm/ug) isolated from 14 day cultured mouse embyro livers.

Incubation was in 0.25 mls 2 x SSC/0.1% SDS at 67°C for 16 hours. Each vial contained 1 27mm filter with 87µg total mouse embryo DNA, purified by isopycnic centrifugation in caesium chloride, and 1 blank 27 mm filter.

FIGURE 44.

Double reciprocal plot of the data from Figure 43. At infinite RNA inputs, the RNA hybridises to 0.28% of the DNA.

Figure 43.

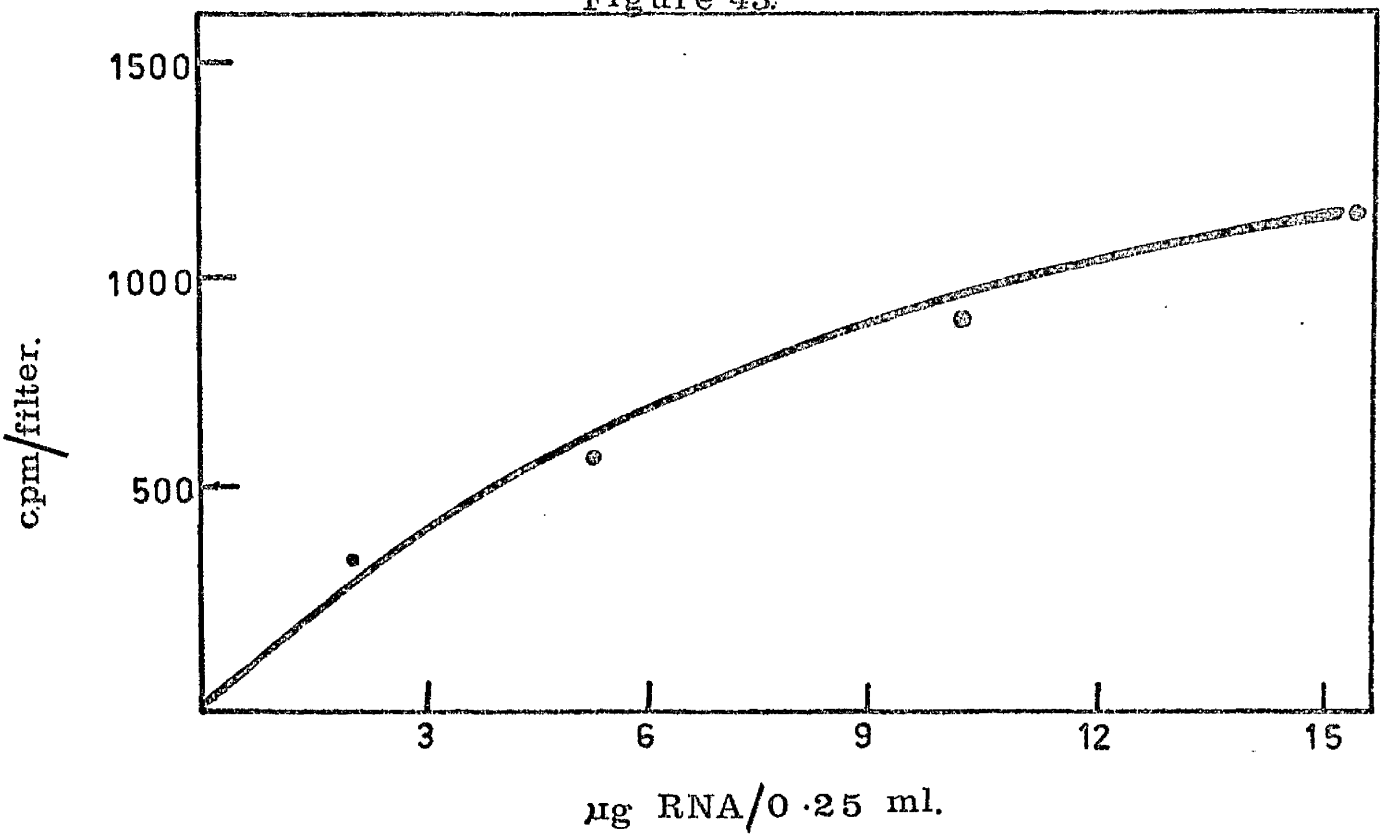


Figure 44.

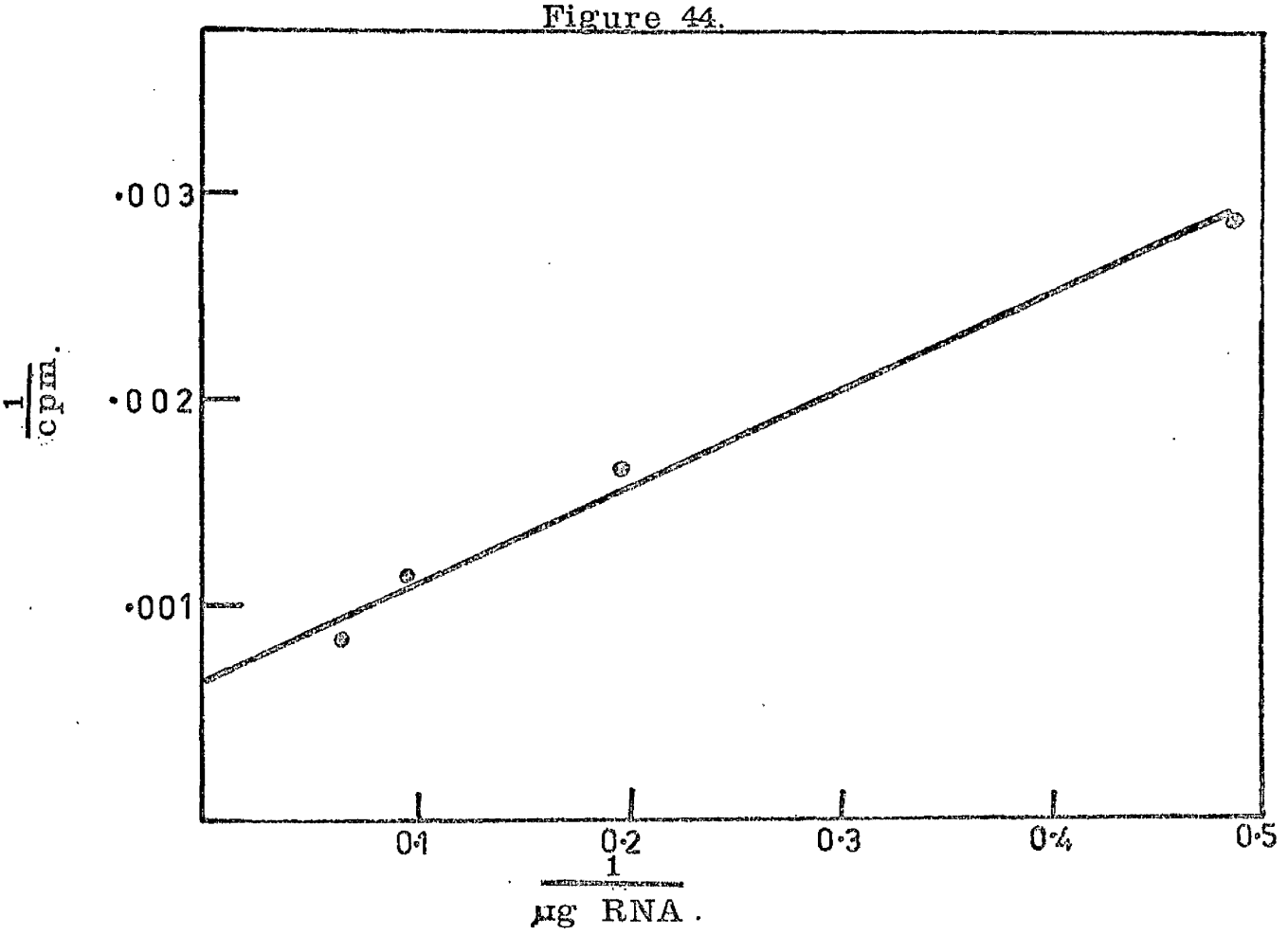


FIGURE 45.

Partial saturation curves for 4 different ^{3}H - methylated 9s RNA preparations hybridised to total mouse embyro DNA purified through caesium chloride. The hybridisation levels are expressed as the amount of ^{3}H - 9s RNA hybridised at each RNA input.

x——x specific activity 2938 cpm/µg.

△——△ specific activity 4480 cpm/µg.

o——o specific activity 3470 cpm/ug.

These hybridisations were in 50% formamide/4 x SSC at 36.5°C for 16 hours as in methods.

△——△ specific activity 4440 cpm/ug.

This hybridisation was in 50% formamide/2 x SSC at 36.5°C for 16 hours as in methods.

FIGURE 46.

Double reciprocal plots for the data of Figure 45. At infinite RNA inputs, the DNA saturation values are:

x——x 0.52%

△——△ 0.18%

o——o 0.15%

△——△ 0.084%

Figure 45.

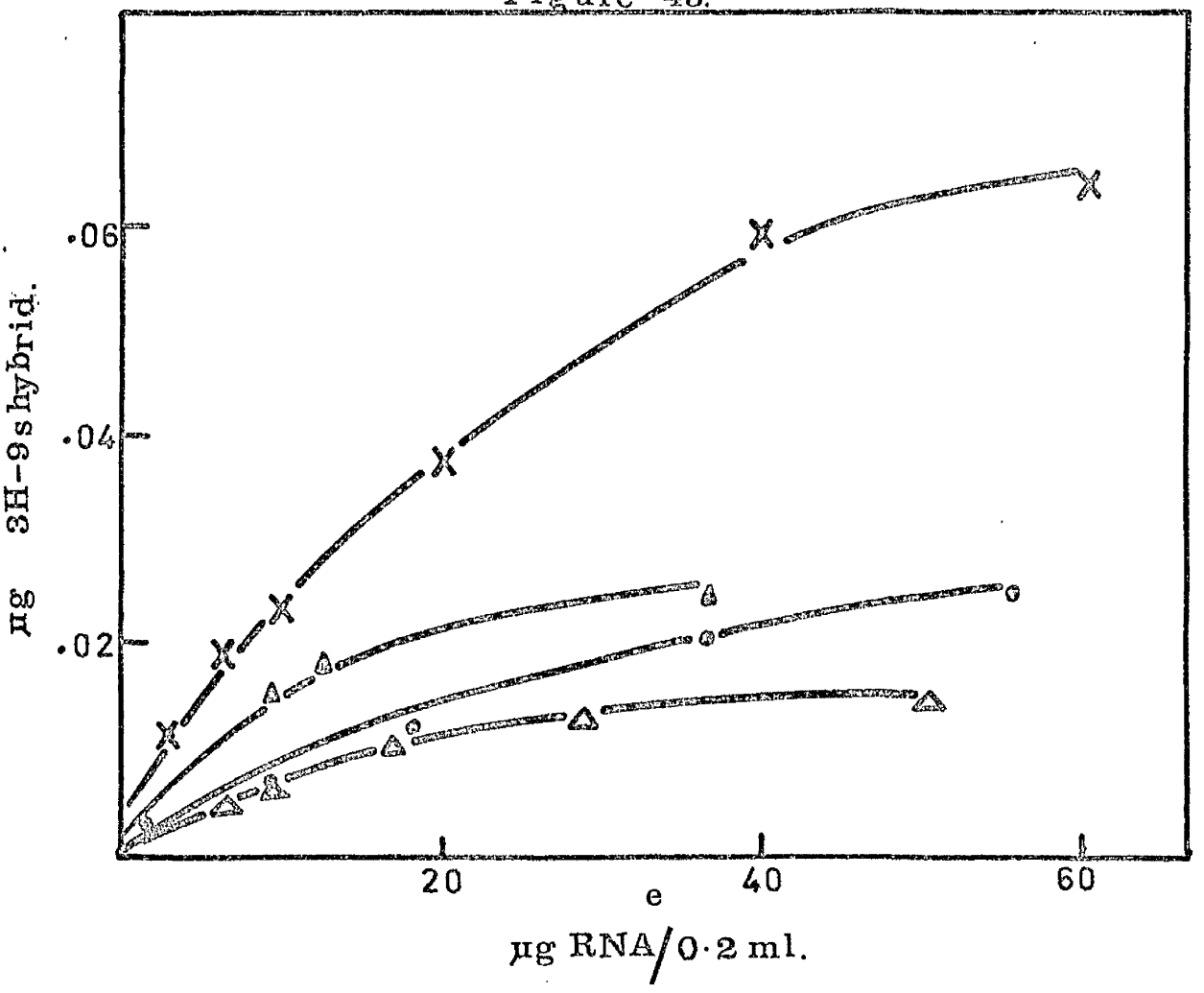
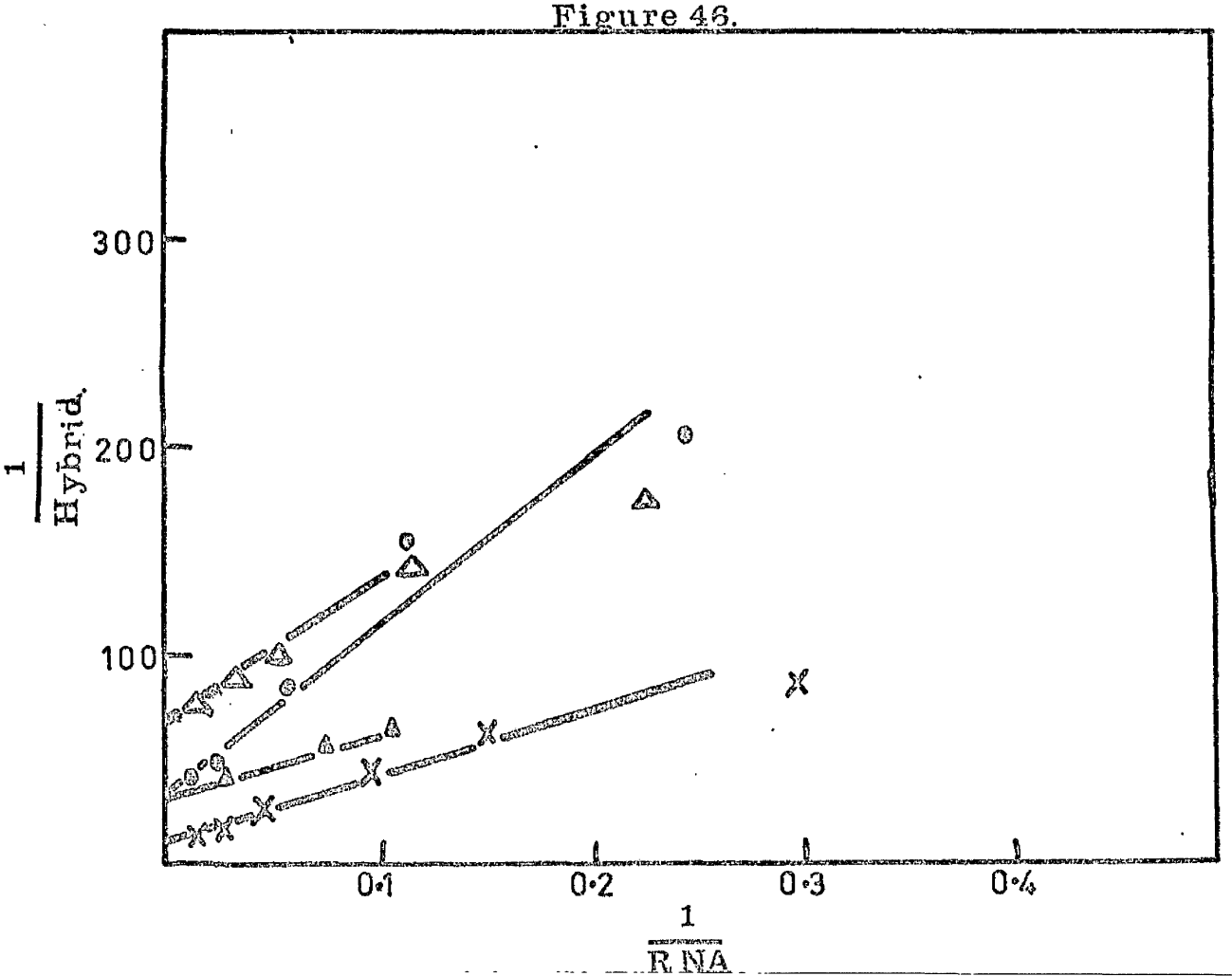


Figure 46.



of the size of 9s (assuming a molecular weight of 170,000 for the 9s RNA; Williamson et al., 1971).

There is no correlation between the amount of input RNA sticking to blank filters and the different hybridisation levels. Neither is there any correlation with the specific activities of the RNAs (Table 6). The saturation values of 0.15% and 0.18% are within the range of variation expected in hybridisations at different times and with different preparations of 9s RNA and total mouse embryo DNA. The lower value of 0.084% most recently obtained may be explained by the higher stringency of the reaction conditions at a lower SSC concentration. This would result in the dissociation of some of the more mismatched hybrids which were stable at higher SSC concentrations.

It is more difficult to account for the difference between these results and the high saturation value of 0.57% obtained from the partial saturation curve for one 9s RNA preparation and the consistently high hybridisation values found using this 9s RNA sample. It is possible that the DNA used in these particular experiments had residual protein or polysaccharide contamination resulting in anomalous sticking of the RNA to filters loaded with DNA but not to blanks. Alternatively, a contamination of the SSC buffer of one of the polynucleotide preparations with divalent metal ions would give more non-specific hybrid formation. The result could also be a true indication of variation in the 9s

TABLE 6.

Comparison of different ^{3}H - methylated 9s RNA hybridisations

Hybridisation medium 36.5°C	Specific activity RNA (cpm/μg).	% input RNA on blank filters	DNA saturation values (%)
4 x SSC/50% formamide	4480	.009	0.15
4 x SSC/50% formamide	2938	.005	0.52
4 x SSC/50% formamide	3470	.019	0.18
2 x SSC/50% formamide	4440	.004	0.084

messenger itself or in contamination with other RNA species in the 9s RNA region.

^{3}H- methylated 9s RNA was also hybridised to E.coli DNA (Table 7). The levels of hybridisation at two RNA inputs were 5.5% and 6.1% of the hybridisation to total mouse embryo DNA. There was therefore very little non-specific hybridisation to bacterial DNA under these annealing conditions.

Hybridisisation of ^{3}H - methylated 9s RNA to DNA s isolated from different tissues.

The partial saturation curves for the hybridisation of ^{3}H - methylated 9s RNA to DNAs isolated from different tissues are shown in Figure 47. The DNAs were isolated as described in the methods and were purified by isopycnic caesium chloride centrifugation. The same preparation of ^{3}H - methylated 9s RNA was used in each experiment. Both the hybridisation conditions and the washing of hybridised filters were standardised so as to eliminate any small variations in bybridisation levels caused by different experimental procedures.

Saturation plateau were approached at RNA/DNA ratios of 1. The curves for total mouse embryo DNA and for mouse sperm DNA were similar. As the levels of counts on blank filters were reproducibly low in each hybridisation, the varying hybridisation levels for the other DNA preparations could not be attributed to differences in the subtracted blank values. Double reciprocal plots for the saturation curves are shown in Figure 48. The

TABLE 7.

Comparison of ^{3}H - methylated 9s RNA hybridisation to E. coli DNA and to total mouse embryo DNA.

Input RNA (µg)	cpm/filter		E.coli DNA / mouse embryo DNA (%)
	mouse embyro DNA	E.coli DNA	
6.76	45, 51	3, 0	6.1
20.28	105, 107	8, 4	5.5

Hybridisation was in 0.2 mls 50% formamide/4 x SSC at 36.5°C. Two filters loaded with mouse embyro DNA and two with E. coli DNA. After hybridisation, the filters were processed as usual.

FIGURE 47.

Partial saturation curves of ^{3}H - methylated 9s RNA to DNAs isolated from different sources. The DNAs were purified by isopycnic caesium chloride centrifugation.

The specific activity of the ^{3}H - methylated 9s RNA was 4480 cpm/ug. The same preparation of 9s RNA was used in each hybridisation. Incubations were in 0.2 mls 50% formamide/2 x SSC at varying RNA inputs at 36.5°C for 16 hours. Each incubation vial contained 2 identical filters containing 20µg DNA/filter and 2 blank filters.

△ ——— △ total mouse embryo DNA.

▲ ——— ▲ mouse sperm DNA.

○ ——— ○ 14 day mouse embryo liver DNA.

x ——— x duck reticulocyte DNA.

FIGURE 48.

Double reciprocal plots of the data from Figure 47. At infinite RNA inputs, the saturation values are

Total mouse embryo DNA : 0.084%

mouse sperm DNA : 0.088%

14 day mouse embryo liver DNA : 0.176%

duck reticulocyte DNA : 0.352%

Figure 47.

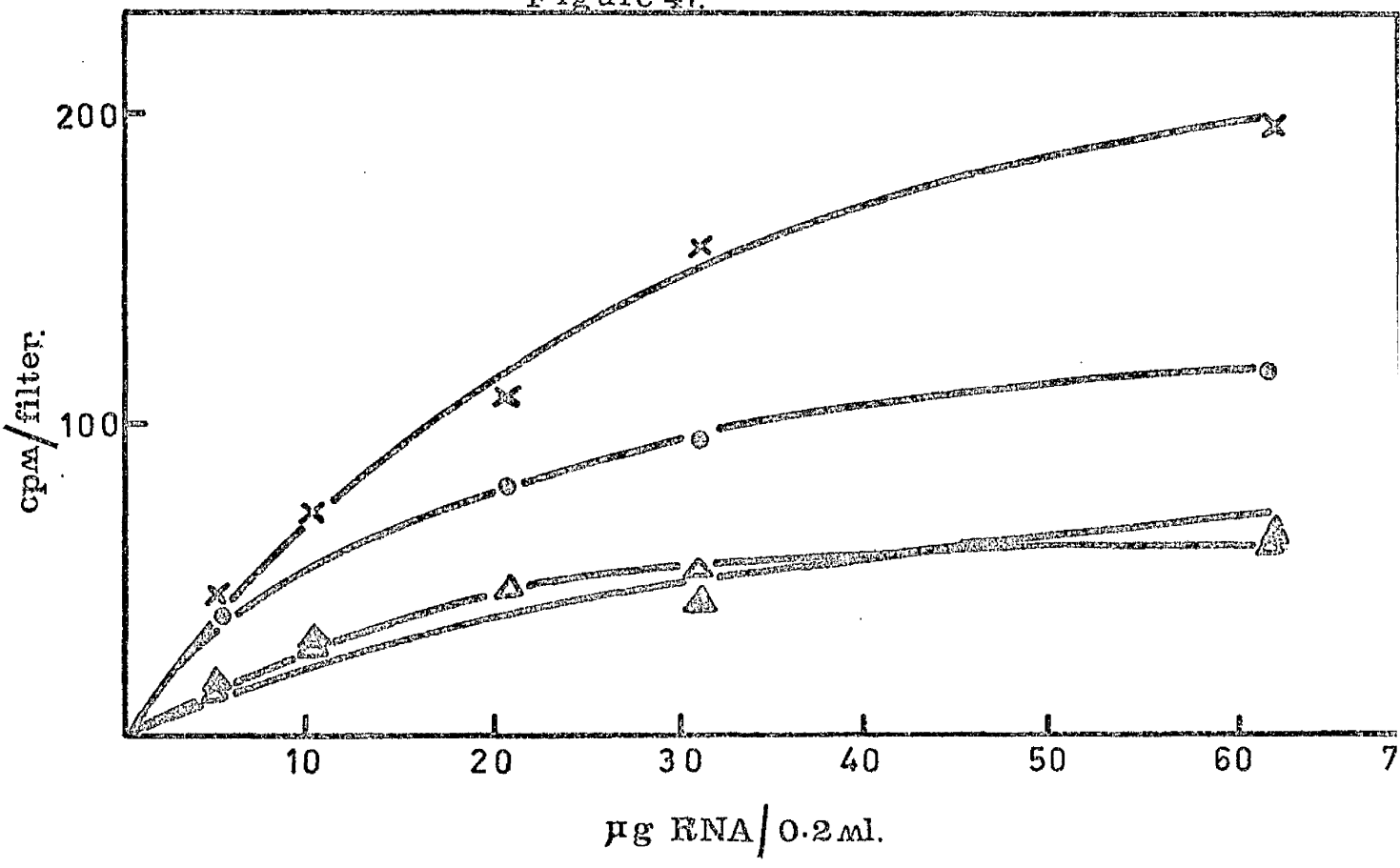
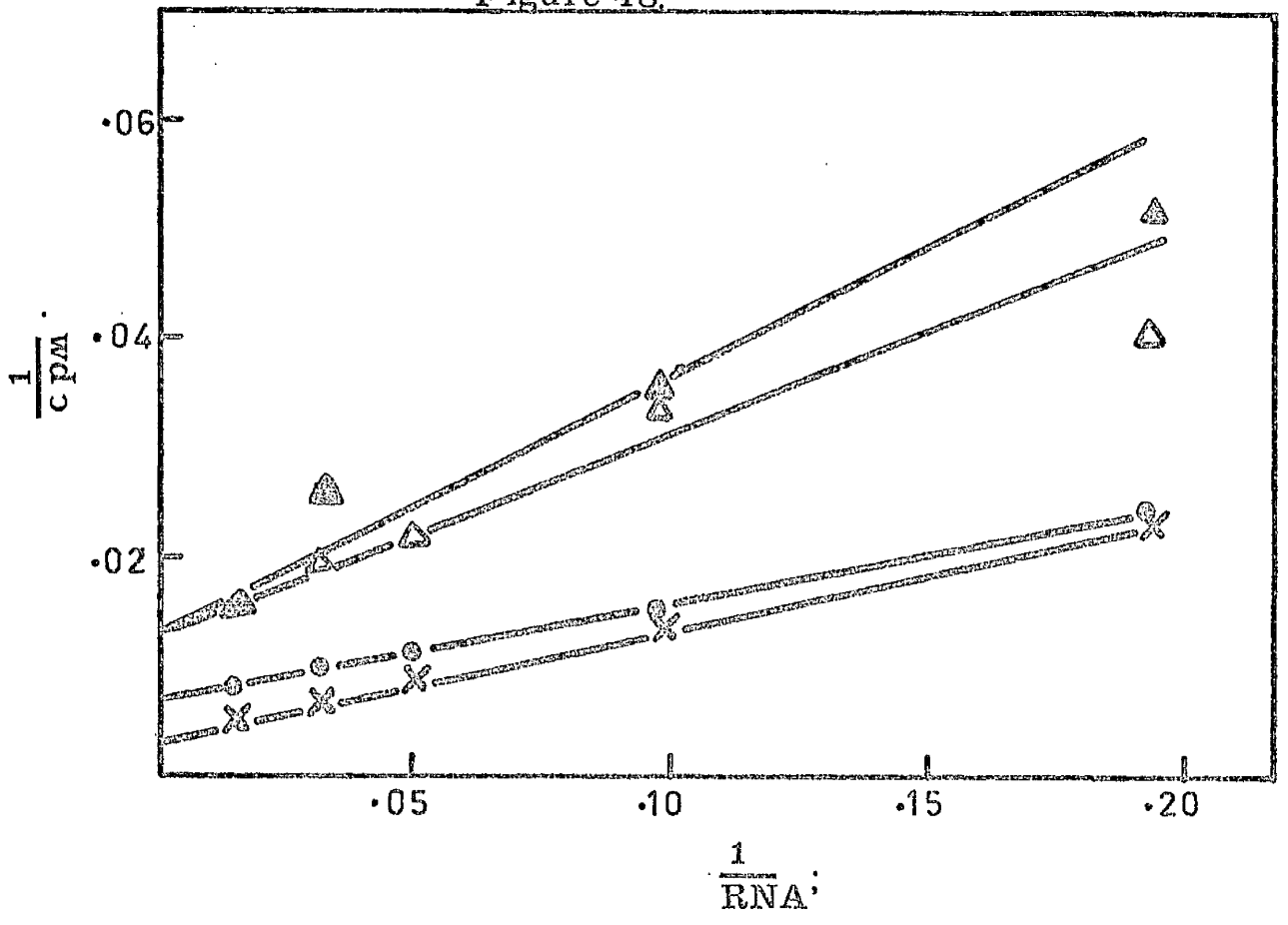


Figure 48.



intercepts at infinite RNA inputs were corrected for 12% DNA loss during hybridisation. The numbers of DNA cistrons in the haploid genomes coding for an RNA of molecular weight 170,000 were calculated from the saturation values (Table 8).

3.9 Effect of varying incubation conditions on the hybridisation conditions on the hybridisation of ^{3}H - methylated 9s RNA.

Hybridisation at different temperatures.

The effect of varying temperature on the hybridisation of ^{3}H - methylated 9s RNA to DNA is shown in Figure 49. The levels of hybridisation were similar between 25°C and 45°C. There was a drop of 48% between the hybridisation levels at 45°C and 55°C, but little further decrease between 55°C and 65°C. The non-specific binding to blank filters rose with increasing temperature; this was 5 times higher at 65°C than it was at 36.5°C. The variation was not sufficient to account for the decrease in hybridisation to DNA filters.

A preliminary partial saturation curve for the hybridisation of ^{3}H - methylated 9s RNA to DNA at 54°C indicated that the approach to saturation was much more rapid and occurred at lower RNA/DNA inputs than the hybridisation at 36.5°C. The curve began to plateau at about one third of the saturation level at 36.5°C.

Hybridisation levels at different formamide concentrations.

The effect of formamide concentration on the level of

TABLE 8.

Multiplicity of cistrons coding for an RNA of molecular weight 170,000 in different haploid genomes.

Source of DNA	Mass DNA/ haploid genome (Daltons)	% DNA complementary to RNA	Multiplicity of cistrons/haploid genome
16 day total mouse embryo DNA	1.5×10^{12}	0.084	3,730
mouse sperm DNA	1.5×10^{12}	0.088	3,890
14 day mouse embryo liver DNA	1.5×10^{12}	0.176	7,750
duck reticulocyte DNA	0.78×10^{12}	0.352	8,100

The mass of DNA in a diploid mouse cell was taken as 3×10^{12} daltons (Vendrely and Vendrely, 1949) and that in a diploid duck cell as 1.56×10^{12} daltons (Mirsky and Ris, 1951).

FIGURE 49

Hybridisation levels of ^{3}H - methylated 9s RNA at different temperatures.

9.1 µg ^{3}H - methylated 9s RNA (3470 cpm/µg) were incubated in 0.2 mls 50% formamide/4 x SSC at different temperatures for 16 hours. Each vial contained 2 filters with 20µg each of total mouse embryo DNA, purified by isopycnic caesium chloride centrifugation, and two blank filters.

FIGURE 50.

Hybridisation levels of ^{3}H - methylated 9s RNA at different formamide concentrations.

20.28µg ^{3}H - methylated 9s RNA (2938cpm/ug) were incubated in 0.2 mls 4 x SSC and varying formamide concentrations at 36.5°C for 16 hours. Each vial contained 2 filters with 20µg each of total mouse embryo DNA, purified by isopycnic caesium chloride centrifugation, and 2 blank filters.

Figure 49.

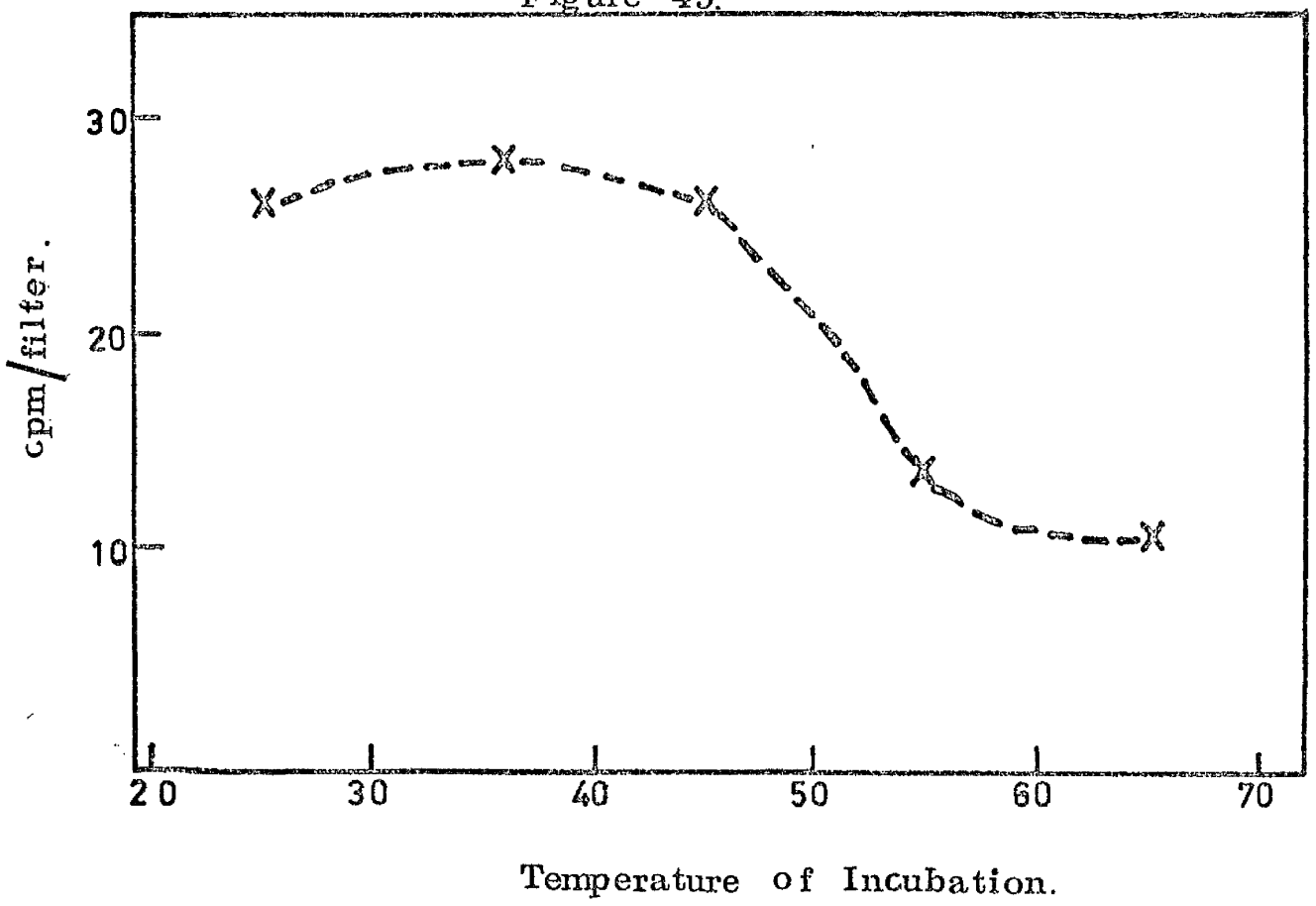
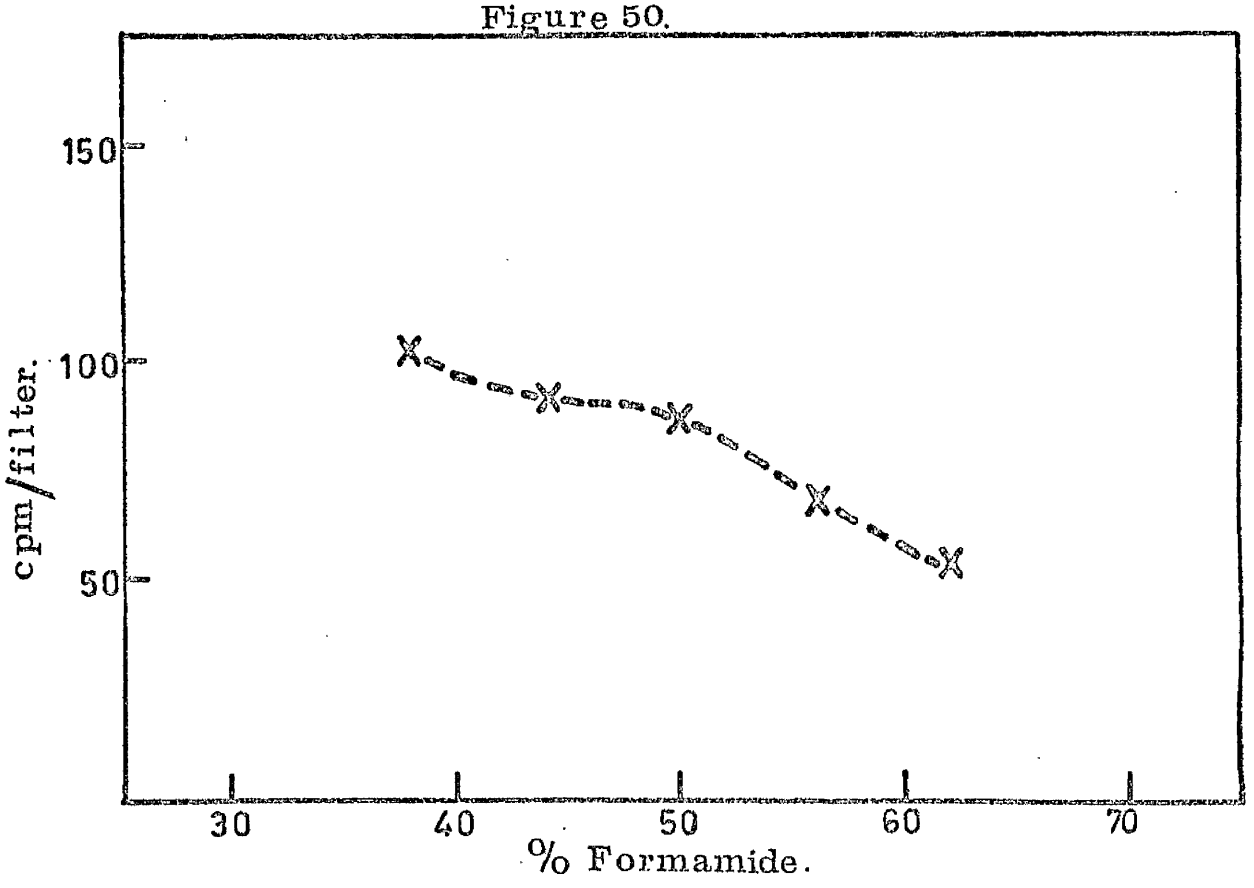


Figure 50.



hybridisation of ^{3}H - methylated 9s RNA to DNA is shown in Figure 50. An increase in formamide concentration from 50% to 74% reduced the hybridisation level by 50%. This increase in formamide concentratio is equivalent to an increase in hybridisation temperature of 17°C, assuming that an increase in formamide concentration of 1% is equivalent to raising the temperature 0.7°C (McConaughy et al., 1969) The non-specific binding to blank filters was relatively constant at the different formamide concentrations.

Time course of hybridisation.

Equivalent amounts of ^{3}H - methylated 9s RNA were hybridised to DNA for varying times (Figure 51). The hybridisation was very rapid and had reached a maximum after 3 minutes. The level of hybridisation did not increase with incubation times up to 70 hours. A similar result was obtained when 20.28µg ^{3}H - methylated 9s RNA was hybridised to DNA for different times.

In both cases, the levels of hybridisation attained correspond to those on the 16 hour saturation curve at equivalent RNA/DNA ratios. The hybridisation did not therefore increase with time towards the eventual saturation level obtained at higher RNA/DNA ratios.

Competition with unlabelled RNAs.

a) Ribosomal RNAs and 9s RNA isolated from EDTA - treated polysomes.

20.25µg ^{3}H - 9s RNA was incubated in the presence of unlabelled ribosomal RNAs or unlabelled 9s RNA at the concentrations

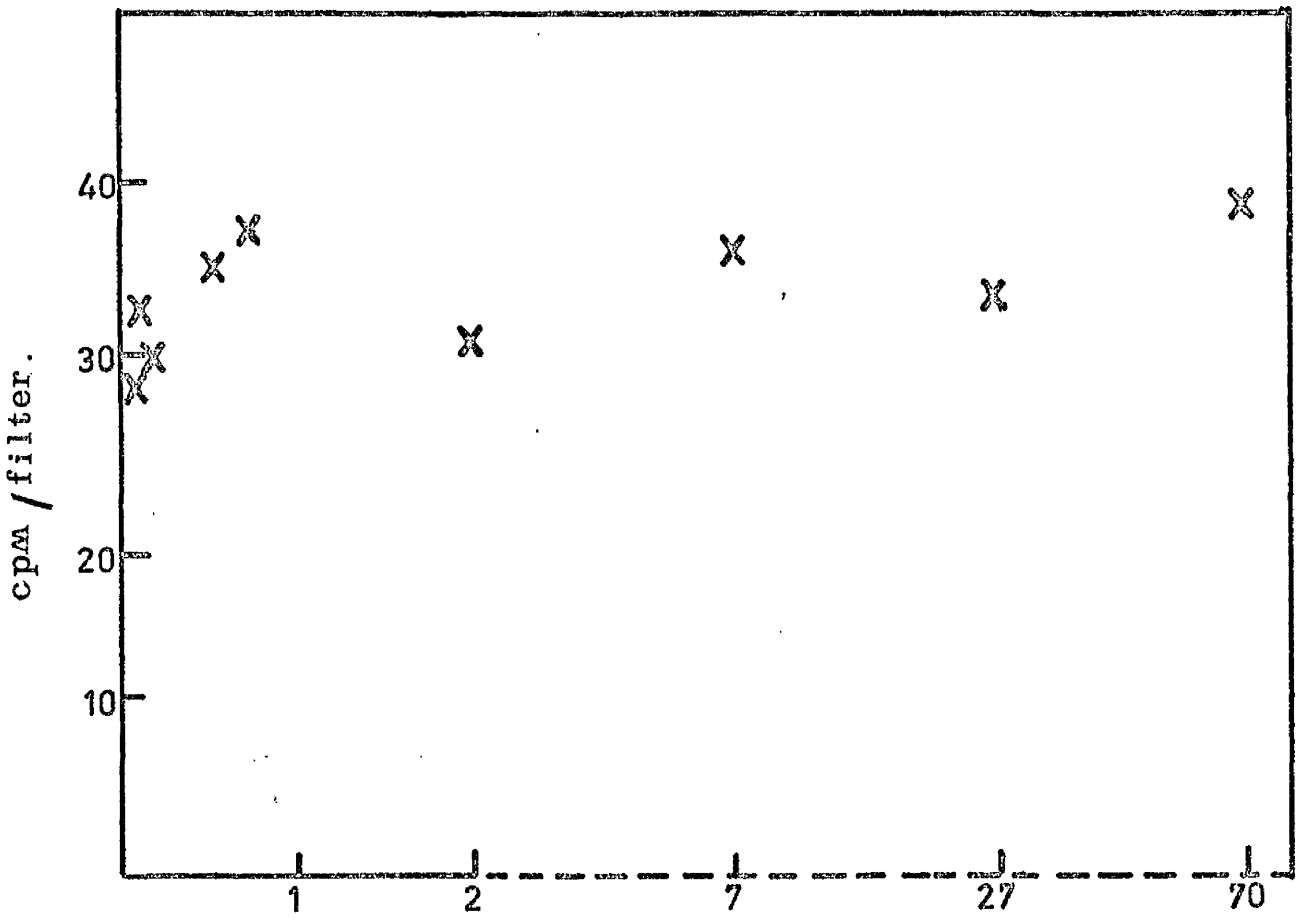
FIGURE 51.

Time course of hybridisation of ^{3}H - methylated 9s RNA.

Each vial contained 9.1µg ^{3}H - methylated 9s RNA (3470 cpm/µg) in 0.2 mls 50% formamide/4 x SSC. Two filters containing 20µg total mouse embryo DNA, purified by isopycnic caesium chloride centrifugation, and two blank filters were incubated in each vial. All components of the reaction mixtures were pre-equilibrated separately at 36.5°C.

Hybridisations were for the specified times at 36.5°C.

Figure 51.



Hours Incubation.

shown (Figure 52). The competition curve with unlabelled 9s RNA shows decreasing hybridisation as the input of unlabelled 9s RNA increases. The competition curve with unlabelled rRNAs shows a sharp drop at low rRNA inputs. The curve levels off at high rRNA inputs. This type of competition curve may be expected in the competition of RNA species such as the rRNAs, which saturate their DNA sites at relatively low RNA inputs, with an RNA such as the ^{3}H - 9s RNA which reaches saturation only at very high RNA/DNA ratios.

b) Theoretical competition curve for 9s RNA.

The competition curves in (a) were performed at subsaturating levels of ^{3}H-9s RNA. Corrections were made to determine the theoretical curve for the competition of an identical unlabelled RNA species at saturating inputs of ^{3}H - 9s RNA. A saturation curve for ^{3}H - 9s RNA was determined by calculating the statistically best fit for the points on the experimental saturation curve. The theoretical competition curve for an identical RNA species was then generated, taking 0% competition at the point on the saturating curve corresponding to 20.25µg input ^{3}H - 9s RNA. The experimental competition points, corrected to saturating inputs of ^{3}H - 9s RNA, lay on the theoretical competition curve (Figure 53), showing more than 9 homology between the two RNA species in the double reciprocal plot (Figu

Repeated hybridisation.

Several identical vials each containing 9.1 µg ^{3}H - 9s RNA were incubated under the usual conditions of hybridisation for 16 hours. The filters were processed and counted. The incubation

FIGURE 52.

Competition of ^{3}H - methylated 9s RNA (2,179cpm/µg) with unlabelled 9s RNA, isolated from the 14s mRNP dissociated from EDTA - treated polysomes, and with unlabelled rRNA isolated from mouse reticulocytes.

The hybridisations were under standard conditions with increasing amounts of the cold RNAs added to vials containing 20.1µg ^{3}H - methylated 9s RNA/0.2 mls 50% formamide/4 x SSC.

x ——— x Theoretical saturation curve for ^{3}H - methylated 9s RNA.

o ——— o Competition with 9s RNA.

△ ——— △ Competition with rRNA.

Figure 52.

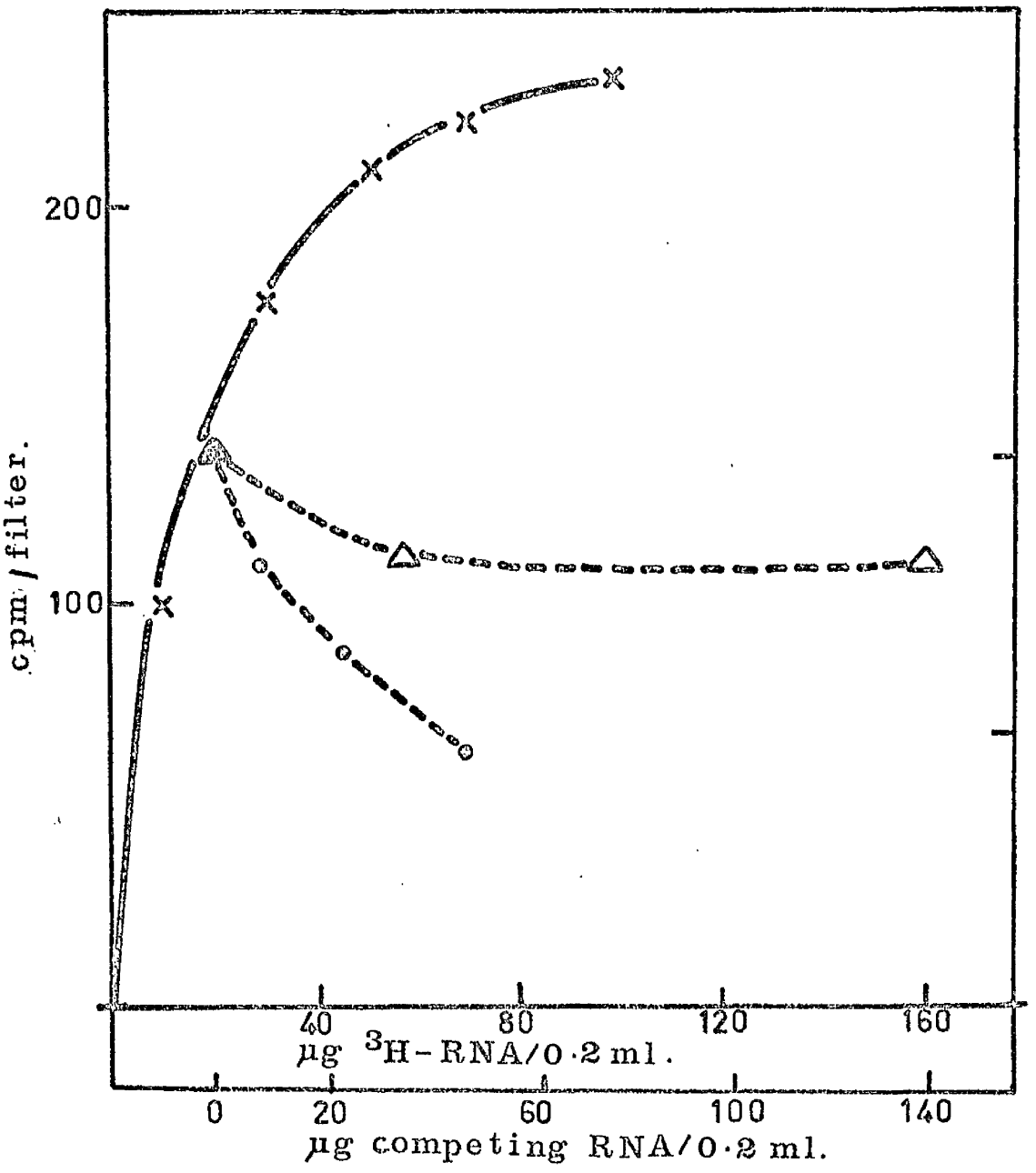

200
100
cpm / filter.
40
80
120
160
µg ³H-RNA/0·2 ml.
0
20
60
100
140
µg competing RNA/0·2 ml.

FIGURE 53.

Competition of ^{3}H - methylated 9s RNA (2,179cpm/µg) with unlabelled 9s RNA isolated from the 14s mRNP, released with EDTA treatment of reticulocyte polysomes.

The data from Figure 52 were corrected for competition with an identical 9s RNA at saturating inputs of ^{3}H - methylated 9s RNA.

——————— theoretical competition curve.

The experimental competition values were also corrected to saturing hybridisation conditions for ^{3}H - methylated 9s RNA (X).

FIGURE 54.

Double reciprocal plot for the data of Figure 53. The intercept on the Y axis gives > 95% homology between the 2 RNA species.

Figure 53.

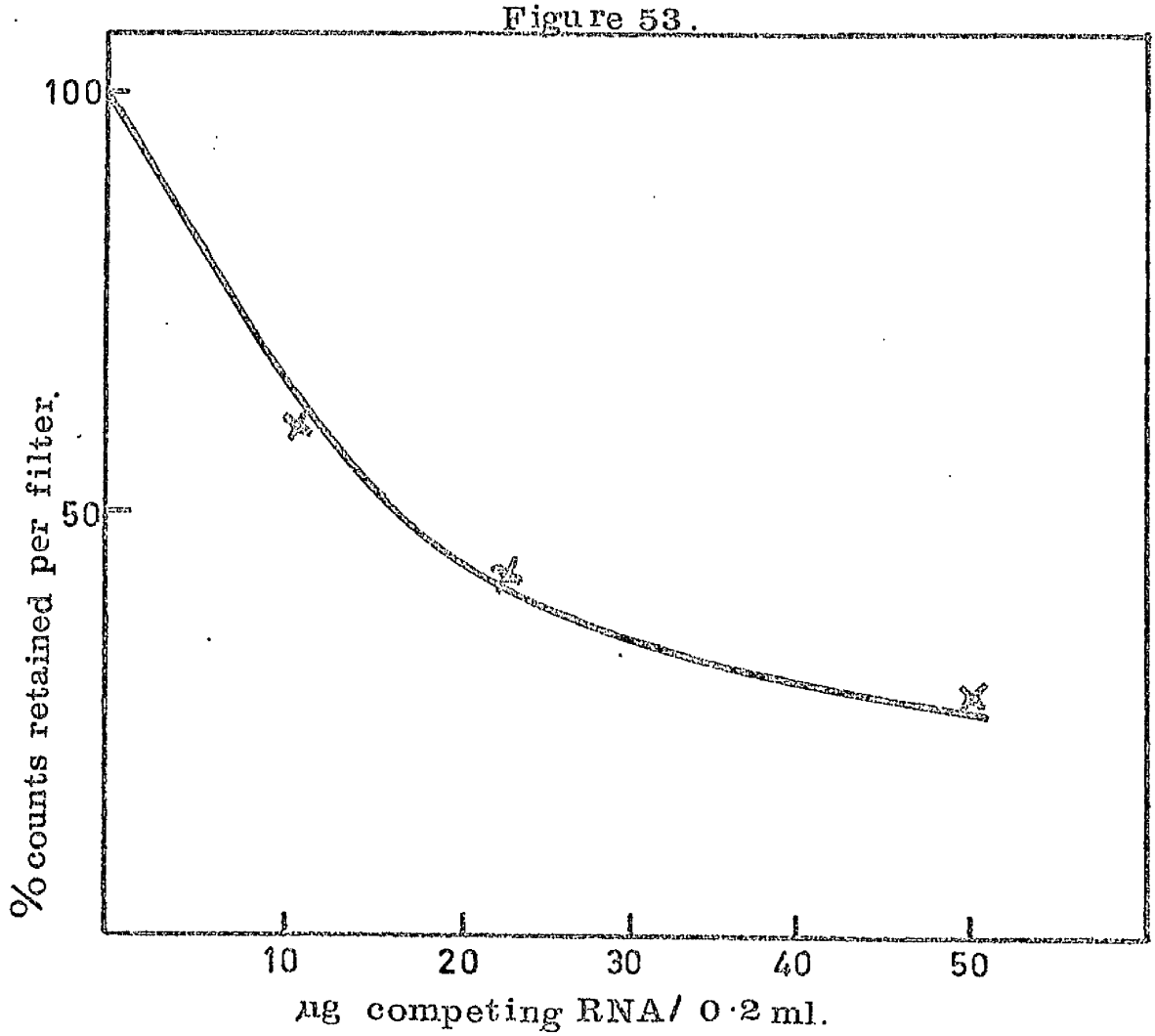
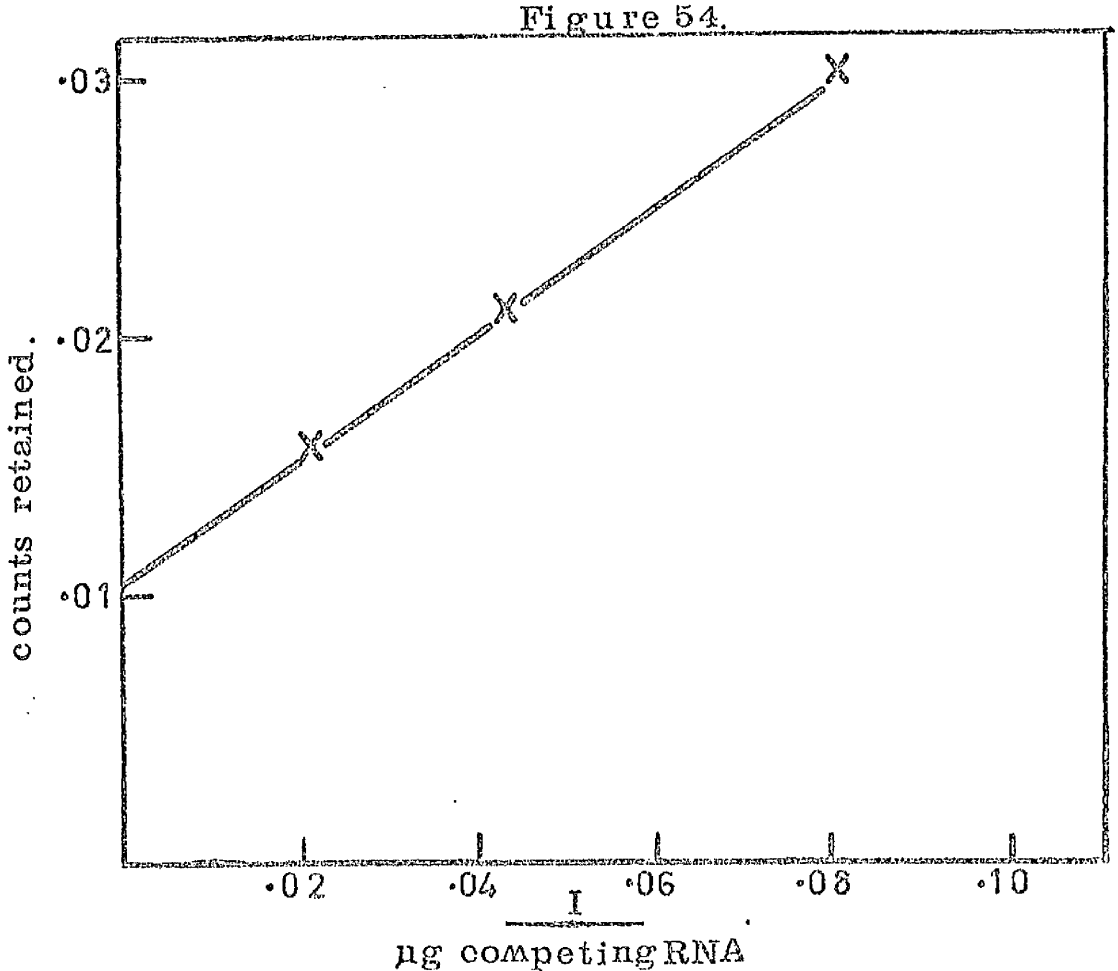


Figure 54.



mixtures were pooled and incubated with DNA and blank filters for a further 16 hours under identical conditions. This procedure was repeated once more. The results showed that the first rehybridisati gave the same level of hybrid formation as in the first incubation. This result eliminates the possibility that the saturation curves in previous experiments were caused by the exhaustion of a small percentage of annealing RNA in the RNA incubation mixture.

The second rehybridisation gave 50% of the initial levels of hybrid formation. This result was not due to a variation in the SSC or RNA concentrations since there was no evaporation at 36.5°C. When a hybridisation medium was pre-incubated for 36 hours before introduction of the DNA filters, the hybrid formed was only 47% of the value for the corresponding hybrid formed during the usual 16 hour incubation. The eventual fall in the level of RNA rehybridising might therefore be due a change in the hybridisation properties of ^{3}H - 9s RNA after 36 hours incubation rather than to the exhaustion of a minor RNA component.

The decrease in the level of hybridisation was not due to absorption of the methylated RNA to the plastic vial since the levelsof radioactivity in the incubation medium were identical before and after hybridisation.

A further possibility was that the RNA had lost its methyl label during incubation. To check this, vials containing varying concentrations of ^{3}H - methylated 9s RNA were incubated for varying times in 50% formamide/4 x SSC at 36.5°C. Aliquots of the incubated

RNAs were analysed on 2.6% polyacrylamide gels. Total mouse RNA was analysed as a control. The results show (Figure 55) that the optical density profiles and the radioactivity profiles were superimposed. Further, there was no drop in the specific activities of the RNAs which had been incubated for longer times. No loss of ^{3}H - methyl groups from the RNA was therefore detected. However, the migration of the methylated RNA was affected by incubation. The ^{3}H - methylated 9s RNA stored at -20°C for 6 weeks had a peak at 9s but there were also counts and optical density in the 12s to 18s region of the gel. Incubation of the ^{3}H - 9s RNA in 4 x SSC/5 formamide at 36.5°C for 18 hours, 36 hours and 70 hours resulted in an increased aggregation of the RNA. After incubation for 70 hours, the counts and the optical density peak ran behind the 28s RNA marker Increasing the RNA concentration during incubation did not affect the degree of aggregation. There was some decrease in aggregation when 0.1% SDS was added to a formamide incubation at 36.5°C and also when the methylated RNA was incubated at 67°C in 2 x SSC/0.1% SDS. Similar results were obtained with another ^{3}H - methylated 9s RNA preparation and a ^{3}H - methylated 5s RNA.

The effect of incubating ^{32}P - 28s RNA under various conditions is shown in Figure 56. After 36 hours incubation, the RNA was degraded to between 18s and 9s, whether it had been incubated in 2 x SSC at 67°C or in 4 x SSC/50% formamide at 36.5°C. After 48 hours, most of the RNA incubated at 67°C ran in the 9 - 4s region but there was no further breakdown of the RNA incubated in

FIGURE 55.

Effect on ^{3}H - methylated 9s RNA of incubation under various conditions at a concentration of 9µg/0.2ml. Different loadings of ^{3}H - 9s - RNA were applied/gel.

(a) Standard total mouse reticulocyte RNA with ^{3}H - 9s RNA immediately after methylation.

(b) ^{3}H - 9s RNA stored at -20°C for 6 weeks in 50% formamide/4 x SSC.

(c) ^{3}H - 9s RNA incubated in 50% formamide/4 x SSC for 16 hours at 36.5°C.

(d) ^{3}H - 9s RNA incubated in 50% formamide/4 x SSC for 32 hours at 36.5°C.

(e) ^{3}H - 9s RNA incubated in 50% formamide/4 x SSC for 16 hours then in 50% formamide/4 x SSC/0.1% SDS for 16 hours.

(f) ^{3}H - 9s RNA incubated in 4 x SSC/50% formamide for 70 hours at 36.5°C.

Electrophoresis was on 2.6% analytical polyacrylamide gels for 20 minutes at 3v/cm then for 1 hour at 10v/cm.

Electrophoresis was at 4°C.

_____, E_{260} ; _____ cpm.

Figure 55.

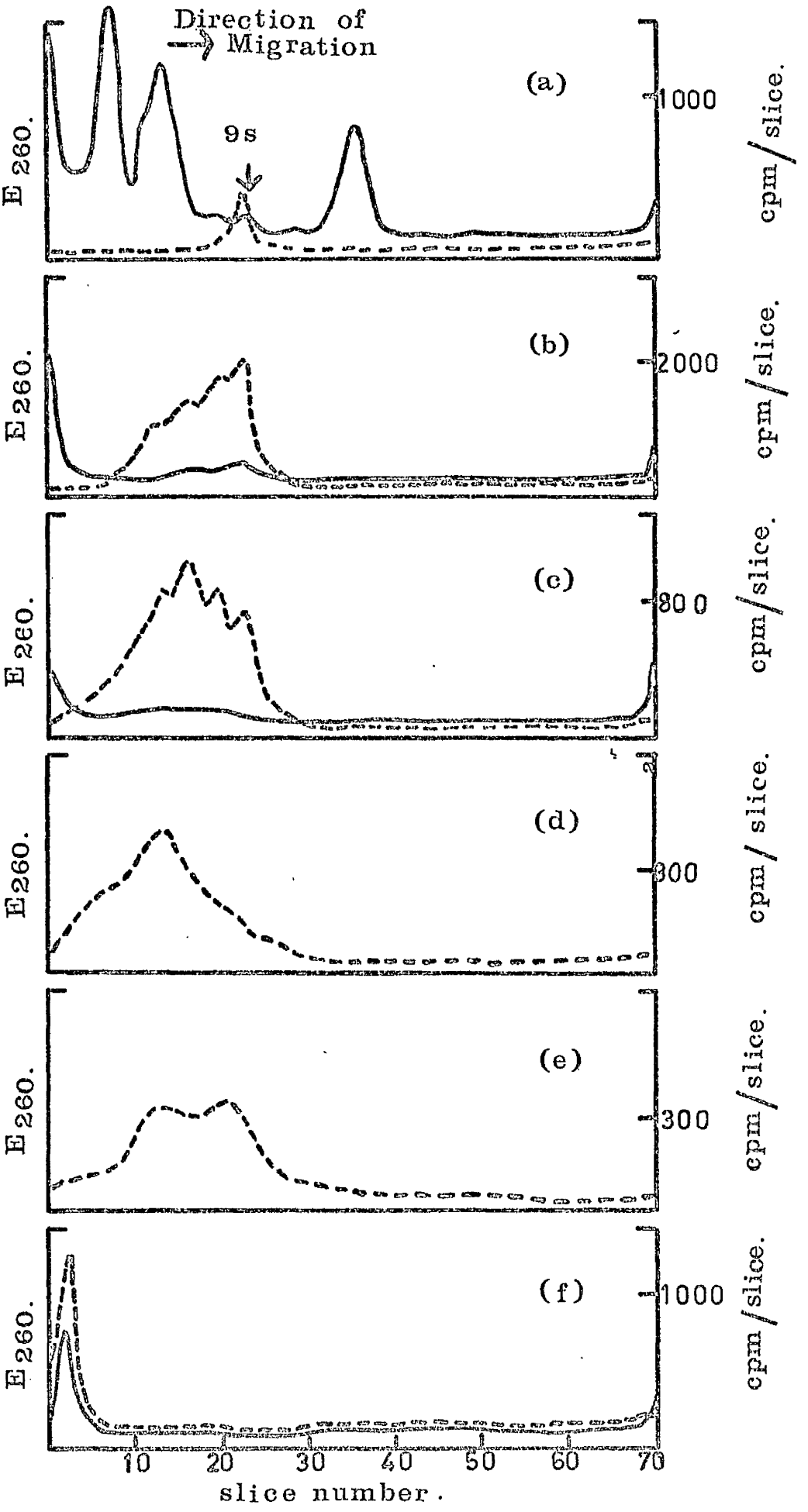

Direction of
Migration
(a)
9s
E 260.
1000
cpm/slice.
(b)
2000
(c)
800
(d)
300
(e)
300
(f)
1000
10
20
30
40
50
60
70
slice number.

FIGURE 56.

Effect on ^{32}P - 28s RNA of incubation under various conditions at a concentration of 0.6µg/0.2 ml.

Different loadings of ^{32}P - 28s RNA were applied/gel.

a) Standard total mouse reticulocyte RNA and ^{32}P - 28s RNA.

b) ^{32}P - 28s RNA incubated in 2 x SSC/0.1% SDS at 67°C or in 2 x SSC/50% formamide for 32 hours.

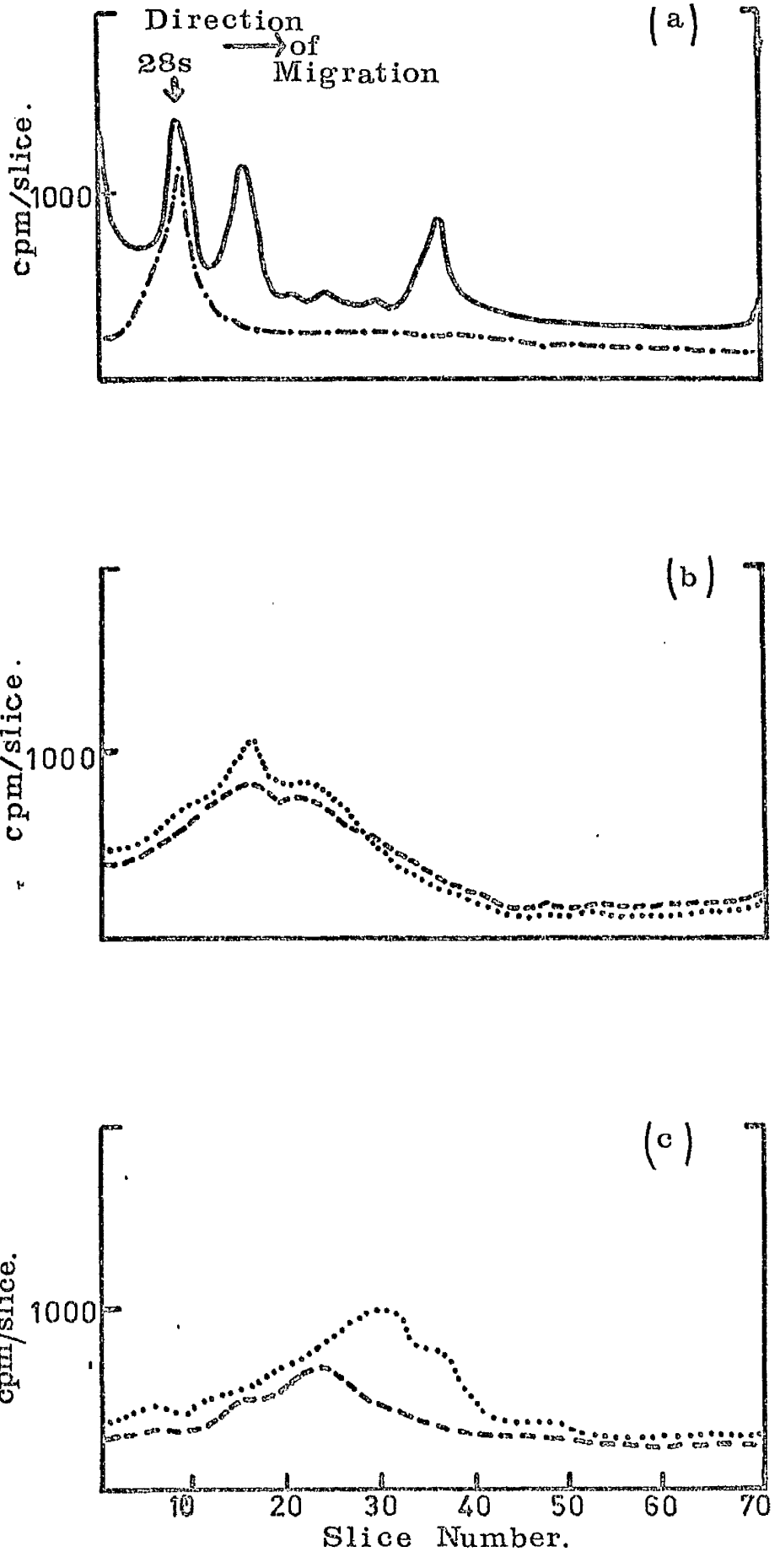
c) ^{32}P - 28s RNA incubated in 2 x SSC/0.1% SDS at 67°C or in 2 x SSC/50% formamide for 48 hours.

Electrophoresis was in 2.6% analytical polyacrylamide gels for 20 minutes at 3v/cm then for 1 hour at 10v/cm.

Electrophoresis was at 4°C.

———— , E_{260} ; — . — , ^{32}P - 28s RNA ; ———— , 67°C incubation; ....., 36.5°C incubation.

Figure 56.


(a)
Direction of Migration
28s
cpm/slice.
1000
(b)
cpm/slice.
1000
(c)
cpm/slice.
1000
10
20
30
40
50
60
70
Slice Number.

formamide. These results are therefore different from the results with methylated RNAs and show that aggregation is only observed when a methylated RNA is incubated in formamide.

Since maximum hybrid formation takes place after very short incubation times, the repeated hybridisation of ^{3}H - methylated 9s RNA was carried out for incubation periods of 2 hours to minimise any effects of aggregation on the hybridisation levels. The results are shown in Table 9. The identical levels of hybridisation after two incubations, each with 40µg DNA, again confirms that the saturation curve is not due to the gradual exhaustion of a minor RNA component. The level of hybridisation drops by 43% on the 4th incubation. This drop may be due to a depletion of a rapidly-hybridising RNA component present in excess during the initial hybridisations. Further incubations with DNA were impossible as the volume of remaining incubation medium was too small. The total amount of RNA hybridised to the DNA in this experiment was 0.1% of the input. This represents a minimum value for the fraction of the methylated RNA capable of hybridisation to DNA under these conditions.

3.10. Characterisation of ^{3}H - methylated 9s RNA hybrids.

Size of hybrids

The RNA was dissociated from a number of DNA filters which had been hybridised to ^{3}H - 9s RNA and subjected to the standard washing, RNasing and counting procedures. After washing in 2 x SSC

TABLE 9.

Effect of repeated hybridisation of ^{3}H - 9s RNA to DNA

Incubation number	cpm/filter
1	29
2	30.5
3	28.5
4	17.5

The first incubation was for 18 hours with 9µg ^{3}H - 9s RNA/0.2ml of 50% formamide/2 x SSC at 36.5°C. The subsequent incubations were for 2 hours under the same conditions.

2 filters, each loaded with 20µg DNA, were incubated/0.2 mls.

at room temperature, the filters were incubated in 1 ml 2 x SCC at 70 for 20 minutes. The percentage precipitability of the RNA after different treatments is shown in Table 10. Only 26% of the counts were acid-precipitable. Although there is no exact estimate of the length of polynucleotide precipitated by acid in the presence of carrier, it seems probable that about 75% of the hybridised RNA is less than 15 - 20 nucleotides long. The material dissociated from the filters was incubated in 0.3N NaOH for 2 hours at 37°C. After this treatment, no counts were acid-precipitable. The 26% of material previously precipitated was therefore RNA. To estimate the size of this fraction, RNA was dissociated from DNA filters as before. It was precipitated by the addition of 2 volumes of ethanol, stored at -20°C for 24 hours then centrifuged at 18,300g. The pellet was dissolved in electrophoresis buffer and analysed on 10% polyacrylamide gels. Cold total RNA and cytidylic acid were added as markers (Figure 57). Most of the counts ran with the 4s RNA, although there was a small shoulder over the 5s RNA region. The size of the RNA in this fraction is therefore 60-120 nucleotides.

It is possible that residual pancreatic ribonuclease on the filters causes further degradation of RNA hybrids at temperatures above 23°C. A sample of ^{3}H - 9s RNA incubated with a number of RNased DNA filters at 70°C for 20 minutes had 100% acid precipitabil both before and after the incubation. However, this experiment does not preclude a limited RNase attack on the 9s RNA. Nor does it

TABLE 10.

Precipitation of hybrid RNA

Treatment	cpm supernatant	cpm precipitate	% precipitation
Ethanol precipitation	805	360	32
Acid precipitation	1180	309	26
alkali incubation then acid precipitation	196	0	0

Dissociated hybrid RNA was precipitated by ethanol precipitation and by acid precipitation.

An aliquot of hybrid RNA was incubated in 0.3N NaOH for 2 hours at 37°C then precipitated with acid.

FIGURE 57.

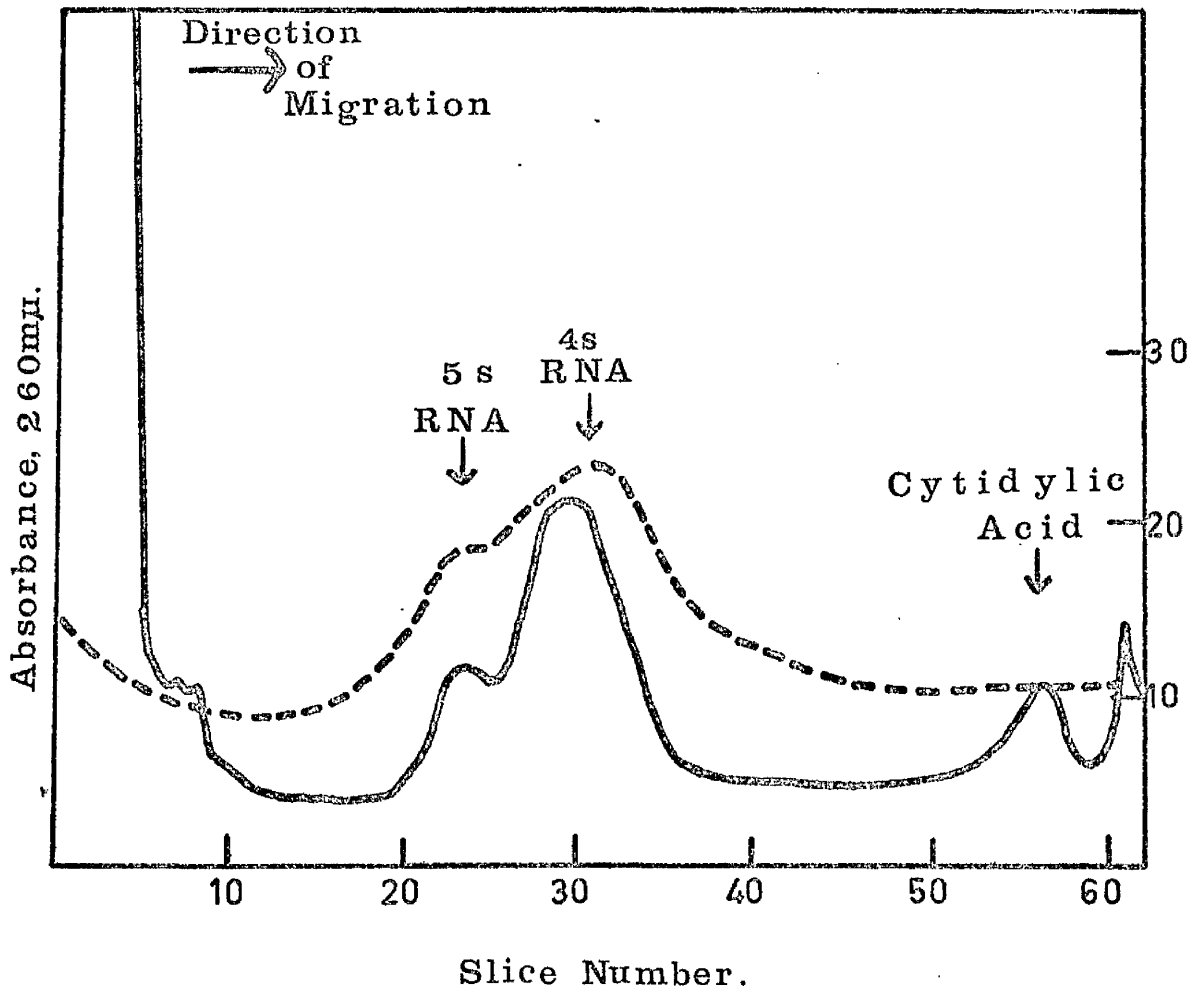
Electrophoresis of ^{3}H - 9s hybrid RNA dissociated from filters by incubation in 2 x SSC at 70°C for 20 minutes followed by precipitation with ethanol and potassium acetate as in methods.

Total cytoplasmic RNA and cytidylic acid were run as markers with the ^{3}H - RNA.

The polyacrylamide gel concentration was 10%. Electrophoresis was carried out in SDS at room temperature for 10 minutes at 3v/cm then for 1 hour at 10v/cm. The gels were sliced and counted as in methods. 4 gel slices were counted in each scintillation vial.

———, E_{260} ; ———— cpm/4 gel slices.

Figure 57.


Direction of Migration
4s RNA
5 s RNA
Cytidylic Acid
Absorbance, 260mμ.
30
20
10
10
20
30
40
50
60
Slice Number.

eliminate a preferential RNase attack on RNA hybridised to the filters rather than on RNA in solution.

A further indication that the RNased hybrids are small is that re-incubation of hybrids in the hybridisation medium under the usual conditions leads to loss of all the RNA from the filters. This indicates that the reaction is readily reversible, as is the case for the hybridisation of small oligonucleotides to DNA

Thermal melting profiles of hybrids

The thermal melting profiles of ^{3}H - methylated 9s RNA hybrids were determined for hybrids formed at the higher RNA/DNA ratios on the saturation curves. The T_m of ^{3}H - 9s RNA hybrid is 45°C in 1 x SSC (Figure 38). Since more DNA is lost from filters when incubated at high temperatures in 1 x SSC than is lost in 2 x SS all subsequent T_ms were determined in 2 x SSC. The T_m of ^{3}H - 9s RNA hybrid in 2 x SSC was raised to 52°C. This difference in T_m is expected from the relationship between the melting temperature and the sodium ion concentration.

The one thermal melting profile of non - RNased hybrid also gave a T_m of 52°C. The hybrid formed between duck reticulocyte DNA and mouse ^{3}H - 9s RNA had a T_m of 55°C (Figure 59). This heterologous hybrid therefore had a higher thermal stability than the homologous hybrid. Since the difference between the T_ms could be due to a difference in the molecular weight of the DNA, the ^{3}H - 9

FIGURE 58.

Thermal melting profiles of ^{3}H - methylated 9s RNA under different conditions. The melting profiles were determined as in methods.

x ——— x Dissociation in 1 x SSC.

o ——— o Dissociation in 2 x SSC.

△ ——— △ Dissociation of non-RNased hybrid in 2 x SSC.

FIGURE 59.

Thermal melting profiles of ^{3}H - methylated 9s RNA hybridised under different conditions.

△ ——— △ Dissociation of hybrid formed with Marmur - prepared DNA.

⊙ ——— ⊙ Dissociation of hybrid formed with duck reticulocyte DNA

x ——— x Dissociation of hybrid formed at $54^{o}C$ with caesium chloride - purified DNA.

Figure 58.

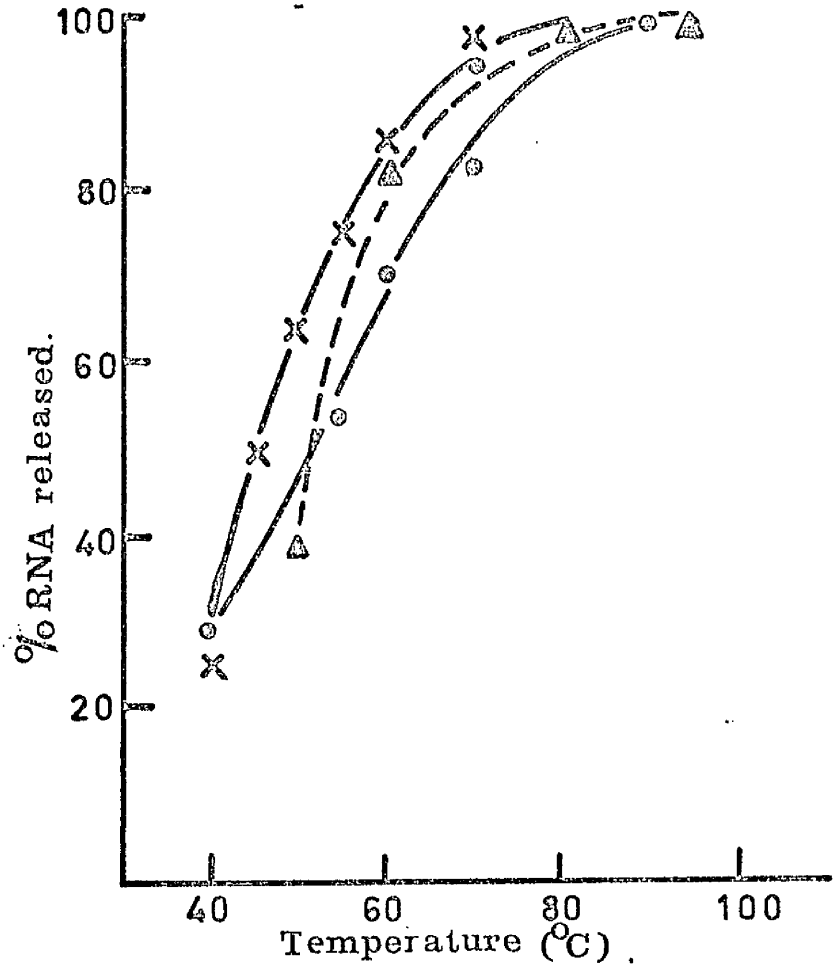
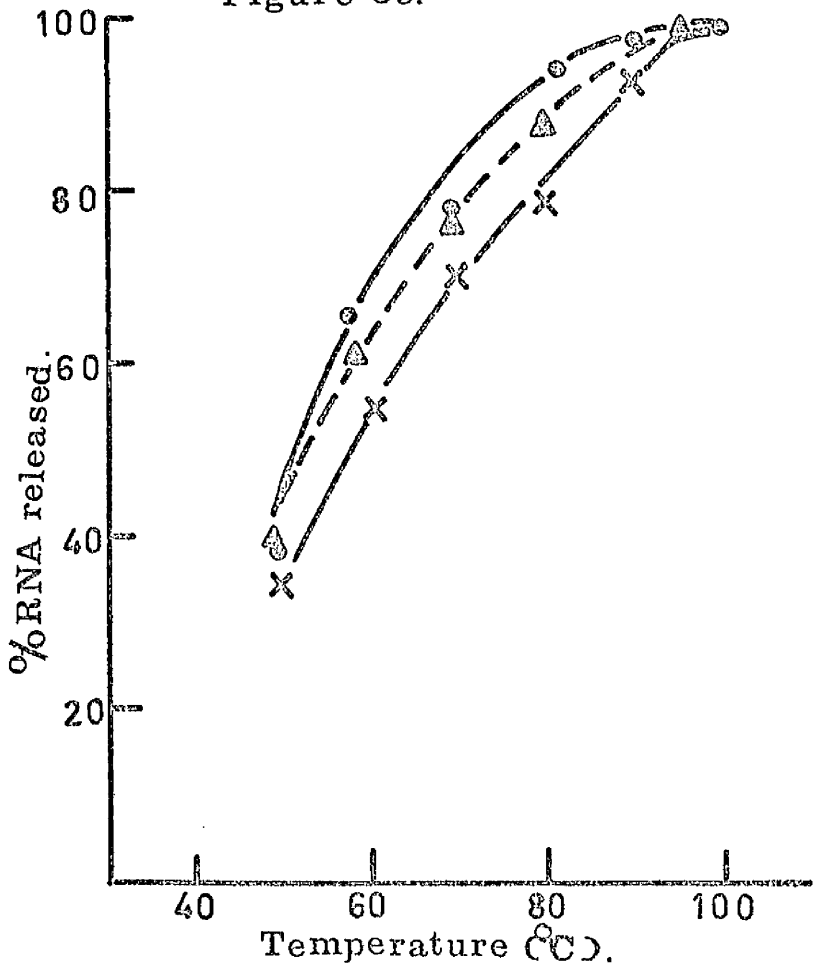


Figure 59.



RNA was hybridised, at a high RNA/DNA input, to high molecular weight mouse embryo DNA prepared by the method of Marmur. The T_m of this hybrid was also 55°C. The difference in melting temperature between the duck reticulocyte DNA and the total mouse embryo DNA was probably due to the smaller size of the mouse DNA.

Hybrids formed at 54°C in 50% formamide/2 x SSC had a T_m of 57°C. Increasing the temperature of incubation therefore increases the stability of the formed hybrids. There was no difference in the T_ms of hybrids formed during the second or third repeated hybridisations with ^3H - 9s RNA.

It is possible that the low T_ms obtained for all the RNA species tested were due to artifacts in the experimental procedure so several variations in the method were tried in preliminary experiments.

A melting curve in 50% formamide/2 x SSC also gave a very low T_m for 9s RNA. The T_m was not raised by incubation of filters with 0.1% DEP to eliminate any residual RNase activity. More thorough washing of filters in chloroform to remove residual toluene also did not change the T_m of the ^3H - 9s RNA hybrids. These experiments would have to be repeated before the possibility of such artifacts causing decreased T_ms was eliminated.

DISCUSSION.

4. DISCUSSION

4.1 Isolation and characterisation of globin mRNA.

4.2 Heterogeneity of 9s RNA from hybridisation data.

4.3 Specificity of hybridisation procedure.

4.4 Factors influencing hybridisation and T_m determinations.

4.5 Analysis of T_m values for 9s RNA.

4.6 Biological interpretations.

DISCUSSION

4.1 Isolation and characterisation of globin mRNA

The existence of a bacterial mRNA fraction, has been confirmed (Jacob and Monod, 1961a, 1961b; Brenner, 1961). The mechanisms for the mRNA - mediated information transfer have since been extensively studied (Review, Geiduschek and Haselkorn, 1969). It has been assumed that the programming of protein synthesis in eucaryotes is analogous to that in bacteria. Until recently, however, there has been no conclusive evidence for the existence of eucaryotic mRNAs. The preliminary identification of histone (Kedes and Gross, 1969), myosin (Heywood and Nwagwu, 1969) and globin mRNAs (Chantrenne et al., 1967; Williamson et al., 1969; Labrie, 1969) has now been reported.

The presumptive globin mRNA was isolated and tested for globin messenger activity in cell-free protein synthesising systems. It has now been shown that 9s RNA, prepared from mouse and rabbit reticulocytes, specifically stimulates the synthesis of the corresponding globins in cell - free protein synthesising systems (Schapira et al., 1968; Lockard and Lingrel, 1969; Laycock and Hunt, 1969). These experiments are the first proof of the validity of the messenger RNA concept in eucaryotes.

The 9s RNA fraction contains the information necessary for translating both the α and β globin chains (Lockard and Lingrel, 1 Lingrel, 1971). Since the size of 9s RNA is approximately that

required for the programming of either the α or β chains of globin, these experiments further prove that the messengers for α and β globins are monocistronic.

The identification of these mRNAs and their purification from the total reticulocyte RNA makes possible experiments on the control of mammalian protein synthesis such as those of Nienhuis et al. (1971) on the controlling factors of globin synthesis in β thalassemia and more general experiments on the specificity of protein factors in mRNA translation. Cohen, Lanyon and Williamson (1971; unpublished results) have shown messenger-specific factors in globin synthesis using mixed reticulocyte and ascites protein synthesising systems.

The absolute proportion of globin messengers in the 9s RNA fraction is not known. This cannot be deduced from the extent of stimulation of globin synthesis in cell-free systems on the addition of 9s RNA, since the efficiency of synthesis is as yet limited by factors other than the input of mRNA. The development of procedures for the isolation of milligram amounts of 9s RNA relatively enriched in globin mRNAs makes possible a more detailed analysis of these messenger components.

Many different cell types have polysome profiles with maxima in the 5 - 6 ribosome region (e.g. Penman et al., 1963; Wettstein et al., 1963). If the correlation between polysome size and length of mRNA such as in the reticulocyte is assumed, many mRNAs may therefo have sizes of 6 - 12s. This conclusion is also inferred from the kn

molecular weights for some proteins, for example histones, if it is assumed that the mRNAs are monocistronic. In order to isolate a 9s RNA fraction enriched in globin mRNA, a system in which globin is the main protein synthesised must therefore be used.

The cells used in the preliminary studies of 9s RNA isolation in this project, were cultures of 14 day mouse embryo livers in which the polynucleotides could be labelled to a high specific activity. Only approximately seventy percent of 14 day foetal liver cells are erythropoetic, but this proportion was increased by harvesting only cells in suspension culture. This eliminates most of the fibroblasts which stick to the walls of the culture vessel (Conkie and Williamson, unpublished results). The livers contain a population of erythroid cells in varying stages of maturation. Since the label will be incorporated into the polynucleotides from the more immature cells, it was possible that much of the radioactivity incorporated into the 9s RNA material was into messengers for other proteins such as the histones.

The reticulocyte present in the circulating blood is more differentiated in that >90% of the protein synthesised is globin (Dintzis, 1958). Further, the absence of non-globin protein synthesis in cells treated with actinomycin D (Fantoni et al., 1968) demonstrates the relative instability of other mRNA species in the more mature erythroid cells. The high proportion of globin mRNA to non-globin messengers therefore made the reticulocyte a more suitable isolation material, although the 9s RNA could only be labelled to a very low

extent in vivo (Chantrenne et al., 1967; Williamson et al., 1971).

The number of globins synthesised by different strains of mice is variable. The Porton strain was chosen in these experiments since this has only two major globin components. Induction of reticulocytosis in these mice gave up to eighty percent reticulocytes in the peripheral circulation. Since the proportion of more immature erythroid cells is very low, the 9s RNA region in a total RNA preparation should not contain unstable non-globin mRNAs more likely to be present in the immature cells.

Contamination of the 9s RNA with degradation products of rRNAs is possible. The low levels of endogenous ribonuclease, however, facilitate the isolation of undegraded RNA species from reticulocytes. The sterilisation of solutions with DEP and the short isolation times necessary for preparation of the 9s RNA also minimise RNase-mediated breakdown of the RNA; the proportion of 9s and 12s RNAs to the rRNAs were the same before and after zonal ultracentrifugation. No detectable breakdown of either the rRNAs or the 12s RNA to 9s RNA had therefore occurred.

The evidence from limited pancreatic ribonuclease breakdown of rabbit 18s and 28s RNAs suggests a selective cleavage of the RNAs at a small number of RNA sites, since this results in a number of discrete RNA bands on 5% polyacrylamide gels (Gould, 1966). One of the first products of 18s RNA breakdown is an RNA running in the 12s region. The presence of a 12s RNA component in reticulocyte preparations might therefore suggest that limited RNA breakdown had

occurred. More extensive RNase action resulted in degradation products not only in the 9s RNA region but also between 9s RNA and 5s RNA (Gould, 1966). These bands were never observed in a typical RNA preparation from mouse reticulocytes. The 7s RNA intermediate between the 9s RNA and 5s RNA species is probably similar to that found associated with rabbit reticulocyte 28s RNA *in vivo* (King and Gould, 1970).

About twenty percent of the anaemic blood contains mature erythrocytes and the reticulocyte fraction itself must consist of reticulocytes at varying stages of maturity. The details of the process whereby reticulocytes mature into erythrocytes, with concomitant loss of polysomes and protein synthetic activity, are unknown; more mature reticulocytes contain fewer polysomes (Rowley, 1965) and the polysomal RNA is eventually degraded into low molecular weight fragments which appear as nucleotides in the extracellular fluid (Bertles and Beck, 1962). It is therefore possible that breakdown products of polysomes from older reticulocytes include the 12s RNA and minor RNA components in the 9s RNA region. The labelling pattern of the 12s RNA is consistent with it being an rRNA breakdown product of this kind (Williamson *et al.*, 1971). RNA prepared from the post - 18,300g supernatant fraction from reticulocyte would include any such breakdown products. These would also contaminate RNA isolated from the polysomes if the rRNA were initially degraded within the ribosomes, although not if the degraded RNA were free in the cytoplasm.

Analysis of the 9s RNA fractions on 6% polyacrylamide gels showed little difference between the 9s RNA region of total polysomal RNA and the 9s RNA cuts isolated from total cytoplasmic RNA by zonal ultracentrifugation. The proportions of the two major RNA bands were more similar in different 9s RNA preparations than were the relative amounts of minor RNA components or the degree of background staining. The 9s RNA in the polysomes seemed to have a smaller proportion of minor bands than the isolated 9s RNAs, although different loadings on the gels make this conclusion tentative.

The two main bands in the 9s RNA region have equal and high specific activities compared to rRNA when the RNA is isolated from anaemic mice injected with ^{32}P-orthophosphate 22 hours before collection of the blood. The labelling pattern therefore suggests that these bands are the α and β globin mRNAs. Their relative migration on the 6% polyacrylamide gels is consistent with the calculated difference of about 5,000 daltons between the coding portions of α and β globin mRNAs. The extraction of the individual bands from 6% polyacrylamide gels for testing protein synthetic activities in the duck reticulocyte cell-free system of Lingrel (1971) will determine whether they do form the α and β globins.

The combination of the zonal techniques already developed for the preparation of 9s RNA and the further fractionation of this RNA on 6% polyacrylamide gels should make possible the isolation of much increased quantities of the two major bands. If they are proven to be the individual globin messengers, such quantitative isolation

of highly-purified mRNAs would make possible more definitive experiments on the mechanisms of globin synthesis, the hybridisation of an mRNA or its sequencing.

Although the estimates of the molecular weight of the 9s RNA in the literature vary widely, they all give values greater than that necessary to code for the globins (Gierer, 1963; Chantrenne et al., 1967; Labrie, 1969; Gaskill and Kabat, 1971; Blobel 1971). These molecular weight determinations are either using sucrose gradient centrifugation or polyacrylamide gel electrophoresis. The determinations by sucrose gradient centrifugation depend on assumptions on the secondary and tertiary configurations of the RNA. Estimates by relative electrophoretic mobilities on polyacrylamide gels assume the mobility of the 9s RNA has a similar dependence on molecular weight to the rRNA markers. This assumption is not always justified, especially with RNAs with low GC contents in low salt buffers or with RNAs in which there are long stretches of base paired double helical regions (Loening, 1969). Further inconsistencie arise from the differing molecular weights assumed for the marker rRNA species themselves.

The molecular weight of the 9s RNA may therefore be determined most accurately when any secondary structure is eliminated. The value obtained using the method of Boedtker (1968) is 170,000 ± 13,000. By this estimate, the RNA has about 70 nucleotides more than is necess to code for the globin proteins.

This is the first conclusive evidence that a mammalian RNA

has a non-coding region. Such extra regions, 60-120 nucleotides long, have been documented and sequenced in several related phage RNAs (Steitz, 1969; Billeter et al., 1969; Adams and Cory, 1970; De Wachter et al., 1971). These non-coding regions are before the initiation codo at the 5′ end of the RNAs. They are very similar in related phages and may be essential for the correct in vivo translation of the RNAs. The extra sequences in the 9s RNA may have a similar function. They may alternatively be necessary for the transport of the mRNA from the nucleus to the cytoplasm, or have a "ticketing" function (Sussman, 1970; Lim and Canellakis, 1970).

The 9s RNA may also be similar to the phage RNAs in having some secondary structure (Williamson et al., 1971). The phage RNAs have a large percentage of secondary structure with loops of 20 base pairs in the coding portions of R17 and QB(Steitz, 1969; Adams and Cory, 1970). There is some evidence that secondary structure regulates the site of initiation of protein synthesis (Steitz et al., 1970; Lodish, 1970). The secondary structure in the 9s RNA might have a similar regulatory function, may protect the mRNA from nuclease action or may be involved either in the transport of mRNA from the nucleus to the cytoplasm (Spirin, 1969) or in polysome binding (Heywood, 1969).

There are now several alternatives for the origin of the minor RNA bands running in the 9s RNA region. It is possible that some are non-globin proteins which are either inactive in the reticulocyte or active at very low levels in the synthesis of, for example, the enzymes in the haem biosynthetic pathway. Analysis of RNA, prepared

from blood containing 10% reticulocytes, on 6% polyacrylamide gels showed a number of minor bands running between the 12s and 9s RNAs. The 9s RNA region also contained more of the minor RNA species. It is therefore more probable, as suggested before, that the 9s RNA region may contain RNA breakdown products from later reticulocytes. These may be ribosomal. Alternatively, they may be different forms of the α and β globin mRNAs as these may change in size or in conformation with age; Resistance of mRNA to nuclease action in young reticulocyte actively synthesising globin might be a function of the secondary structure of the RNAs. A change in this secondary structure might alter the electrophoretic mobility of the mRNAs per se or allow their partial degradation. The non-coding portions of the mRNAs might themselves be degraded if a "ticketing" mechanism regulated the lives o the mRNAs (Sussman, 1970; Lim and Canellakis, 1970). The isolation and characterisation of the 9s mRNAs and the minor RNA components from cells at different stages in the erythroid pathway would differentiate between these alternatives.

The function of the 9s RNA in the membrane-associated fraction from reticulocytes has not been determined. Although the 9s RNA band is less diffuse on 2.6% polyacrylamide gels than is the 9s RNA band in the supernatant fraction, this result has not been confirmed by analysis on 6% polyacrylamide gels. There have been several reports that the membrane - associated RNAs have different turnovers(Burka et al., 1967) and capacities for in vitro protein synthesis (Schreml an Burka, 1969). The membrane - associated polysomes also synthesise

a much larger percentage of non-globin proteins than the supernatant polysome fraction (Bulova and Burka, 1970). If these reports are correct, the membrane-associated 9s RNA may contain little functional, globin messenger. This might be due to a greater content of non-globin mRNAs, rRNA breakdown products or inactivated globin messengers Isolation of this 9s RNA and determination of its protein synthetic activity in the duck reticulocyte cell free protein synthesising system would be informative.

4.2 Heterogeneity of 9s RNA from hybridisation data

The hybridisation of mouse 9s RNA was performed under conditio of RNA excess. In the hybridisation of RNA transcribed from a mouse DNA template, with inputs of RNA up to 200 µg/ml, only hybrids with the intermediate DNA fraction would be kinetically expected to form (Bishop, 1969). The intermediate fraction of mouse DNA re-anneals between C_0t values of approximately 10^{-1} - 10^{2} in 4 x SSC/50% formami 37°C (McConaughy et al., 1969). Only at C_0t values greater than this will the unique sequences begin to reanneal. Although the 9s RNA may represent a hundredfold purification from total cellular RNA, it is probable that hybridisation of 9s RNA was also with the reitera sequences of mouse DNA. C_0t values for the hybridisation cannot be derived on the basis of RNA input and compared with C_0t values for DNA re-annealing since the concentrations of reacting species are totally different.

The saturation curves for the methylated 9s RNA show a slow

approach to saturation. A plateau is not reached even at RNA/DNA ratios of 1:1. This may be partly due to an artifact in the hybridisation of methylated RNA (see section on artifacts), but is still in contrast to saturation curves for single RNA species hybridising to reiterated DNA sequences in mammals. For example; the rRNAs reach saturation at an RNA/DNA ratio of 0.06 (Di Girolamo et al., 1969). Even the gradual saturation curve for the 28s RNA in this study was reached at very low RNA/DNA ratios. 5s RNA saturates at an RNA/DNA ratio of 0.07 (Zehavi-Willner and Combe, 1966). The 5s used for comparative purposes in this study also saturates at equivalent RNA/DNA ratios. In contrast, heterologous populations of RNA species, such as nuclear pulse-labelled RNA, do not approach a saturation plateau, even at RNA/DNA ratios of 30:1 (Paul and Gilmour, 1968).

The gradual approach to saturation for the 9s RNA could have several causes.

(1) The eventual high saturation level masks some specific hybrid formation at low RNA/DNA inputs, more non-specific hybrid being formed at high RNA inputs.

(2) The hybridisation of families of increasing uniqueness increases as the effective C_0t value for the incubation increases with the addition of more RNA.

(3) Heterogeneity of the input 9s RNA.

(1) and (2) will be discussed more fully in a later section.

Heterogeneity of an RNA would be evident not only from the shape of the saturation curve but also from the initial rate of hybridisation (Bishop, 1969) or the time for half the hybrid formation (Purdom et al. 1971). The time course for the hybridisation of 28s RNA shows the reaction in formamide at 36.5°C is 4-8 times slower than that at 67°C in 2 x SSC.

Purdom et al. (1971) have shown that in the Gillespie Spiegelman hybridisation, the time taken ($t_{\frac{1}{2}}$) to reach half the maximum saturation value under conditions of excess DNA, is dependent only on the concentration of the reacting RNA species in solution. The product of the concentration and the half-time of reaction is a constant for each type of RNA; this product may be used as a measure of the base sequence complexity of the RNA. Assuming the kinetic complexity of 5s Xenopus laevis RNA is equivalent to its genomic complexity of 3.8×10^4 daltons (i.e. that one molecule of 5s RNA does not consist of two or more repeated sequences), the complexities of other rRNAs and tRNAs relative to their genomic complexity have been found. This comparison may not be valid for the hybridisation of 9s RNA since the hybrids formed have different characteristics; the RNA of Purdom et al (1971) is hybridising to very similar base sequences in the DNA and the formed hybrids have high thermal stabilities. The 9s RNA may form short, mispaired hybrids with the DNA and the hybrids may have lower thermal stabilities (see later sections). However the calculation does suggest that the 9s RNA is kinetically complex.

At its maximum rate of hybridisation, (62°C in 50% formamide/6 x SSC) the $t_{\frac{1}{2}}$ for Xenopus laevis 5s RNA is 0.8 minutes, at a concentrat of 3µg/ml. The product of $t_{\frac{1}{2}}$ and concentration is 2.4 µg/min./ml. At 36.5°C in 50% formamide/4 x SSC, the $t_{\frac{1}{2}}$ for 9s RNA is 3 minutes at a 9s RNA concentration of 45 µg/ml. The product of $t_{\frac{1}{2}}$ and concentration is 135µg/min./ml and the 9s RNA is therefore kinetically 56 times more complex than the 5s RNA. Since the molecular weight of 9s RNA (17×10^4) is 4.5 times that of the 5s RNA, the absolute kinetic complexity of the 9s RNA would be 56/4.5 = 12.5. This is a maximum value since the $t_{\frac{1}{2}}$ may be less than 3 minutes and the RNA may not be hybridising at its optimum rate.

If the 9s RNA hybridised to a complementary DNA cistron, the kinetic complexity expected would be 2 since the 9s RNA fraction consists of 2 globin mRNAs (assuming they had no internal redundancy).

Further experimental results bearing not only on the complexity of the 9s RNA but also on differences in this complexity in different 9s RNA preparations, are the variations in saturation values for different 9s RNA preparations. The differences cannot be correlated with the extent of methylation of the RNA or the non-specific binding of RNA to blank filters. A possibility was that the variation was due to differential losses of DNA from the filters. This was considered unlikely, since the routine monitoring of the denatured DNA solution, before and after DNA retention on filters, never showed losses greater than 5%. Further, the total DNA lost during hybridisati and washing of filters was only 10-15% with ^{3}H - 14 day mouse embryo

liver DNA which had been isolated by the usual methods.

However, the small size of the DNA used in the hybridisation procedure (4×10^5) is within the size range found to have diminished hybridisation values compared with DNA of higher molecular weight (Melli and Bishop, 1970). This diminished hybridisation was found even after correction for differential losses of DNA during the hybridisation procedure and it was suggested that there was a preferential loss of hybridised DNA when the DNA was small. To test this possibility with caesium chloride-purified DNA, ^{32}P - 28s RNA was hybridised to the low molecular weight DNA and also to Marmur-prepared DNA of higher molecular weight. The preparation of DNA used when the lowest saturation value was obtained for the ^{3}H-9s RNA (0.084%) gave a 10% lower saturation value for ^{32}P-28s RNA than did the Marmur-prepared DNA. This was within the 10-15% estimate for caesium chloride-purified DNA loss from filters. There was therefore no evidence for preferential loss of DNA - ^{32}P - 28s RNA hybrids with the lower-molecular weight DNA.

The different ^{3}H-9s RNA saturation levels might in part be due to variation in the stringency of the hybridisation conditions. The value of 0.084% in 2 x SSC may be quite compatible with those of 0.15% and 0.18% in 4 x SSC if many hybrids were very poor and easily displaced at the lower salt concentration. The value of 0.52% is more difficult to explain except by contamination of the DNA by protein or contamination of the hybridisation medium with heavy metals or divalent cations.

Variation in the level of a minor, rapidly-hybridising RNA component might give such a difference in the saturation levels. Some analysis of contamination may be made by competition experiments with non-radioactive RNAs. The competition curve between ^{3}H - total 9s RNA and unlabelled 9s RNA prepared from EDTA-treated reticulocyte polysome subunits is very close to the theoretical, assuming complete homology between the competing RNA species. The double-reciprocal plot gave 95% competition. Within the limits of accuracy of the method, this suggests that a maximum of 5% of other RNA species, hybridising at intermediate $C_o t$ values, are present in the 9s RNA from total reticulocyte RNA compared with the 9s RNA from polysomes

The competition curve with reticulocyte rRNAs levels off very quickly to 30% competition with ^{3}H-9s RNA. Since the competition was done at subsaturating levels of ^{3}H-9s RNA, the interpretation of this figure is complex. There are two extreme assumptions. The first is that the competition observed would increase proportional to the input of RNA, in which case the competition with rRNA would be extrapolated to almost 50% of the hybrid at saturating inputs of ^{3}H - RNA.

The other is that the levelling off of the rRNA competition curve is caused by the saturation of rDNA sites at very low rRNA inputs. In this case, the 30% competition represents the maximum possible at this DNA input and the % competition due to the rRNA species would decrease at higher ^{3}H - 9s RNA inputs. The level of competition

observed is equivalent to 0.03% of the DNA. This value is approximately similar to the percentage of DNA experimentally found to hybridise with rRNA (Birnstiel et al., 1971) and therefore reinforces the second interpretation. A contamination of less than 1 µg of rRNA in the 20 µg ^{3}H - 9s RNA would give such an effect (i.e. 5% of the ^{3}H - 9s RNA preparation). This is, however, only a minimum estimate of the rRNA contamination in the ^{3}H - 9s RNA preparation.

These interpretations assume that no non-specific competition is occurring. No check was made for non-specific competition with E.coli RNA. The results could also be partly due to contamination of the rRNA with globin mRNA precursors. A report has recently demonstrated a possible 17s RNA precursor to globin mRNA in chick erythroblasts (Maroun et al., 1971). If this were present in reticulocyte 18s RNA, competition with pure globin mRNA would take place. The control experiment, that of competing the ^{3}H - 9s reticulocyte RNA with rRNA from a non-erythyroid mouse tissue, has not yet been done.

Correlation of the results from 9s RNA heterogeneity on gels, its slow approach to and rate of hybridisation and its competition with rRNA would indicate that the 9s RNA is contaminated with some rRNA. More critical experiments on the hybridisation of non-rRNA components would have necessitated the hybridisation of ^{3}H - 9s RNA in the presence of excess amounts of unlabelled rRNAs. As this was not done, the interpretation of some of the other hybridisation result is more ambiguous.

4.3 Specificity of hybridisation procedure.

The temperature for optimum rate of formation of an RNA-DNA hybrid varies with the GC content of the strands and their degree of complementarity. It also depends on the length of the hybrid. This temperature is usually taken as 15-20°C below the T_m of the corresponding DNA (Nygaard and Hall, 1964). Most hybridisations in mammalian systems have used the standard conditions of bacterial hybridisations, 67°C in 4 x SSC. The extend of hybridisation of mammalian non-rRNAs to DNA of the intermediate fraction is a function of the reaction conditions for hybrid formation (McConaughy, 1968) and the washing procedure (Church and McCarthy, 1967a,b). This is due to the possibility of RNA hybridisation to related DNA families. The choice of reaction conditions for these hybridisations is therefore somewhat arbitrary.

The theoretical T_m for the thermal denaturation of mouse DNA may be calculated from the GC content of its DNA by the relationship: T_m DNA = 69.3°C + (0.41 x percentage GC content of the DNA)°C in 0.2 M Na^+ (Marmur and Doty, 1962). This relationship was established for a series of bacterial DNAs of known GC content. The calculation gives a value of 86.3°C for mouse DNA (42% GC) in 0.2 Assuming a linear relationship between log (Na^+) and T_m (Dove and Davidson, 1962), the T_m of mouse DNA in 0.3 M Na^+ (2 x SSC) =

90.3°C. Since the T_m of DNA-RNA hybrids is approximately 4°C lower than the corresponding DNA-DNA hybrids, (Chamberlin and Berg, 1964), the T_m of mouse RNA-DNA hybrids would be 86.3°C in 2 x SSC. The temperature for optimum rate of hybridisation would be 70.3 - 75.3°C. Using incubation conditions of 67°C in 4 x SSC, and pulse-labelled mouse RNA, hybrids with a T_m of 82°C, measured in 2 x SSC, are formed. (Church and McCarthy, 1967). This corresponds to about 6% base mismatching (Laird et al., 1969).

The base composition of mouse reticulocyte 9s RNA is 50.7% GC. (Williamson et al., 1971). This would give a theoretical T_m for 9s DNA-DNA hybrid of 93.8°C in 2 x SSC and a theoretical T_m for 9s RNA-DNA hybrid of 89.8°C. Conditions for maximum rate of hybrid formation would therefore be 73.8 - 78.8°C.

Since the hybridisation of methylated-9s RNA was only possible at low temperatures, due to non-specific adsorption of RNA to the filters at incubation temperatures of 67°C, formamide was added to the incubation mixtures (McConaughy et al., 1969). This non-ionic solvent reduces the stability, and hence the melting temperature, of a hybrid and the optimum conditions for its formation The incubation conditions used were 2 x or 4 x SSC in 50% formamide at a temperature of 36.5°C. Assuming a drop in the T_m of 0.72°C for 1% formamide (McConaughy et al., 1969), these incubation conditions correspond to incubation at 73°C in 2 x or 4 x SSC respectively and are thus similar to the calculated conditions on the basis of GC content for this RNA. Due to the low specific activity of the

9s RNA and the rapid saturation value at the necessarily high RNA inputs, it was not possible to check experimentally that the hybridisation of 9s RNA was performed at its rate optimum.

The experiments performed with ^{32}P - 28s rRNA confirmed that the hybridisation in formamide at 36.5°C is more stringent than hybridisation at 67°C in the equivalent SSC buffer. The extent of hybridisation at 67°C in 2 x SSC was greater, and the T_m of hybrids was 8°C lower than these hybrids which were formed in 2 x SSC/50% formamide at 36.5°C. Although the DNA saturation values for the 28s RNA were similar to other estimates, the very low T_ms (62 - 67°C in 2 x SSC) showed the hybrids were of very poor quality. This could have been due to the contamination of the rRNA with mRNAs of high specific activities which formed mismatched hybrids with related families in the DNA. Alternatively, if the 18s or 28s rDNA cistrons were internally redundant, some mismatched rRNA - rDNA hybrids thus possible might be stable enough at the incubation temperature to preclude the formation of perfectly - matched, stable hybrids. If the rRNA secondary structure was not melted out at the temperature of incubation, this also would hinder perfect RNA-DNA hybrid formation.

The quality of hybrid formed can also be influenced by the washing procedure. The selective removal by pancreatic ribonuclease of some unpaired loops and free ends of RNA stablised by hybrid regions was used first by Giacomoni and Spiegelman (1962).

The conditions of RNase treatment used in bacterial hybridisations have been adopted for the removal of mismatched regions in eucaryotic hybrids. However, RNase action does not discriminate completely between good and bad hybrids. Pancreatic ribonuclease attacks only the C-3′ linkage in a pyrimidine nucleotide to the C-5′ linkage in the adjacent nucleotide. Any unpaired runs of purine bases will therefore not be excised.

The non-reactivity of double-stranded RNA to RNase is not absolute, but depends on the concentration of RNase, temperature and length of incubation. Base-paired sequences less than 10-20 nucleotides long in rRNA are not resistant to RNase action, unless protected by a tertiary structure (Gould, 1966). This approximate minimum length can therefore be taken as the length of hybrid RNA-DNA resistant to RNase action. The length of RNA remaining associated with DNA would be greater if parts of the hybrid included double-stranded RNA loops, also resistant to the action of RNase. The efficiency of excision by RNase of one or two mismatched bases in long hybrid stretches is also unknown. If these bases are protected by the surrounding hybrid regions, the length of "hybrid" resistant to RNase action would again be greater.

Despite these shortcomings, RNase-resistant hybrids, of length about 12 nucleotides, give a good indication of the extent of homology of an RNA to bacterial DNA.

Since a bacterial DNA is one single chain, the presence of repeated sequences of lengths greater than the minimum length for stable hybrid formation, would give the possibility of unstable genetic recombination. Sequence lengths greater than about 12 nucleotides would therefore be improbable (Thomas, 1966).

The E.coli genome is only about 4×10^6 nucleotide pairs and this corresponds statistically to the maximum chain length possible for repetitions of 12 nucleotides or above to be unlikely (Thomas, 1966). Since the probability of an RNA sequence 12 nucleotides long hybridising to a fortuitously similar sequence in the DNA is therefore small, the formation of a short stable hybrid would presuppose that the rest of the hybrid was also identical. However, since the length of the E.coli molecule is close to the maximum chain length possible for non-repetition of sequences, some repetition of shorter sequences will occur. This is shown experimentally with bacterial hybridisations at very low incubation temperatures. Mismatched hybrids were formed (Attardi et al., 1965a).

Since eucaryotic DNA is compartmentalised into separate chromosomes, the possibility of repetitions occurring which are greater than the stable minimum length is increased. The presence of repeated sequences in eucaryotic DNA has been shown by its hybridisat at intermediate C_0t values (e.g. Hoyer et al., 1964). Since parts of the genome have been duplicated during evolution, (Britten and

Kohne, 1968) the probability of similar sequences occurring in different DNA cistrons is much greater. Several short base sequences in an RNA molecule might then form short, stable hybrids with similar DNA regions. These hybrids would be separated by stretches of non-hybridised strands.

Hybridisation of HeLa rRNA to DNA at 70°C in 2 x SSC resulted in the formation of very short hybrids (Attardi et al , 1965b). Only 50% of the hybrids formed were acid precipitable and of this fraction, only a part was of equivalent size to the input RNA. The formation of hybrids of demonstrably poor quality is similar to the hybrids formed by the ^{32}P - 28s RNA from Landschutz cells. The cause may be similarly due to contamination with mRNAs and formation of mismatched hybrids between the mRNAs and the DNA.

Washing of the formed hybrids at high temperatures eluted badly-paired regions and thus increased the size and specificity of the remaining hybrids (Attardi et al., 1965b). High temperature washes have also been used extensively by McCarthy (e.g. McCarthy, 1967) for both ribosomal and non-ribosomal RNA hybrids. After washing at high temperatures, no difference in the T_ms of RNased and non-RNased hybrids was found, so the RNase step was eliminated.

These high-temperature washes are equivalent to increasing the stringency of reaction conditions in that both procedures remove families of hybrids which are not closely related. Increasing the stringency of the reaction conditions during hybridisation is,

however, preferable since there is more likelihood of all the families of very similar sequences then being hybridised. If the HeLa hybrids (Attardi et al., 1965b) had been formed at higher temperatures (74-79°C), it is probable that the hybrids would have been more specific. The importance of temperature of hybrid incubation if hot washes are not used, is also illustrated by the hybridisation of sea urchin total RNA at 60°C in 2 x SSC (Wegnez and Denis, 1970). Even assuming GC content of 42%, the theoretical temperature optimum for hybridisation should be at 70°C or above. Although the hybrids were RNased and washed at room temperatures, their T_m was still 15°C less than that of the native DNA (21% base mismatching).

High temperature washes would be satisfactory if hybridisati were between similar families with only short lengths of mismatched regions. Excision of these short mismatched regions with RNase would then give an underestimation of the hybridisation between these similar families. Washing at different temperatures removes the shorter stretches of hybrids stable at lower temperature and thus gives an estimate of families of different degrees of relatedness.

High temperature washes will not, however, remove long stretches of non-hybridised RNA, if these are contiguous to RNA-DNA hybrids stable at the washing temperature. The extent of homology of the RNA to the DNA will, therefore, be overestimated.

RNasing would remove these long, mismatching regions.

The hybridisation of 9s RNA was performed within the theoretical rate optimum conditions for the GC content of 9s RNA. After hybridisation, the filters were placed in a large volume of 4 x SSC at 36.5°C. There was therefore little chance of non-specific hybrids forming at lower temperatures at the end of the hybridisation. Partly due to the non-specific binding of methylated RNA at high temperatures, and partly in order to observe the range of hybrids formed under these presumed optimum conditions, no high temperature washes were performed. RNase treatment resulted in removal of 50% of the hybrid and in lowering the counts on blank filters to a low and very reproducible level.

That the temperature of incubation was close to the optimum for maximum ^{3}H - 9s RNA-DNA hybrid formation was shown by the variation of hybrid formation with temperature. The maxmimum hybrid formation was between 30°C and 40°C, with a sharp drop at temperatures over 45°C. Increasing the formamide concentration at a fixed incubation temperature of 36.5°C also lowered the hybridisation level at formamide concentrations above 55%. Since the hybrids formed under optimum conditions could contain a high percentage of base mismatching, no conclusions could be inferred from the hybridisation level of ^{3}H - 9s RNA to DNA without analysis of the quality of hybrid formed.

4.4 Factors influencing hybridisation and T_m determinations

The hybrids formed between ^3H - 9s RNA and DNA melted at very low temperatures, even in 2 x SSC. The T_m of the hybrids varies with the salt concentration at which the T_m is determined in the predicted fashion (Dove and Davidson, 1962), being 45°C in 1 x SSC and 52°C in 2 x SSC. This difference also agrees with that found for mouse total RNA-DNA hybrids at these salt concentrati (McCarthy, 1967). The observed T_m is therefore about 35°C lower than that predicted for a fully base-paired hybrid between 9s RNA and its complementary DNA. The T_m of a hybrid is lowered by 1% for every 1.5% base mismatching for mismatching of less than 30% and polynucleotide sequences of less than 50 nucleotides (Laird et al., 1969). An extrapolation of this relationship would give 50% mismatched bases in the 9s RNA hybrid. Another possible explanation for the low T_ms is that the hybrid lengths are very smal

It was possible that the stability of hybrid formation might be affected by chemical modification of the RNA bases, either by methylation or by contact with DEP. Although comparisons in the literature on hybridisation between methylated and non-methylate total RNA preparations show no detectable differences (Smith et al., 1967; Davidson and Hough, 1969), the high proportion of substituted bases in the tRNAs (Madison, 1968) was one possible explanation for the T_m of tRNA - DNA hybrids being 15-20°C lower than predicted (Wegnez and Denis, 1970).

Two comparisons of different preparations of methylated and non-methylated RNA species in this work showed some differences between them. The first was a comparison between a methylated rabbit 5s RNA and a ^{32}P-5s RNA with 25% 4s RNA contamination. Although the methylated 5s RNA was a purer species, the T_m was 14°C lower than that of the ^{32}P - 5s RNA. Since there might be some differences in the hybridisation of 5s rabbit RNA and 5s mouse RNA, despite the identity between different species in the literature, (e.g. Williamson and Brownlee, 1969), the experiment was repeated by comparing the hybridisation of ^{32}P - 5s mouse RNA with part of the same preparation after its chemical methylation. Although the RNA had a specific activity of only 1070 cpm/µg, the T_m of its hybrid to DNA was 7°C lower than that for the ^{32}P - 5s RNA control. The saturation curve also showed a less rapid approach to saturation than did the unmethylated RNA. This might be expected if the kinetics of hybrid formation between mismatched bases were slower than the kinetics of hybrid formation between well-matched base pairs.

Hydrogen bonding between A-T and G-C base pairs optimises the packing of aromatic rings in a double helix and hence the stability due to base stacking. Base methylations which do not adversely affect the hydrogen bonding or base stacking are possible. An example is the 5-methyl substitution in pyrimidines (Szer and Shugar, 1966). The order of in vitro methylation of bases in RNA is similar to the order of reactivity of the individual bases, since

none of the potential methylation sites are strongly protected by the hydrogen bonding in a secondary structure. The order of methylation at pH 7 is N^7 guanine, the most nucleophilic site (65%), N^1 adenine (23%), N^1 cytosine (11%) and N^3 adenine (1%)) (Lawley and Brookes, 1963).

Since both the N^1 of adenine and the N^1 of cytosine are involved in the base-pairing of complementary strands in a double helix (Watson and Crick, 1953a), methylation at these sites would destabilise the helix by interference with the specificity of base pairing. This is shown experimentally by the lack of interactio between 70% methylated N^1 polyadenylic acid and polyuridylic acid under conditions where the unmethylated polyadenylic acid complexes fully with polyuridylic acid (Michelson and Pochon, 1966). The T_m of the methylated polyadenylic acid is lowered by 25°C compared with the unmethylated form. The methylation at N^1 also destroys the ability of polyadenylic acid to stimulate lysine incorporation in a cell-free protein synthesising system (Michelson and Grunberg-Manago, 1964). Although the N^7 of guanine is not involved in G-C base pairing, methylation at N^7 may have a destabilising effect caused by steric hindrance of the methyl group. Anomalous base pairing could also occur since N^7 guanosine is in a more acidic form than unmethylated guanosine and is thus more likely to react with uracil or thymidine (Lawley and Brookes, 1961, 1962). The stability of the complex between methylated N^7 polyguanosinic acid and polycytidyl acid is considerably less than the non-methylated complex (ΔT_m = 21°

Michelson and Pochon, 1966). Similarly, methylation of 70% of the guanine residues at N^7 in DNA resulted in a 14°C lowering of the T_m (Pochon and Michelson, 1967).

Methylation also leads to considerable destablisation of nucleosides and nucleotides (Schapiro, 1968; Lawley and Brookes, 1963; Lawley, 1966). Methylated DNA is destabilised and loses radioactivity by splitting off methylated bases (Lawley and Brookes, 1963). Re-arrangements are more frequent in RNA, N^1-methyl adenine slowly re-arranging to 6-methylamino purine (1% of all methylated bases after 18 hours at 37°C) and N^1-methyl cytosine even more slowly to 1-methyl uracil.

The depurination reaction may occur very slowly in RNA, but the counts in ^{3}H-methylated 9s RNA were 100% acid precipitable after 32 hours incubation at 36.5°C. More convincingly, even after incubation for 72 hours at 36.5°C, all the counts were located over the optical density profile when the ^{3}H-methylated 9s RNA was analysed on polyacrylamide gels.

Further modifications of the 9s RNA might be caused by react with DEP. As discussed already, the isolation of 9s RNA relatively uncontaminated with breakdown products of other species was optimise by using DEP as an enzyme inactivator during isolation of the 9s RNA RNA isolated in the presence of protein and DEP gives biologically-active preparations (Fedoresák and Turtóczky, 1966; Fedoresák et al. 1969 ; Oxenfelt and Åstrand, 1970). Double-stranded nucleic

acids are also not inactivated with DEP (Oberg, 1970). However, Leonard et al. (1970) have recently shown a reaction of adenine with 0.3% DEP, with consequent ring-opening and formation of a carbethoxy derivative. There is also a reaction of DEP with the bases in RNA whenthe RNA is incubated with 6% DEP at 0°C for 20 minutes (Solymosy et al., 1971). This reaction causes RNA inactivation.

DEP also inactivates tobacco mosaic virus RNA at concentrations of 0.6% (Oxenfelt and Åstrand, 1970). Above threshold concentrations of 0.2 - 0.4%, DEP also inactivates tRNAs (Denić et al., 1971). The threshold effect may be due to a relationship between the number of carbethoxylations and the loss of reactivity. Alternatively, it could be due to an increased inactivation effect when DEP exceeds its water-solubility (0.6%) Denic et al., 1971).

During isolation of the 9s RNA, DEP was added at subsaturating levels (0.1%). Since the RNA was also protected by proteins at this stage, reaction of DEP with the RNA at this concentration is unlikely. The final total RNA preparation was stored in DEP-saturated buffer at -20°C for periods up to 1 day. This was to ensure no RNA breakdown occurred before the isolation of 9s RNA by zonal centrifugation. It was possible that the DEP had reacted with the RNA at this stage; extensive reaction of DEP with RNA results in a decreased E_{260}/E_{230} ratio (Solymosy et al.,

1971), but this was not observed with a pure 9s RNA preparation whose E_{260}/E_{230} ratio was > 2. There was therefore no extensive reaction with the 9s RNA although a small extent of reaction would probably not be detected by this criterion; the data of Solymosy et al. (1971) indicate that incubation of 4% DEP with RNA for 20 minutes at 0°C. carbethoxylated 0.4% of the bases. At this level of methylation there was no detectable decrease in the E_{260}/E_{230} ratio. A decrease could be observed after incubating 6% DEP with RNA for 30 minutes at room temperature. In contrast, preparations of total RNA stored in 5-10% DEP for 9 months at -20°C showed a much-decreased $E_{260}/_{230}$ ratio of <1.5. Reaction of the nucleic acids with DEP might have occurred during this period.

No further DEP was added to 9s RNA after its purification, but even a small percentage of ring opening of the RNA bases would be expected to affect the hybridisation of RNA to DNA. Any mismatched hybrids formed might be stable enough to withstand the washing and ribonuclease treatments and yet melt at spuriously low T_ms. An effect of carbethoxylated bases on the elimination of badly-mismatched hybrids might also occur, since extensive DEP reaction results in resistance of RNA to nuclease action (Solymosy et al., 1971).

No control experiments isolating large amounts of 9s RNA for methylation in the absence of DEP were performed due to the difficulty in the interpretation of the results with 9s DNA species

more likely to contain rRNA breakdown products. Some comparison may be made with the results from 14 day mouse embryo livers since the 9s RNA was prepared without contact with DEP; the saturation value is within the same range. No melting profiles were performed on these hybrids so the comparison is of limited value, especially since the identity of the RNAs in these different preparations is unproven.

The results on the methylation of 5s RNA species indicate that the T_m of unmethylated 9s RNA would be $52^oC + 7^oC = 59^oC$. No estimation can be made of any possible effects of DEP in lowering the T_m of the hdyrids.

Even with the correction for methylating the 9s RNA, the T_m is still 28^oC below the theoretical. T_ms obtained with rRNA species, 28s and 5s RNAs, were also low, being $20-30^oC$ below the theoretical values. It is therefore possible that other factors were affecting the determination of T_ms.

The DNA used for hybridisation had a molecular weight of 5×10^4. Although only 15% of the total DNA was lost on incubating filters under the conditions for T_m determination, it was possible that if the DNA were hybridised to RNA it might be preferentially lost from the filter at higher temperatures, even though no preferential loss of hybridised DNA, as suggested by Melli and Bishop (1970) could be detected during incubation and washing of the filters when the hybridisation of ^{32}P - 28s RNA with different DNA preparations was compared. However, 9s RNA hybrid had a T_m

3°C lower when hybridised with the low molecular weight mouse embryo DNA than when hybridised with the Marmur-prepared DNA or the higher molecular weight duck reticulocyte DNA. This suggests that there is some preferential loss of low-molecular weight DNA in hybrid form during the T_m determinations and gives a revised T_m of 77°C for the non-methylated 5s RNA and 62°C for a non-methylated 9s RNA. For 5s RNA of 60% GC base content (Forget and Weissman, 1967), the calculated T_m would be 93.9°C in 2 x SSC. The observed T_m is therefore still 16°C lower than the theoretical, giving 22% base mismatching (Laird et al., 1969). This would not be expected from the fast approach to saturation at low RNA/DNA ratios and from the similarity of the estimated number of 5s DNA cistrons (2080 in a haploid genome) to the other values in the literature (2,000 in Hela, Hatlen and Attardi, 1971; 3,500 in chinese hampster, Amaldi and Buongiorno-Nardelli, 1971). The discrepancy between the observed and expected T_m is even greater for 28s RNA. Although the saturation value also falls well within the range quoted in the literature, the approach to saturation is slow; the discrepancies in this case may be partially explained by contamination with labelled mRNAs.

A possibility for the low T_ms of 9s RNA-DNA hybrids is that they were not being formed under conditions of maximum efficiency. Although it has been assumed from the work of Nygaard and Hall (1964), that the maximum hybridisation rate occurs at 15-2

below the T_m, recent results of Purdom *et al.* (1971) strongly suggest that optimum rates of hybridisation occur at temperatures only 5-10°C below the T_m. The optimum rates for both 5s and 28s RNAs were over 60°C in 6 x SSC/50% formamide. Assuming that the dependence of hybrid T_m on SSC concentration is similar for optimal rates, the optimum rates would be about 50°C in 2 x SSC/50 formamide. The formed hybrids had T_ms very close to theoretical, with no high temperature washing before the T_m determination (Purdom *et al.*, 1971). They were therefore a very good match. Taking into consideration the different GC content of 9s RNA, the optimum temperature for the rate of annealing 9s RNA to DNA would be about 46°C in 2 x SSC/50% formamide.

Hybridisation at lower temperatures would give a higher percentage of mismatched hybrids. A further factor in lowering of the T_ms of the 28s and 5 s RNA - DNA hybrids might be residual secondary structure in the RNA at these low incubation temperatures Even with RNA which was melted out before hybridisation, the reformation of its secondary structure would compete with the formation of imperfectly matched RNA-DNA hybrids and thus diminish the RNA's capacity for forming long stretches of specific hybrids. Double-stranded RNA stretches closely linked to DNA-RNA hybrids might then be resistant to pancreatic ribonuclease. This would give an overestimate of the extent of hybrid formation and also result in low T_ms. Although some of the increase in optical densit

at E_{260} observed on heating rRNAs is due to the breakdown of base stacking within the individual chains, much of it is probably due to double-strandedness and therefore both ribosomal and 5s RNAs would have over 50% double strandedness remaining at 36.5°C in 2 x SSC/50% formamide (Williamson et al., 1971).

Although it was assumed that 9s RNA would have no appreciable double-strandedness since this was thought to be incompatable with its messenger function, its optical melting curve also would show about 50% remaining hyperchromicity under the hybridising conditions (Williamson et al., 1971). That secondary structure is present in viral messengers, and sometimes has a regulatory functio has been shown by Steitz et al. (1970) and Lodish (1970), so it is not inconceivable that some secondary structure in mammalian RNAs might have similar functions.

The instability of the RNased hybrids may partially be due to experimental artifacts. A spuriously-low T_m might result from continued degradation of hybridised RNA by residual positively-charg ribonuclease remaining on the filters throughout the washing and counting procedures. This might also account for the instability of the hybrid when re-incubated in the hybridisation medium alone. The low T_ms of non-RNased hybrids and of hybrids subjected to increasing temperatures before the RNase step would discount this explanation, as would pre-incubation of the RNased filters in DEP before the T_m determination. These experiments were done only once

and therefore the results are only tentative. The same is true for an incubation of ^{3}H-methylated 9s RNA with RNase treated DNA filters where no loss of acid-precipitable material was found. This method of assay would detect only a large amount of RNase activity.

Residual toluene on the filters might also affect the stability of RNA-DNA hybrids, although a preliminary experiment with filters subjected to chloroform washes again gave low T_m values for the hybrids.

Although the T_ms of formed hybrids might therefore be affected by several factors in the method of their determination, there is no proof for this. Conversely, there is some evidence from the work of others, that the combination of hybridisation under sub-maximal conditions and the non-removal of imperfect hybrids by high temperature washes would give results similar to those obtained.

4.5 Analysis of T_m values for 9s RNA

As stated, the 9s RNA hybrid has a T_m of 25°C less than the theoretical, making corrections for the lowering of the T_m for methylated RNA and low molecular weight DNA. If the RNA in hybrid form had a lower GC content than that of total 9s RNA, the theoretical T_m would be lowered according to the relationship established between T_m and GC content (Marmur and Doty, 1962). (Since GC pairs stabilise a double helix more than A-T pairs (Marmur and Doty, 1962), the melting of A-T rich regions in a double helix will occur before regions enriched in GC base pairs (Geiduschek, 1962)). This relationship has been tested with GC contents of down to 26%. The T_m of such a DNA is 80.5°C in 0.2 M Na (Marmur and Doty, 1962). This is equivalent to 83.5°C in 0.3 M Na The T_m of high molecular weight poly A. poly U, measured optically in 1M Na^+, is 70°C (Lipsett *et al.*, 1961). This would be equivalent to about 60°C in 2 x SSC. The T_m of the 9s RNA hybrids could be as low, assuming the hybridised regions contained essentially no GC base pairs.

The very small size of 70% of the hybridised regions is also likely as an explanation of the low T_ms. Since double helices are mainly stabilised by the stacking forces between the bases in the helical structure (Crothers and Zimm, 1964), a decrease in the length of the helix will decrease the free energy/molecule for forming the helical state. The temperature at which the free

energy of helix formation becomes equivalent to the increase in entropy caused by separation of the strands, will therefore decrease with decreasing helix length. This decrease in T_m or decrease in optimum temperature of annealing with decreasing hybrid size has been shown experimentally for the hybridisation of synthetic oligonucleotides to polynucleotides (e.g. Lipsett, 1964) and also with the hybridisation of degraded natural DNA or RNA with single-stranded DNA (Niyogi and Thomas, 1967; McConaughy and McCarthy, 1967 Niyogi, 1969).

Niyogi (1969) found that phage RNA oligomers, of chain lengths greater than 12, formed hybrids with phage DNAs if incubated in 5 x SSC. The optimum temperature of incubation increased with increasing chain length of the oligonucleotides. T7 RNA oligonucleotides, hybridised to T7 DNA (50% GC content), had T_ms of 65°C (oligonucleotide chain length of 13) and 76°C (oligonucleoti chain length of 16) when the T_ms were measured in 2 x SSC. The corresponding figures for hybrids of T2 RNA oligonucleotides and T2 DNA (30% GC content) were 40°C and 57°C. When oligomers of E.coli rRNA were hybridised to E.coli DNA in 2 x SSC, 50 nucleotides were necessary for formation of a stable hybrid at 67°C, 32 nucleotides at 55°C and 17 nucleotides at 44°C (Gillespie and Spiegelman, 1966). These results are for hybrids of approximately 60% GC content and a correction to hybrids of 50% GC content would give temperatures of approximately 62°C, 50°C and 39°C.

Long hybrids are also needed for stabilisation of hybrids formed between mouse DNA oligonucleotides and mouse DNA (42% GC content) at 60°C in 0.5 M KCl (McConaughy and McCarthy, 1967). The T_m of hybrids formed with oligonucleotides of chain length = 20, was 40°C. The T_ms for chain lengths of 60 and 200 were 50°C and 70°C respectively. The discrepancy between McCarthy's results and those of Niyogi are probably due to the increase in genome size between the phages and mammals. In the phage, the hybrids are likely to be perfectly base-paired. Under the conditions of the McConaughy incubation for mammalian hybrids, long stretches of mismatched bases will occur and the stabilities will thus be lower for hybrid stretches of equivalent length to the phage hybrid

The influence of mismatched bases has been generally studied (Bautz and Bautz, 1964; Uhlenbeck, et al., 1971, Laird et al., 19 and a general relationship has been derived between the extent of base mismatching and the lowering of the T_m. The synthesis of complex and exactly defined oligomers of A and U allows the stud of specific, non-complementary bases on the stability of a hybrid (Uhlenbeck et al., 1971). If non-complementary bases are looped out of the helical structure, there is a considerable destabilisati The destabilisation effect is greater for short hybrids. It is, however, less than for two separate helices not joined by a mismatched base. The destabilising effect is comparatively less if mismatched bases are not looped out but are accomodated within

the helix. This is so if the mismatching is between a G and a U since wobble permits some base - base pairing between them. The general relationship derived between the extent of base mismatching and the T_m is still a good approximation for interpretin hybrid results.

Assuming that some base mismatching is responsible for the results on the T_ms of long oligonucleotide sequences, (Gillespie and Spiegelman, 1966; McConaughy and McCarthy, 1967) it can be inferred that the 70% of the 9s RNA hybrid which is less than 20 bases could be a perfect hybrid and still melt at the T_m of 62°C.

This conclusion is less certain since the effect of the 30% hybrid RNA which is 60-120 nucleotides long on the average T_m is also not known. If this were a perfect hybrid sequence of averag GC content, it would have a T_m of over 90°C. Such a high T_m would be detected by a biphasic melting profile for the hybrids. If there were regions of base mismatching of up to 30%, the T_m would be low, but it is difficult to see why RNase should not then attack the mismatched stretches. That is, unless the mismatched sequences included bases modified by either methylation or by incubation in DEP, or stretches of RNA which were double-stranded. In each case attack by RNase might be reduced. A further possibility is that this long sequence is very rich in A and G. This would give a low T_m for the hybrid and resistance to RNase attack on mismatched bases.

The experimental finding that the stretches of bases in stable hybrid are mostly very short, is compatible with the efficiency of hybridisation being dependent on the concentration of RNA in the incubation medium. This is the situation in the hybridisation of very short oligonucleotides to DNA (Niyogi, 1969). This type of equilibrium reaction is found with the hybridisation of pulse-labelled E.coli RNA to DNA under conditions of low stringency (Kennell, 1971). An equilibrium state for the hybrids is supported by the loss of RNase-treated 9s RNA hybrid when re-incubated in the hybridisation medium when no RNA is presen The equilibrium interpretation would also explain the differences obtained between the saturation curves with varying RNA input and with varying time. Varying the input RNA gave a saturation curve approaching a plateau at high RNA/DNA ratios. Double reciprocal plots gave the theoretical saturation at infinite RNA input. Varyi the length of reaction at a subsaturating RNA/DNA input gave a saturation value equivalent to that found for the input RNA on the saturation versus RNA concentration curve. Even after 40 hours incubation the amount of hybrid formed was far short of that at infinite RNA concentration. Although this result may be an artifac caused by the aggregation of the 9s RNA after long incubation in formamide, it could also be due to the hybridisation reaction being reversible. In the reaction RNA + DNA $\rightleftharpoons$ RNA · DNA., the free RNA in solution is therefore in equilibrium with the RNA in hybrid form.

R free $\rightleftharpoons$ R bound .

$\therefore \frac{[R\ bound]}{[R\ free]} = K\ eq.$ Both the rapid time course and the small fraction of RNA bound are consistent with a low value for Keq. This means that a large amount of free RNA is necessary for a certain amount of hybrid formation and, conversely, that the hybrid will be rapidly dissociated in the absence of free RNA. This is the state predicted for the hybridisation of short regions in the RNA to the DNA since the increase in enthalpy for the formation of a short helix is little different from the loss of entropy due to the ordering of the strands.

An equilibrium reaction may be a general feature of hybrids formed by non-ribosomal RNA and DNA under Gillespie Spiegelman conditions and conditions of excess RNA. This is indicated by the comparable results in the time-course curve for RNA synthesised on a mammalian DNA template by DNA-dependent RNA polymerase (Gilmour, 1967). The assumption of reversibility was made by Paul and Gilmour (1966a), although not stated explicitly. A reversible situation has been assumed by Lavallé and Hauwer (1968) for the calculation of hybridisation levels of tryptophan mRNA to phageϕ80 DNA at infinite RNA input. That such an assumption is perhaps unjustified for hybrids containing long sequences of stable base pairs, such as rRNA-DNA hybrids, has been demonstrated by Bishop (1970).

4.6 Biological Interpretations

There are several possible interpretations of the hybridisation data. If the globin mRNA were hybridising to long stretches of complementary base sequences in the DNA, estimation of the saturation values would give the extent of reduplication of these DNA regions. Since the length of the 9s RNA is greater than that needed to code for globin, it is alternatively possible that the high levels of hybridisation are between the non-coding region of the RNA and sequences in the DNA.

If the extra sequences were transcribed from regions in the DNA adjacent to those coding for the globin mRNAs, these extra sequences in the RNA might then hybridise back to many such sequences in the DNA adjacent to other mRNAs. Such a transcription of extra nucleotides might be due to the transcription of regulatory sequences in the DNA adjacent to the globin cistrons. They might alternatively be involved in mRNA transport from the nucleus to the cytoplasm, in ribosome recognition of initiator codons or in the regulation of mRNA degradation, either by giving the mRNA a circular configuration or by "ticketing" mRNA by the stepwise degradation of the extra sequences. Similarities between the sequences transcribed with different mRNAs would then explain the high levels of non-specific hybrid formation.

If the extra sequences were not transcribed with the mRNA but were added enzymically at a later stage, the hybridisation of such sequences would be to many non-specific regions in the DNA. Such non-specific hybridisation might also occur between short stretches of base sequences in the coding portion of RNA to similar sequences in the DNA.

The amount of DNA hybridising to RNA can only be determined if the DNA sites are saturated with the input RNA. Experimentally, saturation curves only approach saturation and the saturation value at infinite RNA inputs has to be derived.

Applying the law of mass action to the equilibrium DNA + RNA $\rightleftharpoons$ DNA · RNA hybrid enables an expression to be derived for the velocity of the reaction for hybrid formation.

$$v = \frac{V_o RNA}{K + RNA}$$

where $K = \frac{K_1}{K_2}$; RNA is the concentration of RNA reacting with the DNA; V is the maximum velocity when the DNA is saturated with RNA. Therefore, from a plot of $\frac{1}{v}$ versus $\frac{1}{RNA}$,

$$\frac{1}{v} = \frac{K}{V_o\ RNA} + \frac{1}{V}$$

V can be estimated from the intercept on the $\frac{1}{v}$ axis (where $\frac{1}{RNA} = 0$, i.e. RNA = ∞).

If the rates are expressed as extent of hybrid formation, the estimate of $\frac{1}{V}$ gives the extent of hybrid formation at infinite RNA inputs. The extent of maximum hybrid formation for RNA-DNA

hybridisations of different rates can therefore be compared. This approach is similar to that of Lavallé and De Hauwer (1968).

The saturation hybridisation of a single RNA to a sequence of bases of the DNA identical to those from which it was transcribed, allows a calculation of the number of cistrons coding for this RNA. This assumes formation of a perfect RNA-DNA hybrid.

Such a calculation is valid for the rRNAs if hybridised under the correct experimental conditions, since the hybrids formed have similar melting temperatures to those predicted and therefore have very few mismatched bases (e.g. Purdom et al., 1971). The DNA family from which the rRNAs are transcribed must therefore be one which does not cross-react with other families in the genome under the experimental conditions used. However, even in the hybridisation of the rRNAs there is some variation in the saturation values obtained For example, those for rat range from 0.04-0.09% (Birnstiel et al., 1971). The discrepancies may be partly due to contamination with mRNA or other RNA species. Annealing at suboptimal temperatures would also give inaccurate results, especially if the RNA still had some secondary structure.

Exact calculation of the number of cistrons coding for the 4s and 5s RNAs which are transcribed from reiterated sequences is difficult to determine since the criteria for formation of well-matched hybrids are not often used to examine the products of the hybridisation reaction. As well as determination of the T_m, the hybr size could be compared with that of the input RNA; the kinetics

of hybridisation could also be examined to see if they were compatibl with the number of RNA species hybridising.

For the hybridisation of a heterogeneous mRNA population, saturation values are less easily interpreted. Many mRNAs will have short sequences in common, resulting from cistron duplications and divergence in evolution or from the evolutionary convergence of parts of proteins having similar active sites or other regions (Sorm and Keil, 1962). Absolute correlations of DNA similarities cannot be made from protein sequences since codon degeneracy in the 3rd triplet base might lead to DNA stretches of slightly different base sequence coding for the same peptide region.

Increasing the temperature and decreasing the salt concentrati during hybridisation will reduce the amount of cross-hybridisations with short stretches of similar base sequence. Hybrids between more related RNA and DNA regions will be selected at higher temperatures. This is shown experimentally in the work of Denis (1966). The hybrids formed by labelled mouse DNA or pulse labelled mouse RNA to mouse DNA at higher temperatures have a higher T_m and are therefor better matched (McCarthy, 1967; Church and McCarthy, 1968). Even under the most stringent hybridisation conditions, the hybrids formed are not perfectly base-paired.

However, a spectrum of different mRNA species will be statistically expected to have many more regions similar to stretches of DNA than will one single mRNA species. The probability of

fortuitous hybridisation will be even less if this single mRNA species has a distinctive base sequence pattern enabling its specific hybridisation to one complementary DNA family. This would be an analogous situation to the hybridisation of the rRNAs.

In the hybridisation of 9s RNA to DNA, cross reaction between the α and β globin mRNAs and their DNA cistrons was expected, even assuming formation of well-matched hybrids. The properties of the hybrids formed between ^{3}H - 9s RNA and DNA are consistent, however, with a high degree of base mismatching. With the reservation that further RNase action on formed hybrids might give spurious results due to RNase sticking to the filters after the washing and counting procedures, the ^{3}H-9s RNA hybrids formed are unstable, having T_ms of 25°C below the theoretical and with sizes ranging from less than 20 nucleotides (70% of the total) to between 60 and 120 nucleotides (30% of the total). The hybrids may not therefore be long stretches of perfect base pairs such as would be expected for the hybridisation of globin mRNAs to their complementary DNAs. Any biological significance of the saturation values in terms of globin cistron redundancy is therefore unlikely.

The most closely corresponding of the DNA saturation values would give 3730-8005 DNA cistrons coding for 9s RNA in a haploid total mouse embryo "genome". The values are similar for the sperm genome and are higher for the haploid genomes of 14 day mouse embryo livers and duck erythroblasts. These higher values may not be

experimentally significant as they fall well within the range of values obtained for the different 9s RNA preparations.

The similar saturation values and stability of hybrids formed between homologous mouse-mouse and heterologous mouse-duck RNA - DNA hybridisations again indicate that the hybridisation is not to long DNA sequences similar to those transcribing the globin mRNAs, since the amino acid sequences of duck globins are approximatel 30% different to those of mouse (Scherrer, personal communication). Hybrids formed between mouse globin mRNA and duck globin DNA cistrons should therefore be fewer, assuming there was no relative amplificatio of these cistrons in the duck. They should also have lower thermal stabilities.

Theoretically, the redundancy of globin genes calculated is possible in terms of the cellular DNA content. In the 20% redundant fraction of mouse DNA, a possible 1750 genes the size of 9s RNA could each be reiterated 10,000 times. This is a minimum estimate for some since this fraction comprises a wide range of redundancy. The unique fraction alone could code for 4.35 million genes the size of a cistron for 9s globin mRNA. Since the number of cellular proteins is very much smaller, these calculations show a possibility for much amplification. Since much of the DNA may be quiescent or have a regulatory function, the possibility of extensive gene duplication cannot be calculated without more precise estimates of the percentage of the DNA which does not code for protein mRNAs.

The preliminary experiments of Bishop (personal communication) indicate that a highly-labelled 9s RNA isolated from cultured duck erythroblasts hybridises to DNA at $C_o t$ values corresponding to cistrons only duplicated to a small extent (<20).

The only other hybridisation of possible eucaryotic mRNAs is that of the histone mRNAs in sea urchins (Kedes and Birnstiel, 1971). The 9s mRNA was annealed to DNA under conditions of vast DNA excess. It hybridised with high efficiency to repetitive DNA sequences with a $C_o t_{\frac{1}{2}}$ of between 15 and 40. This represents a repetition frequency of about 400 for the DNA coding for histone messengers. The 9s RNA hybridised in 2 x SSC at 67°C (Gillespie Spiegelman technique) to DNA of high GC content, in accordance to a calculated high GC content for histone messengers. The T_m for the filter hybrid was higher than that for total DNA and this was again consistent with a high GC content for the hybrid. These results indicate that the hybridisation was to sequences very similar to those coding for the histone mRNAs.

These results differ from those on the hybridisation of globin mRNA. Since the histone messengers were probably of similar GC content (about 50%) to the globin messengers, the conditions for formation of hybrids with a high percentage of matched base pairs should be similar. Yet the hybrids formed by the histone messengers show a very high fidelity of base pairing to DNA relative to those formed by the globin mRNAs.

One possible explanation is that the histone DNA cistrons are very highly conserved whereas the globin mRNA cistrons have diverged through changes in the 3rd triplet bases although this is unlikely since the T_1 digest pattern of 9s RNA is relatively simple (Labrie, 1969; Williamson, personal communication).

A more probable explanation is the difference in $\frac{RNA}{DNA}$ ratios in these two experiments. The histone hybridisation was at very low RNA/DNA inputs whereas the globin hybridisation was at very high $\frac{RNA}{DNA}$ ratios. The possibility of an equilibrium hybridisation of short stretches of the globin 9s RNA to DNA at high RNA/DNA inputs must therefore be considered.

The 9s RNA hybridisation is specific for eucaryotic DNAs since there is negligible hybridisation to E.coli DNA. This does not exclude the hybridisation being non-specific with eucaryotic DNAs. Britten and Roberts (1971) have computed the possibilities of chance sequences of a given length being identical for the hybridisation of 2 DNA strands. For hybrids of 20 matched base pairs, there is a probability of 10^{-12} of a chance relationship between them, assuming all sequence permutations have equal probability. This would give a background hybridisation of 0.3% in the hybridisatio of 2 DNA strands of 3×10^9 nucleotide pairs which were unrelated except by chance. In the hybridisation of a 9s RNA to one of these strands, the background hybridisation would be reduced to 5×10^{-8}%, assuming a kinetic complexity of 2 for the 9s RNA. If the hybrids

formed were shorter, or had mismatched base sequences, the background hybridisation would be up to 6 orders of magnitude greater.

However, these calculations of probability do not assume any non-fortuitous relationship between base sequences in the DNA and RNA. These relationships do exist since mammalian DNA has been duplicated during evolution. Homologies of base sequences occurring between the sequences in 9s RNA and the mouse genome might therefore be frequent enough to account for the observed hybridisation levels. Such hybridisation of short base sequences might be an equilibrium reaction, giving hybrids with properties similar to those observed.

It is more difficult to account in this way for the heterologo hybridisation between mouse 9s RNA and duck DNA since the hybridisatio level was as great as in the homologous reaction and the hybrids formed were as specific. Families of repeated sequences common to different rodent species are found. These have similar, low thermal stabilities (50-60°C) after hybridisation under conditions of low specificity (0.1 M phosphate, 50°C; Rice, 1971). The heterologous reaction with mouse DNA is under more stringent hybridisation conditions and with a more distantly related species. Cross hybridisation would therefore be less probable.

An alternative explanation for the similar homologous and heterologous hybrids formed is that the hybrids are formed between the non-coding region of the 9s RNA and the DNA. Even the most accurate and lowest estimate of the molecular weight of 9s RNA

(170,1000 ± 13,000; Williamson et al., 1971) corresponds to a base sequence over 60 nucleotides longer than the 435 or 453 needed to code for the α and β globin chains respectively. These extra nucleotides may arise or be functional in several different ways.

The extra sequences might be transcribed from a part of the genome concerned with the co-ordinate regulation of mRNA synthesis such as is envisaged by Britten and Davidson (1969). Such "receptor genes" would be contiguous to the genes transcribing messenger sequences and might be simultaneously transcribed with them.

The model involves a large number of similar receptor genes responsible for the simultaneous activation of a distinct class of mRNAs. These would be activated by a specific inducer, such as a hormone. The activation would be mediated through the translation of a class of repeated RNAs interacting with the receptor genes. Many different sets of receptor genes are envisaged. However, it is difficult to see any advantage in transcribing a non-coding region required only for the regulation of mRNA translation and the transport of this non-coding region to the cytoplasm. The alternative theories for the role of extra sequences in mRNAs are more attractive since they postulate that the extra sequences are involved in the control of mRNA processing and translation. There would therefore be a biological significance for extra nucleotide sequences attached to cytoplasmic globin mRNAs.

The globin mRNAs may have at each end regions of self-complementary nucleotides giving them circular configurations, with a resultant resistance to attack by exonucleases. Many reticulocyte polysomes do have a circular conformation (Mathias et al., 1964). This would explain the relative longevity of globin mRNAs when compared to other messengers.

The mRNAs may have sequences at the 5′ ends necessary for recognition of the initiator codons. Non-coding sequences of 60 - 120 nucleotides precede the initiation codons in the RNA of bacteriophages Qβ (Billeter et al., 1969), MS2 (De Wachter et al., 1971) and R17 (Steitz, 1969; Adams and Cory, 1970). These sequences are very similar and in the case of MS2 and R17, there are fewer base differences between them than in the coding portion of the RNAs. This points to a very specific role for these sequences, perhaps in the formation of a specific secondary or tertiary structure for the correct initiation of protein synthesis (Steitz, 1969; De Wachter et al., 1971).

It is unclear how analogous is the situation for bacterial or eucaryotic mRNAs. Bacterial mRNAs may not have similarly long pre-initiation sequences. The existence of long pre-initiator sequences similar for different bacterial mRNAs would imply that bacterial RNA would anneal to repeated sequences in the bacterial DNA. This is not found experimentally. Perhaps a long and specific initiator sequence is not needed in bacterial systems due to the clos spatial coupling between transcription and translation. This does no

exist in eucaryotic systems so a control mechanism similar to that in phages might be necessary.

Much of the high molecular weight heterogeneous nuclear RNA seems to be rapidly turned over within the nucleus. If it is assumed that some is potentially functional mRNA, a post-transcription method of selecting this mRNA population and delivering it to the cytoplasm is necessary. Such a stepwise mechanism in the selection of transcribed RNAs for translation has been proposed to be cellularly more efficient than their differential transcription (Scherrer and Marcaud, 1968).

Adenine-rich polynucelotides have been found in rat liver microsomes (Hadjivassiliou and Brawerman, 1966). These were about 10s in size and were not attached to other RNA molecules. Similar purine-rich polynucleotides are found in ascites cell nuclei (Edmonds and Caramela , 1969) and in the nuclei of mouse liver (Lim et al., 1969; Lim et al., 1970a). Their synthesis was co-ordinated with nuclear RNA synthesis in the mouse liver nuclei. There is now evidence that in HeLa nuclei, poly A sequences 100 - 200 nucleotides long are covalently bound to the heterogeneous, high molecular weight nuclear RNA (Edmonds et al., 1971; Darnell et al., 1971). Since poly A sequences were also found associated with the rapidly-labelled polysomal RNAs from the cytoplasm (Edmonds et al., 1971; Darnell et al., 1971), a mechanism postulating that these poly A sequences adjoined mRNA sequences in the high molecular weight nuclear RNA and thus facilitated their processing for mRNA export to the

cytoplasm was proposed (Edmonds et al., 1971).

Polypurines were also found associated with RNAs in the soluble and microsome fractions from mouse liver ribosomes (Lim et al., 1970a, 1970b), associated with rabbit globin mRNA (Lim and Canellakis, 1970) and in the rapidly-labelled polyribosomal RNA component from mouse sarcoma 180 ascites cells (Lee et al., 1971). Similar mechanisms for the transport of mRNAs from the nucleus to the cytoplasm were therefore proposed.

There was no convincing evidence on whether the poly A sequences were transcribed with the total RNA or whether parts were added enzymically after transcription. Some of the poly A is actinomycin D-sensitive (Lim et al; 1970a, 1970b; Edmonds et al., 1971; Darnell et al., 1971). One mechanism of synthesis may theref be an enzyme - mediated synthesis from ATP (Heere and Holbrook, 1969).

An alternative, or secondary, function for these sequences in determining the translation lifetime of their mRNAs by a ticketing mechanism (Sussman, 1970) has been proposed by Lim and Canellakis (1970). The adenine-rich polymer associated with rabbit reticulocyte 9s RNA is unlikely to be a coding portion of the mRNA both due to its base composition, and the correlation of its length (50-70 nucleotides) with the length of the mRNA extra to that necessary for globin translation. Lim and Canellakis (1970) showed a diminution in the amount of this polypurine in the 9s RNA associated with the light polysomes or the monosomes. Since there is some evidence that the smaller polysomes are found in older reticulocytes (Evans and

Lingrel, 1969b), they concluded that a part of the polypurine might be removed on completion of each round of mRNA translation.

Different lengths of polypurines attached to other mRNAs might explain the differences in their stabilities; the most unstable mRNAs might have few extra sequences. For example, the actinomycin D - sensitive poly A, associated with free mouse liver polyribosomes, is about 20 nucleotides long. This might therefore mean the mRNAs were unstable. In contrast, the actinomycin D-independent poly A, associated with membrane - bound polyribosomes, is 3 - 5 s long. Its association with membrane-bound mRNA might make the RNA relatively stable (Lim et al., 1970a, 1970b).

These proposed functions and properties of the extra nucleotide sequences in the mRNAs would explain some of the hybridisation results with 9s globin mRNAs.

If the extra sequences were control regions translated with the mRNAs, the high levels of hybridisation would be due to the hybridisation of the translated receptor genes attached to the mRNA back to many such similar genes in the DNA. The hybridisation to similar, not identical, control DNA sequences would account for the poor quality of the hybrids formed. If the control sequences were conserved during evolution relative to the coding sequences, the hybridisation to duck DNA would be explained. The high levels of nuclear RNA hybridisation compared to cytoplasmic RNA (Denis, 1966; Shearer and McCarthy, 1967) would not fit this model unless it were assumed that the regulator sequences are synthesised in vast excess

compared to the amount of mRNA transcribed or that they were split off from the mRNA before it reached the cytoplasm. The hybridisation of adenine-rich regions in the mRNA which were concerned with either mRNA transport or translation would give a more convincing biological explanation for several of the characteristics of globin mRNA-DNA hybrids.

The polypurine regions associated with the globin mRNAs may not be transcribed from DNA. The existence of complementary pyrimidine stretches in the DNA is therefore unproven. Even if most of the polypurine regions were, however, synthesised enzymically, they could base pair with any pyrimidine stretches in the DNA. The work of Kubinski et al. (1966) and Szybalski et al. (1966) has shown that poly G forms stable hybrids with DNA, as does poly U. The hybridisation of poly U means that the DNA must contain A - rich regions and therefore by implication, T-rich regions but these authors could not detect any hybridisation between poly A and DNA in 1 x SSC.

Darnell et al. (1971) have shown that the portions of mRNA which do hybridise to DNA at low $C_o t$ values have an adenine content of greater than 50%. They concluded that either the poly A regions were themselves hybridising to the DNA or that they were attached to stretches of RNA hybridising with reiterated DNA and were not enzymically removed (Beers, 1960).

Although Lee et al. (1971) found that poly A was selectively retained on millipore filters at temperatures up to 24°C, it is

improbable that this was the explanation for the A - rich hybrids. They were formed at elevated temperatures and any binding of poly A to the filters would have been detected by adsorption of counts onto control filters containing no DNA. The retention of poly A sequences on filters previously treated in Denhardt's medium (1967), as in the experiments with globin mRNA, is unknown.

The hybrids formed by polypurines would have T_ms of about 65°C in 1 x SSC (Marmur and Doty, 1959) if they were composed of long stretches of adenine bases. The T_ms would be raised if there were guanosines in the sequence but would be much lower if the hybridised sequences were very short. Any mismatched regions would be resistant to the action of pancreatic ribonuclease.

The properties of the short, unstable hybrids formed by the globin mRNAs and DNA would therefore be predicted if the hybridisation were between polypurine non-coding regions and DNA. It is even possible that the 30% of the hybridised RNA, 60-120 nucleotides long, would be an undegraded, mismatched poly AG fragment. It is also possible, however, that this represents the hybrid formed by the rRNA contaminant in the 9s RNA preparation. Even if the hybridisation of the rRNA were less efficient at the temperature of annealing, it might be expected that it would form more stable and longer hybrid stretches than would the mRNAs.

Hybridisation of ticketing fractions of the globin messengers might be an explanation for some of the variation in hybridisation levels found for the different mRNA preparations. The proportion of

polypurines associated with the mRNAs from polysomes of different size classes differed by a factor of 2 (Lim and Canellakis, 1970). It is possible that the mouse reticulocytes did vary in the proportions of old and young reticulocytes in different batches. The content of polypurines in different batches might then also vary.

A methodological explanation might involve differential recovery of poly A sequences after the phenol extractions of total RNA at high salt concentrations (Lee et al., 1971).

9s mRNA preparations of different ages and sizes would also account for the minor variations between different 9s RNA preparations on 6% gels. If it were assumed that mRNAs for the same proteins had non-coding regions that were conserved relative to the coding sequences during evolution, the results would also explain the similar hybridisation between mouse globin mRNAs and mouse and duck DNAs. This is assuming that the non-codin regions are transcribed from the DNAs. Similar proportions of pyrimidine-rich regions in duck and mouse DNA must be postulated if the extra sequences were synthesised enzymically.

Assuming the hybridising regions were 80 nucleotides long, comprising 70 adenines and 10 guanines (Lim and Canellakis, 1970), their specific activity would be about 40% of the 9s RNA (50% GC content) when the calculation is made for the relative proportions of bases methylated by dimethyl suphate. The calculation for the number of DNA base sequences, 80 nucleotides

long, corresponding to the proportion of the genome hybridised is from approximately 100,000 (0.082% DNA hybridised) to 600,000 (0.54% DNA hybridised). Assuming the base sequences were 20 nucleotides long, the numbers range from 400,000 to 2,400,000. These are maximum estimates, which do not take into consideration that at least 50% of the hybrid formed may be ribosomal. Whether these numbers could be biologically possible, due to duplicated ticketing regions or large numbers of pyrimidine - rich regions is an open question.

The hybridisations performed with 9s RNA from 14 day mouse embryo livers gave quantitatively similar results to those for the methylated 9s mRNAs from reticulocytes. The uridine label would exclude any detection of hybrid RNA which was predominantly poly A, although the attachment of poly A regions to the RNA - DNA hybrid cannot be eliminated. However, it is unclear whether the two 9s RNA preparations were identical since the 9s RNA prepared from 14 day livers was more likely to be contaminated with other messengers such as those for histones. Analysis of the size and stability of the hybrid formed by this 9s RNA would have permitted a more conclusive comparison of the hybrids formed by the 9s RNAs from the different sources.

SUMMARY.

Summary.

1. The identification, isolation and characterisation of a mammalian mRNA required choice of a cell type which synthesises only one or a few proteins. The mammalian reticulocyte synthesises globin almost exclusively. This cell type can be isolated in large quantities and has low endogenous ribonuclease activity, thus making it a suitable system for the isolation of large amounts of undegraded RNA. Preliminary experiments indicated that the reticulocyte RNA component sedimenting at 9s had many of the characteristics expected of the globin mRNAs.

2. The characterisation of an mRNA which can only be labelled to a small extent *in vivo* and which is only about 1% of the total RNA requires the development of large-scale isolation procedures. Preliminary experiments designed to circumvent the difficulty of labelling reticulocyte 9s RNA *in vivo* involved the isolation of ^{3}H-uridi 9s RNA from cultures of 14 day mouse embryo liver cells. The technique of preparative polyacrylamide gel electrophoresis was adapted for the isolation of pure 9s RNA from total 14 day mouse embryo liver nucleic acids. The 14 day mouse embryo liver may contain cell types synthesisin 9s mRNAs for other proteins. Contamination of any globin 9s RNA with breakdown products of other RNA species was also likely since the livers contain some endogenous ribonuclease.

3. 9s RNA was therefore isolated from mouse reticulocytes. The development of techniques for separation of large amounts of RNA on

the zonal ultracentrifuge combined with the use of diethyl pyrocarbonate as an RNase inactivator permitted the isolation of large amounts of 9s RNA.

Preliminary experiments used the M.S.E. B-XV zonal rotor for the isolation of a fraction enriched in the 14s mRNP particle dissociated from polysomes by EDTA. The RNA isolated from the 14s mRNP particle was further purified by preparative gel electrophoresis.

A 9s RNA fraction was also isolated from total reticulocyte RNA. Since isolation of undegraded RNA was more routine using this method, conditions were developed for the isolation of a 9s RNA fraction, uncontaminated with RNAs of other size classes, after a single fractionation of the total RNA in the M.S.E. B-XIV zonal rotor.

4. The RNA was analysed for purity on 2.6% polyacrylamide gels. The microheterogeneity of the RNA in the 9s RNA region was analysed on 6% polyacrylamide gels. There were two major and several minor bands in the 9s region. There was some variation in the relative amounts of the minor bands in the 9s RNAs isolated from different batches of reticulocytes.

5. The molecular weight of the 9s RNA was determined by analytical ultracentrifugation. The value obtained was 170,000, equivalent to about seventy nucleotides greater than the theoretical estimate for RNAs coding for the α or β globins. Determination of the molecular weight by relative electrophoretic mobilities on analytical polyacrylamide gels gave a higher value, 225,000, thus demonstrating that the 9s RNA had different migration properties compared to the rRNA

marker species.

6. Isolation of large amounts of pure 9s RNA made possible the study of the hybridisation of this messenger - enriched RNA fraction to DNA. Since 9s RNA is only labelled to a small extent in vivo, it was chemically methylated with tritiated dimethyl sulphate. In this way, specific activities of up to 5,000 cpm/μg were obtained.

Techniques were developed for the hybridisation of dimethyl sulphate-labelled RNA to DNA. Such chemically-labelled RNA gave a higher background sticking to blank filters. This was reduced to less than 0.01% of the input RNA by hybridising at low temperatures in the presence of formamide. Background levels were further reduced by pre-incubating all filters in Denhardt's medium and by extensive purification of the RNA.

7. ^{3}H - methylated 9s RNA was hybridised to DNA by an adaption of the method of Gillespie and Spiegelman. At intermediate $C_o t$ values, the ^{3}H - 9s RNA hybridised to over 0.1% of the total DNA, although there was some variation in the saturation values for different 9s RNA preparations. The hybridisation levels were similar for total mouse embryo DNA and mouse sperm DNA. They were higher for 14 day mouse embryo liver DNA and for duck reticulocyte DNA, though within the range of values for the different 9s RNA preparations. Similar saturation values were obtained when hybridising in vivo - labelled 9s RNA from 14 day mouse embryo livers to total mouse embryo DNA.

Although saturation was only approached at high RNA/DNA inputs, the time course of hybridisation was rapid. This suggests that there are only a limited number of components hybridising in the 9s RNA region. Successive hybridisation of the same RNA solution to different DNA filters gave identical hybridisation levels. The hybridisation was not therefore due to the exhaustion of a minor hybridising RNA component in the RNA.

8. Various control experiments were performed. There was little hybridisation to E.coli DNA, showing the hybridisation was specific for eucaryotic DNAs. The competition with unlabelled 9s RNA was close to that predicted. Competition with unlabelled reticulocyte 18s and 28s RNAs indicated some contamination of the 9s RNA with rRNA breakdown products. Less than half the observed hybridisation could be due to rRNA hybridisation.

9. The characteristics of the ^{3}H - 9s RNA - DNA hybrids were investigated. The T_ms of the hybrids were much lower than predicted. Control experiments on the hybridisation of methylated and non-methylat ^{32}P-5s RNAs showed that methylation lowered the hybrid T_ms by about $7^{o}C$. However, the T_ms of the 5s and 9s RNA hybrids were still lower.

10. It is therefore improbable that the high hybridisation values are due to the hybridisation of the globin mRNAs to reduplicated globin genes. The specific hybridisation to eucaryotic DNAs might be due to the hybridisation of short stretches of the 9s RNA to homologous regions in the DNA, related either by chance or by ancestral cistron

duplications and subsequent divergence. Alternatively, the hybridisatio
may be between a non-coding region of the RNA and related DNA sequences.
Hybridisation of the polypurine regions found in globin mRNA to DNA
would account for many of the observed properties of the formed hybrids.

Abbreviations.

RNA	Ribonucleic acid.
DNA	Deoxyribonucleic acid.
mRNA	Messenger RNA.
tRNA	Transfer RNA.
rRNA	Ribosomal RNA.
mRNP	Messenger ribonucleoprotein particle.
A,C,G,U,T.	The bases adenine, cytosine, guanine, uracil and thymine, respectively.
PPi.	Inorganic pyrophosphate.
EDTA	Ethylenediamine tetra-acetic acid.
Tris	2-amino-2-(Hydroxymethyl)-propane-1:3-diol.
1 x SSC	0.15 M NaCl; 0.015 M sodium citrate, pH 7.0.
BSS	Hank's balanced salts solution (Paul, 1965).
DEP	Diethyl pyrocarbonate.
DMS	Dimethyl Sulphate.
E_{260}	Absorbance, 260mμ.

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