STUDIES ON THE PROTEINS OF RAT LIVER HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN PARTICLES.

by

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TO TRISH.

Νυνάκα μου.

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Abbreviations.

Standard abbreviations are in general as recommended in the "Instruction to Authors", revised edition of the Biochemical Journal (Biochem. J. (1978) 169, 1 - 27). In addition, the following abbreviations have been employed:-

bisacrylamide	NN'-methylene bisacrylamide
cpm	counts per minute
cyclic AMP	Adenosine 3′- 5′monophosphate
cyclic GMP	Guanosine 3′- 5′monophosphate
EDTA	Ethylenediaminetetraacetate
DNAse	Deoxyribonuclease
Hepes	N-2-hydroxethyl-piperazine N-2-Ethane-sulphonic acid
hnRNA	heterogeneous nuclear ribonucleic acid
hnRNP	heterogeneous nuclear ribonucleoprotein (particles)
HTC cells	Hepatoma tissue culture cells
IEF	Isoelectric focussing
NEPHGE	Non-equilibrated pH gradient electrophoresis
OD_{260nm}	optical density at 260nm
RNAse	ribonuclease
SDS	sodium dodecyl sulphate
TEMED	N N N' N' tetramethylethylenediamine

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SUMMARY

1. HnRNP particles were prepared by either low salt (pH8·0) extraction, or sonic lysis of purified nuclei. These complexes exhibited a characteristic sedimentation coefficient (40S) and buoyant density (1·39g.cm^{-3}). Possible artefactual protein adsorption to the particles during their preparation was looked for by the addition of exogenous labelled proteins. Under normal circumstances, no adventitious binding could be observed. Approximately 22 polypeptides, dominated by a group of 4 major and 3 minor "core polypeptides" of molecular weight 30,000-45,000, were detected when the particle proteins were fractionated by electrophoresis on one dimensional SDS-polyacrylamide gels. No ribosomal proteins or histones were present.

Two-dimensional analysis, employing isoelectric focussing (IEF) in the charge separation dimension, revealed the neutral and acid polypeptides as a complex mixture, but failed to resolve the major core species. An optimised non-equilibrated pH gradient electrophoresis technique (NEPHGE) was employed to circumvent the short-comings of the IEF-based system. Under these conditions the core polypeptides could be resolved into three chains of spots. In total over 90 species could be resolved by two-dimensional protein fractionation.

An examination of the tissue and species specificity of these proteins by two-dimensional electrophoresis revealed a general conservation of the core species, but a number of minor differences were detected in the neutral and acidic regions of the profile.

2. The core polypeptide microheterogeneity was examined in an attempt to determine its possible causes. Four of the detected polypeptides were significantly phosphorylated *in vitro*. These corresponded to the most acidic component of each of four groups of polypeptides.

It was suggested that phosphorylation may be the cause of at least some of the core protein heterogeneity. Tryptic peptide mapping of each individual core protein, by *in situ* iodination and trypsin digestion, suggested that core polypeptides consisted of four proteins each of which was modified by post-translational modification.

3. Two enzyme activities, found associated with 40S hnRNP particles, were investigated. Preliminary charcterisation of the possible RNA processing enzyme, Poly-(A)-polymerase, revealed that the major part of this activity resided in the non-particulate region of the gradient, and was not bound to the hnRNP particles.

Evidence for the association of a protein kinase activity with 40S hnRNP particles was obtained by sucrose density gradient analysis and gel exclusion chromatography. In both cases, a clear peak of kinase activity remained associated with the particle, but losses during the purification steps suggested that the enzyme was only loosely bound. The loss appeared to be part of a general degeneration of the complex during purification.

The endogenous phosphorylation of hnRNP particles was found to be stimulated by cyclic AMP, polyamines and manganese ions. Examination of the protein profile of hnRNP particles after *in vitro* phosphorylation in the presence of $[\gamma\text{-}^{32}P]$-ATP, revealed that a number of polypeptides were phosphorylated. These included some components of the core proteins and a number of acidic species. One acidic species of molecular weight 32,000 and pI 3·5, which was not abundant enough to be detected by staining, was particularly strongly phosphorylated. No quantitative differences in protein phosphorylation could be observed in the presence of cyclic AMP, polyamines or Mn^{++} ions and attempts to show that the phosphorylation occurred *in vivo* were inconclusive.

INTRODUCTION

1. ORGANISATION OF THE EUKARYOTE NUCLEUS.

In prokaryotes, transcription and translation are closely linked, and the translation of an mRNA molecule can frequently be observed before its transcription is complete. In such a system, the levels at which control of gene expression can occur are limited. Some prokaryote controls have been described which operate at the level of the ribosome, but the most important mechanism appears to be that of transcriptional control, as originally outlined by Jacob and Monod (1961).

In eukaryotes, there are considerable barriers between transcription and translation. Firstly, there are physical barriers in the form of the eukaryote nuclear membrane, and the distance which mRNA must migrate before it reaches the ribosomes. Secondly, there are chemical barriers in that the initial RNA transcripts must undergo extensive processing before they can function as messages. These barriers create an extra level at which the eukaryote cell can control gene expression. The most likely candidates for the exertion of such controls are nuclear proteins, and perhaps the most likely of these are the proteins which associate with nuclear RNA transcripts to form what are known as heterogeneous nuclear ribonucleoprotein particles (hnRNP). This thesis presents an analysis of rat liver hnRNP, and draws particular attention to a kinase activity which could play a role in the control of mRNA processing.

Before describing the experimental work on these proteins it is intended to review, briefly, the components of the eukaryote cell nucleus, the synthesis and processing of mRNA and the structure of nuclear ribonucleoprotein particles.

1.1. The Nuclear Envelope, Nuclear Lamina and Nuclear Pores.

The nuclear envelope is a double membrane whose inner cisterna is continuous with the endoplasmic reticulum. The nuclear envelope differs from the endoplasmic reticulum by the presence of pore complexes, which provide a direct channel of communication between the nucleus and

the cytoplasm.

The pore complexes appear to be distributed non-randomly across the nuclear surface (Severs et al, 1976), and the pore density of a nucleus varies with the tissue source. Thus, amphibian oocytes have many times the nuclear pore density of, for example, the mature erythrocyte of the same organism. (Scheer, 1973).

Transport across the nuclear membrane, presumably via the nuclear pore complexes, seems to be highly controlled. Certain species of RNA, for example, can exhibit a strictly vectorial transport, while other RNA species shuttle back and forth across the nuclear membrane (Goldstein, 1974). How this selectivity is brought about is unknown.

Associated with the nuclear membrane is a salt-resistant lamina meshwork (Aaronson & Blobel, 1975; Scheer et al, 1976) consisting of three main polypeptide species of mol. wt. 60,000 to 72,000 (Dwyer & Blobel, 1976). The relationship between these species and those associated with the nuclear "matrix" (Berezney & Coffey, 1974) is unknown. However, the possibility that the nuclear matrix and the nuclear lamina are different expressions, or morphotypes, of the same subnuclear apparatus has been raised (Faiferman & Pogo, 1975).

1.2.Chromatin.

1.2.1. General Structure.

Eukaryotes have their interphase genomic DNA associated with both histones and non-histone chromosomal proteins, in an ordered structural complex widely known as chromatin. The basic repeating unit of chromatin is the nucleosome or ν-body. This consists of a stretch of DNA approximately 140 base pairs long, arranged in a superhelix of 1·75 turns, around the outside of an octomeric histone core, (Finch & Klug, 1977). Each octomer consists of two copies of each of the histones H_2A, H_2B, H_3 and H_4, which are believed to exist as duplicate tetramer components.

Each nucleosome is separated from its nearest neighbours by a stretch of "spacer" DNA between 20 and 40 base pairs long. The length of this spacer region appears to depend upon transcriptional status, (Lohr et al, 1977), cell type, (Compton et al, 1976), and the length of time since replication (Murphy et al, 1978).

X-ray crystallography of chromatin reveals the nucleosomes as disc-like structures about 100Å in diameter and 57Å in height (Finch et al, 1977). Higher orders of chromatin structure arise from the coiling of linear arrays of nucleosomes. Thus, the chromatin fibre (100Å in diameter) coils into a solonoidal structure around 300Å in diameter (Finch & Klug, 1976), although some authors prefer to consider this type of fibre as consisting of "superbead" subunits of 8 nucleosomes per superbead (Renz et al, 1978).

1.2.2 The Heterochromatin/Euchromatin Paradigm.

The existence of two forms of interphase chromatin has been known for over half a century, (Heitz, 1928). Heterochromatin is genetically inert, the sites of RNA synthesis being localised in the diffuse euchromatin regions, (Littau et al, 1964). The quantity of euchromatin varies widely from tissue to tissue. In the mouse thymus lymphocyte it constitutes 20% of the total nuclear DNA component (Littau et al, 1964), while in the mouse hepatocyte it forms 90% (Yasmineh & Yunis, 1970).

The correlation between euchromatin and "active" (transcribing) chromatin is one that was drawn from the data of Littau et al (1964), and many other authors. In the case of the thymus lymphocyte, the small euchromatin content of the nucleus is approached by the quantity of DNA believed to be expressed as RNA (10-20%) in these cells. However, investigation by a number of authors on other systems has lead to the conclusion that the majority of euchromatin, like heterochromatin,is transcriptioanlly inert. Indeed, in the case of the mouse hepatocyte, only 6-7% of the

total genome is expressed, whereas nearly 90% of the same genome is present as euchromatin (Grouse et al, 1972). Transcriptionally active chromatin is thus a relatively small fraction of the nuclear euchromatin, and may represent as little as 2% of the total genome, (Aziz et al, 1979).

1.2.3 Structure of Transcriptionally Active Chromatin.

The chromatin of a differentiated cell is probably fixed structurally early on in differentiation, and maintained in a particular conformation during the "life" of that cell. Thus, by using a variety of probes, investigators have been able to detect significant differences between the structure of transcriptionally active genes, and transcriptionally inert genes, which are expressed in other situations.

An early approach to the question of structural differences between active and inactive chromatin has been the use of exogenous RNA polymerases in cell free chromatin transcription studies. The apparent selectivity of the RNA polymerase for regions believed to be transcribed in vivo, has been interpreted as being the result of the fine structure of the chromatin in these active regions (Axel et al, 1973; Gilmour & Paul, 1973).

Nucleases have been widely employed as probes of the structure of active chromatin. Staphylococcal nuclease appears to distinguish no differences between active and inactive chromatin (Axel et al, 1973), however, both pancreatic DNAse (DNAse I) (Weintraub & Groudine, 1976), and spleen DNAse (DNAse II) (Gottesfeld et al, 1974) appear to selectively digest active regions of the chromatin template, perhaps indicating structural differences in these regions from the rest of the chromatin.

The use of DNAse I in this sort of experiment has been particularly fruitful. Weintraub and Groudine (1976) have shown that a very brief digestion of chick erythrocyte nuclei with this enzyme, selectively attacks globin sequences. In a similar study, Garel and Axel (1976) showed that ovalbumin gene sequences in the chick oviduct were particularly sensitive

to this enzyme. Garel et al, (1977) have also shown that the chromatin configuration which lends itself to this particular nuclease sensitivity, is a feature held in common between gene sequences which are transcribed, even at rates as low as a few molecules per day.

Electron microscopic examination of active chromatin regions has revealed that all non-nucleolar transcriptionally active sites in Drosophila retain the typical nucleosomal periodicity (Laird et al , 1976). However, in the case of the milkweed bug (Oncopeltus fasciatus) the DNA/chromatin packing ratio of non-ribosomal transcription units is slightly smaller than in the inactive regions, indicating a degree of longitudinal expansion as a consequence of transcriptional activity, (Foe et al, 1976).

On the basis of EM studies (Foe et al, 1976) and DNA/chromatin packing ratio, that portion of the genome which codes for ribosomal RNA (rDNA) is not packed into nucleosomes. Nevertheless, the nuclease sensitivity of this fraction of the genome shows a sensitivity characteristic of DNA organised as nucleosomes, (Mathis & Gorovsky, 1976), and it remains possible that the differences are more artifactual than real. Other regions of high transcriptional activity, (e.g. the amphibian lampbrush chromosome) also appear to be devoid of nucleosomal structures (Scheer, 1978). There may be correlation, therefore, between the degree of transcriptional activity and the frequency of nucleosomes.

1.2.4. Chromosomal Proteins.

1.2.4.1. Histones.

Histones are highly basic proteins involved in the organisation of the coarse structure of eukaryotic interphase chromatin. Histones H_2A, H_2B, H_3 and H_4 are believed to be components of the nucleosome, whilst H_1 is probably located on inter-nucleosomal DNA regions, linking the DNA on either side of a single nucleosome (Gaubatz et al, 1978) or

linking non-adjacent nucleosomes (Christiansen & Griffith, 1977). H_1 may thus act to condense the chromatin template, and in this context its absence from regions of high transcriptional activity, such as Drosophila polytene chromosome puffs (Jamrich et al, 1977), is particularly interesting.

The histones undergo post-synthetic modification such as acetylation (H_1, H_2A & H_4), phosphorylation (all species), methylation (H_3 & H_4) and ADP-ribosylation (H_1), (See Isenberg, 1977). These modifications may have some role in the induction or repression of the transcription of genes (e.g. Tsai et al, 1976; Axel et al, 1975). Moreover, the high degree of conservation of the sites of modification, as well as overall structure of the histones, argues for their involvement in processes common to all cells.

1.2.4.2 Non-Histone Chromosomal Proteins.

Non-histone chromosomal proteins (NHCP) are broadly defined as all nuclear proteins other than the histones. NHCPs thus include structural proteins such as actin (Lestourgeon et al, 1975) and nuclear matrix components (Miller et al, 1979), and numerous enzymes, such as protein kinases (Kish & Kleinsmith, 1974), phosphoprotein phosphatases (Kleinsmith, 1978), histone methylases (Paik & Kim, 1971), RNA methylases (Wei & Moss, 1977), DNA methylases (Cato et al, 1978), RNA polymerase I, II and III (Chambon, 1974) and DNAse polymerases (Weissbach, 1977). In addition, various functionally ill-defined sub-fractions of NHCP have been isolated, such as DNA tight-binding proteins (Pederson & Bhorjee, 1975) and chromatin "scaffold" proteins (Laemmli et al, 1978; Campbell et al, 1979), which are potentially important components of nuclear structure and function.

The properties of the polypeptides associated with particular

subnuclear structures such as hnRNP particles, snRNP particles and the nuclear matrix will be discussed elsewhere. However, the proteins associated with chromatin bear special mention since there is evidence for the presence of controlling elements amongst their number.

Two dimensional fractionation of chromatin proteins has revealed their considerable complexity (Yeoman et al, 1973; MacGillivray & Rickwood, 1974). Evidence for the role in the control of gene expression has come from their apparent tissue and species specificity (Davies et al, 1972; Elgin & Bonner, 1972), although many of the proteins are held in common by widely divergent sources (Elgin & Bonner, 1970). However, the validity of using electrophoretic analysis to detect gene regulatory proteins has rightly been questioned (Pederson, 1974b, inter alia), mainly because the quantities of these regulatory proteins will be, by necessity, particularly low. The use of more sensitive immunological techniques (e.g. Chiu et al, 1974) has failed to resolve this difficulty.

One property expected to be held in common by the proteins likely to control gene expression is that of rapid turnover. The majority of chromatin proteins exhibit a slow turnover rate (Seale, 1975), however, two other subsets of proteins with a moderate turnover ($t_{\frac{1}{2}}$ = 5hrs.), and a rapid turnover rate ($t_{\frac{1}{2}}$ = 30min.) have been detected (Djondjurov et al, 1979, 1980). The possibility that the former group may be hnRNP particle proteins has been raised (Djondjurov et al, 1980), however, the intimate association of nascent hnRNP with the chromatin upon which it was transcribed makes the separate identification of the proteins involved particularly difficult.

A potentially rewarding approach to the study of NHCP involved in the control of gene expression, has been the use of in vivo transcription systems, employing exogenous RNA polymerase, and a chromatin template,

the protein components of which could be modified by reconstitution techniques. Early experiments using a variety of rabbit tissues as a source of NHCPs suggested that the type of RNA produced was dependent on the source of the NHCP used in the reconstitution template (Gilmour & Paul, 1970). Other experiments using chick oviduct (O'Malley et al, 1972) and synchronised cultured cells (Stein & Farber, 1972) showed a similar transcriptional dependence upon hormonal status and the stage of the cell cycle, respectively.

The use of cDNA probes to obtain a finer resolution and quantitation of the mRNA transcribed by E.Coli RNA polymerase, underlined the dependence of specific transcription on the source of the NHCP. However, a number of features cast considerable doubt on their reliability. Firstly, the fidelity of reconstitution of the chromatin template is difficult to evaluate. Secondly, the DNA used for the template is likely to contain "nicks" which will function as false initiation sites for RNA polymerases. Thirdly, E.Coli RNA polymerase which was used in many published analyses has been shown to give spurious translation products in the presence of cDNA probes.

One particularly interesting group of non-histone chromosomal proteins is the so called "High-Mobility-Group-Proteins" (HMG), (Johns, 1964). These are low molecular weight species which bind to chromatin and histones (Shooter et al, 1974; Goodwin et al, 1975), and have been assigned a structural role in the nucleus. Recent evidence has strongly implicated two of the HMG proteins in the maintenance of an "active" gene conformation. HMG proteins 14 and 17 have been found associated with actively transcribing genes (Weisbrod & Weintraub, 1978; Weisbrod et al, 1980). Various reconstitution experiments using HMG-depleted nucleosome preparations, and purified HMG 14 and HMG 17, have shown that the DNA of these nucleosomes is more sensitive to DNAse I

digestion than those without HMG 14 and HMG 17, indicating a more open ("active") conformation. This is the first indication of direct involvement of a defined NHCP in any aspect of the control of gene expression.

1.2.5 Structure of Genomic DNA.

The fine structure of eukaryotic structural genes is rapidly becoming more fully understood. It is now known that the majority of genes are not colinear with their final product. The genetic information encoded in the genomic DNA is interrupted, within a given gene, by transcribed, non-expressed "intron sequences". The gene thus becomes a mosaic of expressed,and non-expressed,regions which are ultimately brought together by processing of a precursor RNA molecule (See Fig. 1).

The typical intron/exon arrangement(shown in Fig 1b) has so far been described for rRNA genes (Wellauer & Dawid, 1977; Glover, 1977; Pellegrini et al, 1977), tRNA genes (O'Farrell et al, 1977; Knapp et al, 1978) and the genes of many proteins, for example, mouse, rabbit and human globin (Van Ooyen et al, 1979), human globin (Lawn et al, 1978), chicken ovalbumin (Breathnack et al, 1978), chicken conalbumin (Cochet et al, 1979), chicken ovomucoid (Catterall et al, 1979), Xenopus vitellogenin (Wahli et al, 1980), rat insulin (Lomedico et al, 1979), various immunoglobulin genes (Tonegawa et al, 1978, inter alia) and some adenovirus 2 genes (Maat & van Ormondt, 1979). The number of introns in these genes vary from one, for rat insulin II (Lomedico et al, 1979) to 33, for Xenopus vitellogenin (Wahli et al, 1980).

A few eukaryotic and viral genes appear, however, to lack intron sequences e.g. Adenovirus polypeptide IX (Alestrom et al, 1980), the yeast mitochondrial gene for cytochrome C (Smith et al, 1979) and sea urchin histone genes (Schaffner et al, 1978).

With the development of efficient DNA sequencing techniques, (Sanger et al, 1977; Maxam & Gilbert, 1977), and their application to the analysis of structural genes, there has been a recent trend towards scrutinisation of important DNA regions for the potential controlling elements. The three main sites investigated have been:- The 5′terminus and distal flanking sequences, the intron/exon boundary and the 3′terminus of the genes.

1.2.5.1 The 5′Terminus and 5′Flanking Sequences.

At the 5′terminus of a variety of genes, there are a number of common (and,therefore,interesting) sequences. Although there is no firm evidence on the initiation sites for the majority of genes so far examined, it is known that the Major-late transcription units of Adenovirus 2 begin at the first capped nucleotide of the mature mRNA (Ziff & Evans, 1978). By extrapolation from this result, the point of initiation of transcription can be mapped for a number of genes.

A comparison of the 5′termini of ovalbumin, conalbumin, adenovirus early-, and late-genes has revealed some common features (Benoist et al, 1980). The most consistent feature is an octet of bases, based on the model sequence:

$$5' \quad \overset{T}{\underset{A}{\ldots TATATAT \ldots}} \quad 3'$$

which maps between 25 and 32 nucleotides distal to the cap sequence. This region, known as the "Hogness Box", has been compared to a similar structure found in prokaryotic DNA, the "Pribnow Box", and may be the promotor sequence of these genes.

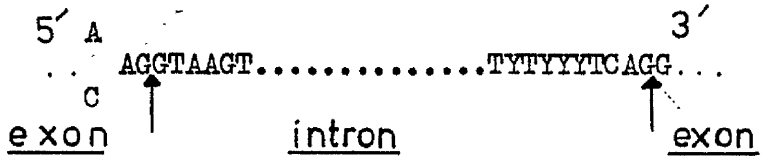
Cloned deletion mutants of sea urchin histone genes (Grosschedl & Birnstiel, 1980), from which this sequence is absent, are transcribed after microinjection into oocytes,but the 5′terminus of the mRNAs generated are heterogeneous. Birnstiel suggests that this region

probably acts as a guide for the RNA polymerase II, such that initiation is in the correct frame.

Although sequences distal to the Hogness Box appear not to be required for specific initiation of transcription *in vitro* (Wasylyk *et al*, 1980), several other regions of homology have been observed. Two blocks of DNA mapping at 67-71 and 83-93 nucleotides from the cap sequence appear common to the Adenovirus 2 Major-late gene and conalbumin. Regions of vague similarity to these are found in comparable positions in the adenovirus 2 early gene EIA and ovalbumin. These may represent more subtle controlling elements for the transcription of these genes.

1.2.5.2 The Intron/Exon Boundary.

A number of authors have recently published a comprehensive list of Intron/Exon boundary sequences, which has lead to the derivation of a model "consensus" sequence for both the 5′intron/exon boundary and the 3′intron/exon boundary (Benoist *et al*, 1980; Lerner *et al*, 1980). The thirty-six 5′regions and thirty-seven 3′regions so far examined all show considerable homology with a putative model sequence:



The possible role of this homology in the mechanism of RNA "splicing" is discussed elsewhere (Section 3.2). In order to investigate the possibility of more extensive homology between introns in the same gene, Robertson *et al*, (1979) and Benoist *et al*, (1980) have sequenced ovalbumin gene DNA fragment containing 3 introns and 4 exons. No significant similarities in the bulk of the intron sequences were observed. These authors, and others who employed an intron deletion approach (Thimmappaya & Shenk, 1979; Hamer & Leder, 1979), conclude

that only the region immediately around the intron/exon junction are required for effective RNA splicing.

1.2.5.3 The 3′ Terminus.

Examination of the 3′ terminus of a number of genes (Benoist et al, 1980) has revealed a number of features of interest. Firstly, the exact 3′ terminus is confused by the presence of a number of adenosine residues, (between one, in SV40 early mRNA (Reddy et al, 1979) and four in mouse dihydrofolate reductase (Nunberg et al, 1980)). The sequence immediately prior to the 3′ terminus appears to be based on the model sequence:

5′ TTTTCACTGC 3′

and between 14 and 30 nucleotides upstream from the 3′ end there is a sequence common to virtually all mRNAs so far examined. This particular sequence homology (5′AATAAA3′) present in all but the P. Milaris histone H_1 gene (Busslinger et al, 1979) was first noted by Proudfoot and Brownlee (1976). Its absence from the non-adenylated histone H_1 gene may be a clue to its function in vivo.

1.2.5.4 Arrangement of Genes on Chromosomes.

The arrangement of certain gene clusters has been delineated for a number of groups of repeated genes e.g. tRNA (Clarkson et al, 1979), rRNA (See Maden, 1971) and histones (Lifton et al, 1978). Recently evidence has been obtained for the fine structure of non-reiterated genes such as globin (Fritsch et al, 1980), ovalbumin (Heilig et al, 1980), and vitellogenin (Wahli et al, 1980). This data suggests that duplication of gene sequences is a widespread phenomenon, and that not all duplication events lead to fully expressed genes. In the case of ovalbumin, "echo genes" have been noted distal to the real ovalbumin gene, which

have considerable homology with the true gene (Heilig et al, 1980). The ovalbumin "pseudogenes" appear to be constitutively expressed at low levels in the chick oviduct.

A gene duplication of the Xenopus vitellogenin gene (Wahli et al, 1980) has lead to a further understanding of the way in which intron and exon sequences are elaborated during evolution. Comparison of the two similar genes has shown that the exon sequences "evolve" by point mutation, while intron sequences diverge by deletion events. Similar comparison of the rat insulin genes (Perler et al, 1980) has lead to the conclusion that the exon sequences remain relatively stable blocks, which are embedded in an evolutionarily mobile background of intron sequences. This type of model has interesting evolutionary implications.

2. NUCLEAR RNA.

RNA exists in eukaryotic cells in a number of different classes, which can be distinguished on the basis of their physical and chemical properties. Most RNA species are functionally expressed in the cytoplasm. Ribosomal RNA and tRNA are structural and mechanical components of the translational apparatus, while mRNA is the template upon which the nascent protein chain is synthesised.

In the nucleus, however, precursors to the cytoplasmic species (pre-mRNA or hnRNA, pre-rRNA, pre-tRNA) co-exist with the mature species, and with RNA species which have a nuclear function (e.g. snRNA). Since the major part of this thesis is concerned with pre-mRNA and its maturation, only this species, and those other nuclear species which may be involved with its maturation (i.e. snRNA) will be considered in detail.

2.1. HnRNA.

HnRNA, a designation proposed by Warner et al (1966), refers to the characteristic heterogeneity exhibited by this species of RNA with respect to its size. The possibility that at least part of the total hnRNA population is the direct precursor of cytoplasmic mRNA was initially put forward on the basis of a number of common features held between these species.

The polyadenylation of the 3′termini (Adesnik et al, 1972), internal methylation and 5′capping (Wei & Moss, 1975) of both mRNA and hnRNA support the idea that hnRNA may contain precursors of mRNA. Pulse-chase experiments have indicated that approximately 2% of labelled hnRNA may enter the cytoplasm as mRNA after lengthy chase periods (Brandhorst & McConkey, 1974). More recent experiments using cultured Drosophila cells (Levis & Penman, 1977) have revealed the presence of distinct hnRNA populations which exhibit a variety of decay rates. The polyadenylated hnRNA behaved as two kinetic components, with half-lives of 20 and 180 minutes, whereas the non-polyadenylated hnRNA (which constituted 80% of the pulse labelled material) decayed rapidly with a half-life of 10-15 minutes. The majority of pulse-labelled polyadenylated mRNA appeared during the first 30 minutes of the chase and correspond to about 14% of the loss of radioactivity from the hnRNA. Levis and Penman estimated that 40% of the total hnRNA population gave rise to cytoplasmic mRNA. These results were largely confirmed by Berger and Cooper (1978) who demonstrated the presence of two similar subsets of polyadenylated hnRNA in human lymphocytes. In this case, mRNA sequences appeared to be derived from the rapidly turning over class.

The best evidence for the presence of precursor mRNA molecules in the total hnRNA population has come from molecular hybridization

analysis, employing purified cDNA copies or cloned segments of the relevant gene. This type of analysis has been successful in the delineation of high molecular weight RNA species for globin (Bastos & Aviv, 1977; Neissing, 1978), ovalbumin (Roop et al, 1980), immunoglobins (Brack et al, 1978; Gilmore-Hebert & Wall, 1978), serum albumin (Strair et al, 1978) and Xenopus laevis vitellogenin (Ryffel et al, 1980).

In the case of X. laevis vitellogenin there is particularly good evidence for the presence of processing intermediates in the hnRNA population (Ryffel et al, 1980). The use of "R-loop" analysis involving vitellogenin hnRNA precursors and cDNA probes, specific for vitellogenin mRNA sequences, has revealed the presence of transcripts containing non-expressed intron sequences (See 1.2.5). While a number of R-loops revealed 12 introns in such transcripts, some contained fewer than 12, and were interpreted as processing intermediates. It seems likely that a distinct population of hnRNA molecules become mRNA molecules by virtue of correct processing and subsequent transport. The large size of the nuclear mRNA precursors in hnRNA are probably due to the presence of intron sequences in the initial transcripts.

2.2 SnRNA.

For more than a decade it has been known that low molecular weight RNA species occur in the nucleus (Weinberg & Penman, 1968). These include, besides tRNA and 5·8s RNA which are destined for transport into the cytoplasm, a group of nucleus restricted RNA species collectively known as snRNAs (small nuclear RNAs).

Early variations in the nomenclature of snRNA species now seem to have been resolved. The most frequently employed system is that of Busch et al (1971) which designates the six major species U_1 to U_6. All six species are nucleoplasmic in location, with the exception of U_3,

which has a nucleolar origin (Zieve & Penman, 1976). These species are between 90 and 220 nucleotides in length, contain internal methylated guanosine, uridine and adenosine residues (Ro-Choi & Busch, 1974), 5′trimethylated guanosine "cap" moieties, and pseudouridine (Busch et al, 1971). There are no detectable polyadenosine tracts on these species (Frederickson et al, 1974), and kinetic data shows that they are not metabolic breakdown products of hnRNA (Weinberg & Penman, 1969). U_1 and U_2 have been sequenced in their entirety (Ro-Choi et al, 1972; Ro-Choi & Busch, 1974; Reddy et al, 1974).

The considerable metabolic stability of some snRNA species (Weinberg & Penman, 1969; Hellung-Larsen et al, 1974), and their nuclear location have tended to suggest a role for these molecules in the regulation of gene expression. However, no direct involvement of any of these species in any nuclear process has been observed, although a wide range of suggestions have been made. The possibility that these species may be involved in the maturation of other RNA molecules (e.g. pre-rRNA or hnRNA) has been raised by a number of authors (Busch et al, 1971; Chambon, 1977; Lerner et al, 1980), and a limited measure of experimental support for this postulate has been obtained (See 3.2). The exact role (or roles) of these RNA species is still, however, a matter of conjecture.

3. PROCESSING OF EUKARYOTIC NUCLEAR pre-mRNA.

3.1 General Aspects.

The vast majority of eukaryotic primary gene transcripts are subjected to extensive processing in the generation of the mature RNA species. Splicing, capping, polyadenylation and internal methylation of the products of RNA polymerase II transcription combine in a well controlled process, by which the ultimate nature of the mRNA to be transported from the nucleus is decided.

Fig. 1.

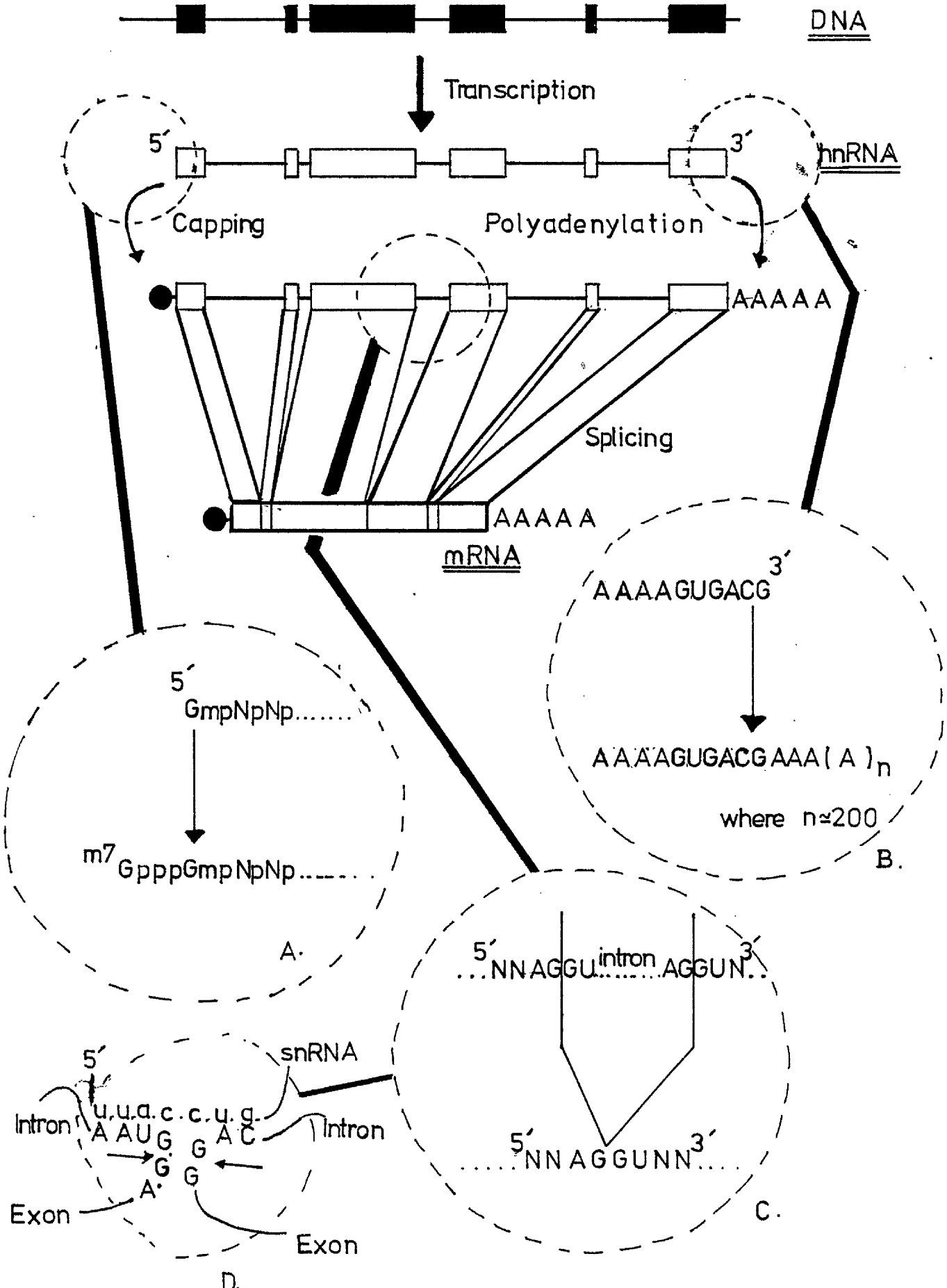
Processing of HnRNA.

A) - 5′capping of pre-mRNA.

B) - 3′polyadenylation.

C) - Splicing.

D) - Possible involvement of snRNA molecules in the location of splice junctions.


DNA
Transcription
5′
3′
hnRNA
Capping
Polyadenylation
AAAAA
Splicing
AAAAA
mRNA
AAAAGUGACG 3′
AAAAGUGACGAAA(A)n
where n≈200
B.
5′
GmpNpNp.....
m7GpppGmpNpNp.....
A.
5′ ...NNAGGU intron AGGUN... 3′
5′ .....NNAGGUNN..... 3′
C.
5′
snRNA
Intron
u.u.a.c.c.u.g.
A A U G A C
Intron
G G
G G
A
Exon
Exon
D.

3.2 Splicing.

The reality of the existence of "genes in pieces" has lead to a re-appraisal of the current concepts of transcription and RNA processing. The discovery that the primary transcripts of a number of genes (See Introduction 2.1. pg.15)contain both intron and exon sequences has indicated that the non-expressed intron sequences are removed post-transcriptionally. The consequent joining of the non-contiguous exon sequences has become known as "splicing".

Perhaps the most attractive model for the mechanism of the splicing reaction is one employing small nuclear RNA (snRNA) molecules. The possible involvement of these species in RNA metabolism had been suggested by several workers (Busch et al, 1971;Deimel et al, 1977). The most recent suggestion combines a modicum of experimental data with a summary of current intron-exon boundary sequence data (Lerner et al, 1980).

Evidence for the existence of these snRNA species as ribonucleoprotein has been obtained over the last 10 years (Enger et al,1974; Rein et al,1971) Raj et al (1975) defined a U_1-containing ribonucleoprotein particle, containing 10 polypeptides,two of which were phosphorylated. Lerner and Steitz (1979) employed antibodies produced by patients suffering from the auto-immune disease systemic lupus erythematosus to isolate these ribonucleoprotein particles. Two particular species of antibody were employed:anti-RNP,which forms immuno-precipitates with RNP complexes containing U_{1a} and U_{1b} snRNAs,and anti-Sm which precipitates ribonucleoprotein complexes containing U_2,U_4,U_5 and U_6,as well as U_1 containing species. Both antibodies precipitate the same 7 polypeptides. It seems, then, that snRNP particles exist as ribonucleoproteins containing 7-10 polypeptides and probably a single snRNA molecule. Based on a comparison of intron/exon boundary sequence data with the 5 ' end of U_1 snRNA Lerner et al (1980) have suggested a model for the possible

involvement of these snRNP particles in the splicing of RNA molecules.

Fig. 1d shows the possible mode of action of these snRNP particles in the splicing event. The 5′ terminus of U_1 snRNA is believed to hybridise to regions on either side of the intron sequence, and thus generate a double stranded RNA region. The dsRNA molecule thus formed is then cleaved (by an unknown mechanism), and the exon sequences in the mRNA, (and possibly the ends of the intron sequence (Arnberg et al, 1980; Halbreich et al, 1980)) are rejoined. A similar mechanism is envisaged for the maturation of E. Coli pre-tRNA by RNAse P, and the possibility that the snRNPs are themselves sequence selective RNAses has been raised (Lerner et al, 1980).

Splicing appears to be an absolute requirement for the production of stable RNAs. The failure of the SV40 deletion mutant d1-2350-I, which lacks precisely one intron, to produce stable RNAs transcripts, supports this fact (Gruss et al, 1979). However, the introduction of a β^{maj} globin intron sequence into the mutant, at the point of deletion and in the correct orientation, restores its ability to produce RNA transcripts (Gruss & Khoury, 1980). It thus seems likely that introns are functional elements in the production of most stable mRNAs.

3.3. Capping.

The addition of a "cap" structure to the 5′ terminal sequence of HnRNA is one of the earliest RNA processing events, (Salditt-Georgieff et al, 1980). Between 40% and 60% of HnRNA molecules, and virtually all mRNAs are capped. The standard cap moiety is a methylated guanosine residue, which is linked to the 5′ end of the RNA molecule by an unusual 5′ to 5′ linkage. There are 3 main cap structures, all of which are variants of the general formula: $^{5'}$GpppXpYp....

Cap 0, which is observed only in lower eukaryotes, (Sripati et al, 1976), is unmethylated at both positions 1 and 2. Cap I is methylated in the 2-0 ribose position of base 1. Cap II is a methylated derivitive of the Cap I structure, again by virtue of a 2-0 ribose methylation, this time of base 2.

Mainly Cap I structures are found in hnRNA molecules, the addition of the second methyl group, to form Cap II, seems to be a cytoplasmic event(Perry & Kelley, 1976; Frederici et al, 1976). In keeping with this observation is the known cellular distribution of the enzymes responsible for the various capping reactions. The three enzymes involved in cap formation, (RNA guanyltransferase, RNA (guanine-7-) methyl transferase and RNA (nucleoside-2-) methyl transferase), have been most extensively studied in the vaccinia virus system (Ensinger et al, 1975; Monroy et al, 1978). However, whilst both RNA guanyltransferase and RNA (guanine-7-) methyl transferase have been observed in HeLa nuclear extracts (Wei & Moss, 1977) and, perhaps significantly, in association with hnRNP particles (See 4.3.3.1), (Basjzar et al, 1978), RNA (nucleoside-2-) methyl transferase has only been characterised in HeLa cell cytoplasm (Keith et al, 1978).

The capped ends of eukaryotic mRNAs appear to have an important role in translation, possibly being involved in the formation of the initiation complex (Both et al, 1975). Other data (Furuichi et al, 1977) suggests that capped RNAs are more stable than uncapped RNAs under certain conditions, and this may, in part, account for the requirement for caps in translation.

3.4 Internal RNA Methylation.

Internal methylation of both hnRNA and mRNA seems to be an exclusive distinction of the eukaryotic system. There are very few

methylated bases (usually m^6A or, occasionally, m^6C) in hnRNA (4-6 per molecule) and fewer still in mRNA (1 or 2 per molecule), (Salditt-Georgieff et al, 1976), and these modifications are assumed to be limited to non-coding regions. The function of internal RNA methylation in hnRNA and mRNA is unknown.

3.5 Polyadenylation.

Between 20% and 30% of all hnRNA molecules (Jelinek et al, 1973), and between 70% and 90% of all cytoplasmic mRNA molecules contain poly-(A) tracts, of between 200-250 nucleotides, attached to their 3′ ends (Brawerman, 1974). Unlike internal oligo-(A) tracts (25 nucleotides long), which are the product of transcription of the DNA template (Edmonds et al, 1976), 3′ polyadenylation is a post-transcriptional event. The poly-(A) is probably added sequentially, since no free poly-(A) molecules can be detected after short (45 second) labelling periods (Jelinek et al, 1973), and the reaction is independent of RNA synthesis, since actinomycin D, whilst abolishing hnRNA synthesis, fails to prevent the polyadenylation of pre-existing hnRNA.

Poly-(A) addition to pre-mRNA takes place on the chromatin under the catalysis of the enzyme poly-(A) polymerase (EC.2.7.7.19), (Rose et al, 1977). En route to the cytoplasm, the poly-(A) tract is extended by the addition of 6-8 extra adenosine residues (Diez & Brawerman, 1974). The extent of polyadenylation also seems to be dependent on the levels of a nuclear poly-(A) specific endonuclease (Müller et al, 1974 and 1977; Matts & Siegel, 1979).

The control of polyadenylation may play an important role in the control of maturation of hnRNA. The investigation of the effects of oestradiol on quail oviduct poly-(A) endonuclease and poly-(A) polymerase (Müller et al, 1974), and the investigation of the effects

of the cell cycle on the same enzymes in a lymphoma cell culture system. (Müller et al, 1977) both seem to point to the control of polyadenylation by manipulation of the poly-(A) endonuclease. These results are confirmed for the hepatic poly-(A) endonuclease under the influence of corticosterone and amino acid starvation. (Matts & Siegel, 1979).

Rose and Jacob (1979, 1980) have shown that poly-(A) polymerase is a phosphoprotein. The equivalent enzymes from hepatocytes and hepatoma appear to vary in their degree of phosphorylation; hepatoma poly-(A) polymerase being more highly phosphorylated. In vitro phosphorylation appears to elevate the enzyme's template capacity and primer requirement, and more poly-(A) chains are synthesised (Rose & Jacob, 1980). These authors interpret these data as an indication that the post-translational modification of poly-(A) polymerase plays an important role in the control of this enzyme's activity in vivo.

The role of polyadenylation in vivo is unknown. Roles in mRNA nucleocytoplasmic transport (Darnell et al, 1971), mRNA stability (Kwan & Brawerman, 1972) or even RNA splicing (Bina et al, 1980) have been suggested, although none have, so far, been entirely validated.

4. HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN PARTICLES.

The association of proteins with nascent hnRNA has been known for almost 20 years (Gall & Callan, 1962). The role of these proteins in the controlled selection, processing and transport of mRNA sequences in the nucleus has been the subject of increasingly intense research.

4.1 Ultrastructural Investigations.

The most successfully employed systems for the ultrastructural investigation of transcription products have been the lampbrush chromosomes of amphibian oocytes and the giant polytene chromosomes

of dipteran salivary glands. Electron microscopic analysis of Triturus christatus oocyte lampbrush chromosomes clearly displays the beaded structure of the primary transcript (Malcolm & Sommerville, 1974)., whilst later biochemical evidence in a similar system (Malcolm & Sommerville, 1977) defined these beads as ribonucleoprotein particles.

In rat brain, electron microscopic analysis of perichromatin fibres and hnRNP particles,isolated by sonication of purified nuclei , displayed the similarity of these structures, particularly with respect to size and distribution (Devilliers et al, 1977).

From these, and other electron microscopic data, it is clear that hnRNA exists as ribonucleoprotein from the earliest moments after transcription, and that this ribonucleoprotein form of hnRNA (hnRNP particles) can be isolated from nuclei in a condition which approximates to the in vivo situation. Biochemical dissection of these hnRNP particles has further characterised their properties, and has provided data consistent with the idea that proteins associated with the hnRNP particle are important in the post-transcriptional processing of hnRNA.

4.2 Biochemical Investigation of HnRNP Particles.

4.2.1 Preparation of HnRNP Particles.

HnRNP particles have been prepared from many different tissues and cell types, by a number of different techniques. Basically, two approaches have been used:- 1). Extraction of particles from purified nuclei (e.g.Samarina et al, 1968; Ishikawa et al, 1969),or, 2)the isolation of particles released by nuclear disruption e.g. in hypotonic buffers (Moule & Chauveau, 1968; Raj et al, 1975), sonic disruption (Pederson, 1974a; Stevenin et al, 1974), detergent treatment (Stevenin et al, 1970), DNAse digestion of nuclei (Faiferman & Pogo, 1975) or french pressure cell disruption. (Faiferman & Pogo, 1970).

The isolation of two basic types of hnRNP particle has been reported. In cases where the endogenous nuclear RNAse levels are low, and where care has been taken to reduce contamination by exogenous RNAses, long RNA transcripts (approaching 200S (Pederson, 1974b)), complexed with a number of proteinaceous "beads" can be isolated. This so-called "poly-particle" preparation is most frequently isolated by sonic disruption of tissue culture cell nuclei (e.g. HeLa cells), or nuclei from tissues containing low levels of RNAse (e.g. brain). When high endogenous RNAse levels are present (e.g. rat liver), hnRNP particles are usually isolated as "monomer" subunits, which consist of a small piece of RNA (5-15S) coupled with the typical compliment of hnRNP particle proteins (See 4.3.). The inter-bead RNA which separates each monoparticle from its neighbour appears to be sensitive to RNAse digestion, and is probably the site of cleavage in the conversion of polyparticles to monoparticles (Samarina et al, 1968; Pederson, 1974a).

4.2.2 Properties of the Isolated HnRNP Particle.

Table 1 outlines the major physical and biochemical properties of hnRNP particles. It is clear from a consideration of this table that monoparticles and polyparticles are very closely related. They both exhibit characteristics peculiar to this class of ribonucleoprotein particle, which are frequently used as diagnostic aids for their identification. A characteristically high protein to RNA ratio, for example (4:1),as shown by buoyant density centrifugation and straightforward biochemical analysis, compares with the considerably lower value (1:1) for ribosomes.

Another striking feature is the considerable similarity of properties between hnRNP particles from widely different sources (compare, for example, rat liver hnRNP particles (Samarina et al, 1968)

Source	Sedimentation Coefficient.	Buoyant Density.	RNA Size.	No. of Proteins.	Ref.
Rat liver	38S up to 200S	1·39 g.cm^{-3} 1·39 g.cm^{-3}	8 S 15–20 S	45–50 45–50	A
HeLa cells	76S (-200S)	1·43–1·45 g.cm^{-3}	20–60 S	12–25	B
Slime mould	55S	1·41–1·43 g.cm^{-3}	15 S	-	C
Wheat embryo	-	1·4 g.cm^{-3}	15–30 S	-	D
Sea urchin embryo	-	1·4–1·55 g.cm^{-3}	10–30 S	-	E

A - Northemann et al, (1977, 1978).

B - Pederson (1974a).

C - Firtel & Pederson, (1975).

D - Ajtkhozhin et al, (1975).

E - Alfageme & Infante (1975).

Table 1. Properties of Isolated HnRNP Particles.

(buoyant density 1·4 g.cm^{-3}; size 30-200 S; RNA size 5-20S) with the equivalent particles from higher plants (Ajtkhoshin et al, 1975) (buoyant density 1·4 g.cm^{-3}; size 30-150S; RNA size 15-30S). It is perhaps the most interesting feature of these particles that super-imposed on this basic universallity of properties, there is some variety in a number of aspects of the properties of specific components of the hnRNP particles.

4.3 Components of HnRNP Particles.

4.3.1 Properties of the RNA Component of HnRNP Particles.

HnRNP particles contain two distinct sub-populations of nuclear RNA. HnRNA is the main component, representing about 10-20% of the mass of the hnRNP particle. The hnRNA component is characterised by its radiolabelling kinetics and sensitivity to actinomycin D (Pederson,1974), size distribution (Pederson, 1974; Samarina et al, 1968), base composition (Georgiev & Samarina, 1971), sequence complexity (Firtel & Pederson, 1975; Kinniburgh & Martin, 1976b; Maundrell & Scherrer, 1980), and the presence of a number of structural elements such as poly-(A) tails (Quinlan et al, 1977), oligo-(A) tracts (Kinniburgh & Martin, 1976a), oligo-(U) tracts (Kish & Pederson, 1977), double-stranded regions (Calvet & Pederson, 1977, 1978) and nuclear restricted sequences (Martin & McCarthy, 1972).

The second variety of nuclear RNA associated with hnRNP particles is the so-called snRNA species (See 2.2). These species are distinguished from the hnRNA component by a considerably slower turnover (Augenlicht, 1978). On polyacrylamide gels, 5 or 6 species can be seen (Deimel et al, 1977; Northemann et al, 1977; Guimont-Duchamp et al, 1977; Howard, 1978; Seifert et al, 1979). The presence of this slowly turning over component was originally predicted by Sekeris and Neissing (1975) who envisaged a structural role for these species.

However, recent work by Steitz and her co-workers (Lerner et al, 1980) has shown that these snRNA species can exist as separate ribonucleoprotein particles (snRNP's) which may be involved in the splicing of pre-mRNA (See section 3.2.).

4.3.2. Protein Components of hnRNP Particles.

4.3.2.1. General Considerations.

Proteins represent approximately 80% of the total mass of the hnRNP particle (Samarina et al, 1968, inter alia). Protein-protein interaction within these particles must therefore be considerable. Wide discrepency has persisted among workers in this field as to the precise number of polypeptide components in the hnRNP particle. Estimates vary from one (Samarina et al, 1968; Krichevskaya & Georgiev, 1969) to in excess of 90 (Maundrell & Scherrer, 1979).

In the majority of studies, however, the dominant feature displayed on one-dimensional SDS polyacrylamide gel electrophoresis analyses of hnRNP particle polypeptides is a group of 3-4 polypeptides of molecular weight 30,000 to 45,000 (Pederson, 1974; Karn et al; 1977; Beyer et al, 1977). While polypeptides of less than 25,000 seem to be absent from the protein profile, the basic pattern is elaborated by a variable quantity of minor polypeptides ranging from 45,000-150,000.

The discrepencies described in the literature for the polypeptide components of hnRNP particles prepared from different tissues and cell lines may, in part, be explainable by a degree of tissue specificity. While the major ("core") polypeptides appear to be conserved between species (Noll & Lukanidin, 1977; Karn et al, 1977; Brunel & Lelay, 1979), the minor species vary considerably. The difficulties of comparing analyses performed by different workers, frequently using different techniques and with different experimental objectives

considerably complicates this issue. Thus, Beyer et al, and Pederson (1974) describe very different minor protein components despite the fact that they both isolate hnRNP from Hela cells.

Two-dimensional fractionation techniques have recently been employed in the investigation of these polypeptides (Pagoulatos & Yaniv, 1977; Karn et al, 1977; Beyer et al, 1977; Maundrell & Scherrer, 1979; Suria & Liew, 1979; Brunel & Lelay, 1979). These analyses have lead to a fuller understanding of the complexity of the hnRNP particle polypeptides, and has revealed a number of interesting aspects, particularly with respect to possible post-translational modification of these polypeptides (See below).

4.3.2.2. HnRNP Particle "Core" Polypeptides.

The hnRNP particle "core" polypeptides are probably structural in function. Their predominance amongst the polypeptide components of the hnRNP particles (Beyer et al, 1977, inter alia) coupled with their high basicity (pI 8·0-10·0) (a feature held in common with ribosomal proteins and histones), a high level of glycine in their amino-acid composition (Karn et al, 1977; Beyer et al, 1977; Fuchs et al, 1980) and the presence of an unusual methylated arginine derivative, $N^{G}N^{G}$ dimethyl arginine, (Karn et al, 1977; Fuchs et al, 1980) point towards a structural role for these polypeptides.

The work of Fuchs et al (1979, 1980) on the core polypeptides of brain hnRNP particles suggests the presence of four highly derivatised polypeptides. Although the exact nature of the modification is unknown, neither methylation nor phosphorylation seem to account for the full range of modification (Brunel & Lelay, 1979; Fuchs et al, 1980). There has been a suggestion that glycosylation may be a significant feature of the core polypeptides (Fuchs et al, 1980)

although no firm data is available on this point. Neither acetylation nor ADP-ribosylation have been reported in hnRNP particle proteins.

The core polypeptides are also believed to be components of chromatin (Suria & Liew, 1979). However, the formation of hnRNP during the synthesis of hnRNA, and the difficulty in removing the nascent ribonucleoprotein during chromatin preparation, means that core proteins are likely to be present in preparations of chromatin, but are not necessarily chromatin proteins.

4.3.2.3 Minor Polypeptide Components of HnRNP Particles.

Very few of the minor polypeptide components of hnRNP particles have been ascribed functions. A number of these polypeptides have been observed to be "shared" with other nuclear compartments, such as nucleosol (Stevenin et al, 1978; Pagoulatos & Yaniv, 1978), chromatin (Stevenin et al, 1978; Suria & Liew, 1979) and the nuclear matrix (Faiferman & Pogo, 1975; Herman et al, 1978). One specific polypeptide, of molecular weight 110,000, appears to be common to both hnRNP particles and cytoplasmic mRNP particles (Schweiger & Kostka, 1980). These investigators suggest that this protein may be involved in nucleo-cytoplasmic transfer. Pagoulatos and Yaniv (1978), have tentatively identified actin as a component of CV1 monkey kidney hnRNP particles, although this may be due to contamination of these particles by actin polymers.

The 75,000 poly-(A) binding protein associated with polysomal mRNP particles, may also be a component of HeLa cell hnRNP particles (Kish & Pederson, 1975). It has been suggested that this polypeptide may be responsible for the transport of poly-(A) containing mRNAs from nucleus to cytoplasm (Schwartz & Darnell, 1976).

ENZYME ACTIVITY	SOURCE	REFERENCE
POLY-A SYNTHETASE	RAT LIVER	Neissing & Sekeris, (1972).
"CAPPING" ENZYMES	RAT LIVER	Bajszar et al, (1978).
RIBONUCLEASE	RAT LIVER	Neissing & Sekeris, (1970). Molnar et al, (1978).
dsRNA SPECIFIC RNASE	RAT LIVER	Molnar et al, (1978).
	HELA CELLS	Rech et al, (1979).
PROTEIN KINASE	RAT LIVER	Schweiger & Schmidt, (1975). Karn et al, (1977).
	HELA CELLS	Blanchard et al, (1977).
PHOSPHOPROTEIN PHOSPHATASE	HELA CELLS	Periasamy et al, (1977).

Table 2. Enzymes Associated With HnRNP Particles.

4.3.3 Enzyme Activities Associated with HnRNP Particles.

Table 2 lists the enzyme activities associated with hnRNP particles. These enzymes can be considered as being of two types:- potential RNA processing enzymes and potential controlling enzymes.

4.3.3.1 Potential RNA Processing Enzymes.

It is likely that the proteins associated with hnRNA in the hnRNP particle will include enzymic activities which may play a role in the processing of the RNA component of the hnRNP particle. The known extent of RNA processing has been outlined elsewhere (Section 3), and a consideration of Table 2 reveals a number of enzyme activities which could play a part in this processing.

Perhaps the most probable processing enzyme activities are the poly-(A) polymerase activities (Neissing & Sekeris, 1972) and the "capping" enzymes, guanyltransferase, N^7- and 2-O methyl transferase (Bajszar et al, 1978).

In addition to the poly-(A) polymerase activity, Neissing and Sekeris (1975) have detected other homoribopolymerase activities, capable of synthesising guanosine, cytosine or adenosine polymers. The significance of this finding is not known. A recent report (Rose et al, 1980) has suggested that the 75,000 poly-(A) binding protein (Kwan & Brawerman, 1972) may be identical to poly-(A) polymerase. Whether it is the detection of the poly-(A) polymerase activity of the known poly-(A) binding component of hnRNP particles (Kish & Pederson, 1975) that has been observed on hnRNP particles has yet to be clarified.

The processing potential of most of the ribonucleases so far discovered in association with hnRNP particles is fairly limited. The exonuclease activities described by Neissing and Sekeris (1970)

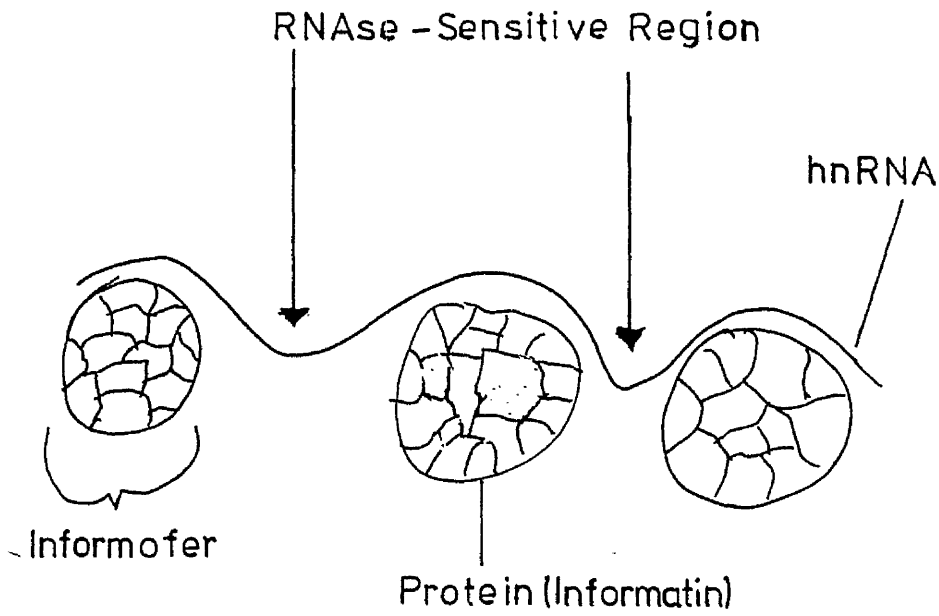
and by Molnar et al, (1978) are probably not important in RNA processing. However, the double-stranded RNA specific RNAse activities observed in both Hela cell hnRNP particles (Rech et al, 1979) and rat liver hnRNP particles (Molnar et al, 1978) are of particular interest. The distribution of double-stranded RNA regions on the hnRNP particle (Molnar et al, 1975), and their possible lack of association with proteins (Calvet & Pederson, 1978), coupled with the possible importance of double-stranded RNA regions in the delineation of specific RNA splice points (Lerner et al, 1980; Section 1.2.5.), has lead to speculation on the possible role of these hnRNP particle associated enzymes.

4.3.3.2. Potential Controlling Enzymes.

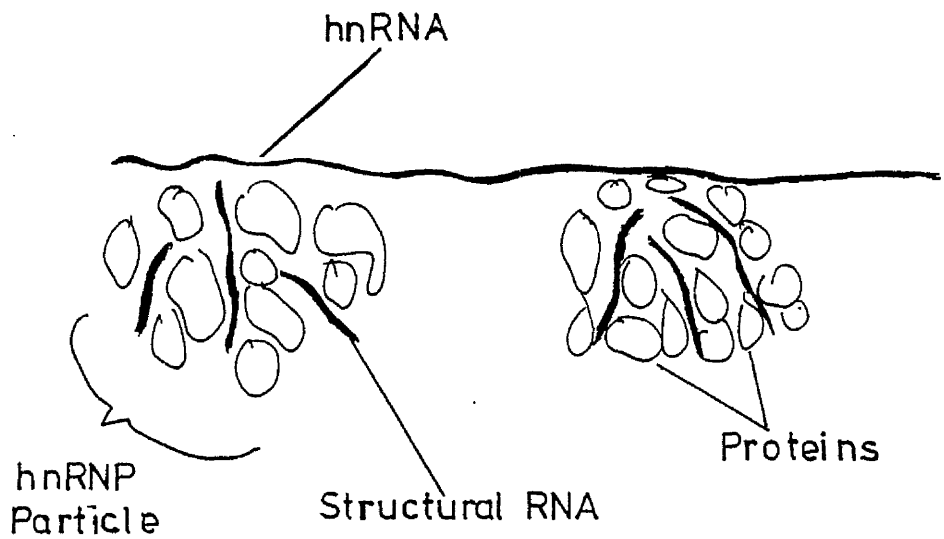
Phosphorylation of proteins is thought to play a role in the control of many processes. Phosphorylation of non-histone chromosomal proteins can be correlated with elevated rates of RNA synthesis (Kleinsmith, 1974), and both RNA polymerase I (Hirsch & Marcello, 1976) and RNA polymerase II (Kranias et al, 1977) exhibit increased rates of RNA synthesis after phosphorylation. Indeed, one of the enzyme activities associated with hnRNP particles (poly-(A) polymerase) appears to be stimulated by phosphorylation (Rose & Jacob, 1980). It is tempting to view the protein kinase activities of the hnRNP particles as possible regulatory enzymes.

The most well defined kinase activity associated with hnRNP particles is that described for the Hela cell system (Blanchard et al, 1975, 1977). Protein kinase activity seems to be "balanced" by the presence of a protein phosphatase activity (Periasamy et al, 1977) which is distinct from non-specific alkaline phosphatase activities possibly described for nucleoli (Olson et al, 1976). Partial purification

Fig. 2 MODELS FOR THE STRUCTURE OF HnRNP PARTICLES.



A. Informatin Model (Samarina et al, 1968).



B.(Sekeris & Neissing, 1975)

of the kinase activity (Periasamy et al, 1979) has revealed the similarity of this kinase activity to nuclear kinase NKI from rat liver (Thornburg et al, 1977). Whether partially purified,or on intact particles, the kinase activity appeared to be unresponsive to cyclic AMP, cyclic GMP or calmodulin,and displayed a pH optimum at pH 8.5 (Blamchard et al, 1977). Chromatography on Sepharose suggested a molecular weight of 48,000 for the kinase,and SDS polyacrylamide gel electrophoretic analysis of the Sepharose derived peak suggested the presence of two polypeptides of 25,000 and 28,000 indicating a possible dimeric form.

The kinase phosphorylates two of the hnRNP particle proteins of molecular weight 28,000 and 37,000 (Blanchard et al, 1977). On autoradiograms of two-dimensional gels (Brunel & Lelay, 1980),these appear as characteristic strings of ^{32}P labelled spots. A comparison of the polypeptides phosphorylated in vivo with those phosphorylated in vitro by this kinase activity,shows that they are similar (Brunel & Lelay, 1980) and peptide mapping reveals that the same tryptic peptides are phosphorylated (Lelay et al, 1978).

4.4 Models of the Structure of HnRNP Particles.

The earliest model for the structure of hnRNP particles was based on the interaction of the hnRNA component with a single 40,000 polypeptide species (informatin), (Samarina et al, 1968), (Fig.2.A). The informatin molecules thus formed proteinaceous "beads" around which the hnRNA was wound. Subsequent work by these,and other (notably Russian),investigators has been dedicated to the enshrinement of this model (Georgiev & Samarina, 1971; Kul'gushkin, 1977),and a number of demonstrations of the spontaneous RNA-free formation of informofer complexes have been made (e.g. Lukanidin et al, 1972).

However, the majority of workers in this field have rejected the "informofer" model. The heterogeneity of the protein components, for example, (See 4.3.2) speaks against such an oversimplified model, and the use of increasing salt concentration, detergents and limited RNAse digestion (Stevenin & Jacob, 1972; Stevenin et al, 1973; Stevenin & Jacob, 1974) has provided evidence for an interior location of the hnRNA component.

The structure of hnRNP particles is probably more complex than either model mentioned above. The use of limited RNAse digestion, and the subsequent analysis of the hnRNP particle RNA remaining (Augenlicht, 1978; Augenlicht et al, 1976) has shown that in HT29 cells, 10-25% of the RNA is protected from digestion, presumably by protein. Further, the RNA protected has a mean length of 26 nucleotides, and was a associated with a 40,000 polypeptide and two 66,000 polypeptides. However, the use of proteases as probes for the structure of hnRNP particles (Northemann et al, 1979a) has suggested that the main "core" polypeptides are protected in the hnRNP particles, and that a limited number of polypeptides are preferentially digested, indicating a more exposed location on the particle. This protection of the core polypeptides appeared to be dependent on the presence of the RNA components of the hnRNP particle, implying an ordered structure for these particles. RNAse and protease digestion analyses thus indicates that the RNA and core polypeptides are interacting within an outer "coat" of other protein components.

Recent data has been obtained from biochemical and electron-microscopic analyses of hnRNP particles for a degree of specificity in the interaction of proteins and nascent RNA. Augenlicht (Augenlicht, 1979; Wahrmann & Augenlicht, 1979) has shown that the sequences protected from limited RNAse digestion are enriched in certain nucleotide

sequences (notably AGC, GGC, AGGC and GACC), indicating a non-random association of the protein component with the RNA component. Beyer et al (1980) have shown dramatically that the ribonucleoprotein particles derived from D. Melanogaster non-nucleolar transcription units, are arranged in a non-random fashion along the nascent transcript. The electron-microscopic data could have considerable repercussions on our concepts of the structure of hnRNP particles, since an unforseen degree of sequence specificity must now be incorporated into the current models.

A further complexity which has emerged in recent months is the possible involvement of snRNA, as distinct from snRNP particles, in the processing of pre-mRNA (See 3.2). Whether all, or only a limited subset of hnRNP particles, contain an snRNP component is unknown. The possibility that hnRNP particles are a mixture of different morphotypes has been suggested by a number of authors (Houssais, 1977; Stevenin et al, 1979). However, Stevenin and co-workers (1979) raised the possibility that a subfraction of the 30S monoparticle hnRNP particles arise from artefactual reaggregation of sub-particles, dis aggregated by endogenous RNAse activity (See Discussion).

Finally, the very existence of the hnRNP particle per se has been questioned by Pogo and co-workers (Long et al, 1979). Sequential DNAse and salt extraction of isolated nuclei appears to locate rapidly labelled hnRNA in the nuclear matrix fraction. The exact nature of this interaction is unknown, and it is difficult to relate this data to the earlier work linking the perichromatin fibre with the hnRNP particle (See 4.1), (Malcolm & Sommerville, 1977).

4.5. Alterations in the Components of hnRNP Particles.

A potentially fruitful approach towards the investigation of the role of hnRNP particle components is to study the effects of agents which interfere with the normal mechanisms of gene expression. Thus, natural phenomena such as viral infection and hormonal stimulation of target tissues, or the use of drugs which interfere with nuclear metabolism, have been investigated with regard to their effects on hnRNP particles.

Perhaps the simplest approach in this vein, however, is to study the tissue and species specificity of the polypeptide components of hnRNP particles. Many workers have compared one-dimensional SDS gel profiles of the particle proteins of various species and tissues (e.g. Karn et al, 1977; Pederson, 1974a, inter alia) with the general conclusion that although the major structural proteins are highly conserved, the majority of tissue specificity resides with the higher molecular weight species (40,000-120,000). The use of antibody preparations prepared against the hnRNP particles of one species (usually rat) to investigate this question, has revealed a steady, phylogenetic relationship of decreasing cross-reactivity for species more distantly removed from the original source (Noll & Lukanidin, 1978).

Knowler (1976) found that administration of oestradiol to immature female rats resulted in an eight fold increase in the incorporation of (^{3}H)-uridine into uterine 30S hnRNP particle RNA. Similarly, Pederson (1974b.), in an investigation of the effects of glucocorticoids on various nuclear components of adrenalectomised rats, observed a marked increase in the incorporation of (^{3}H)-orotate into hnRNP particle RNA after hydrocortisone administration. This increase was combined with an increased synthesis of acidic hnRNP particle proteins, while there was no detectable effects on the synthesis of chromosomal

proteins under the same circumstances. The effects of viral infection on the components of hnRNP particles has been investigated in a number of systems. Stanton and Holoubeck (1977) observed that an early increase in RNA synthesis, induced in Human Amnion U cells by infection with polio virus, is accompanied by an increased incorporation of amino acids into non-histone nuclear proteins, particularly the main 40,000 mol.wt. polypeptide of 30S hnRNP particles. Two-dimensional protein fractionation of hnRNP particles prepared from SV40 infected CV1 monkey kidney cells reveals the presence of four polypeptides not present in the control condition (Pagoulatos & Yaniv, 1978). However, the use of an IEF-based two-dimensional fractionation which is inadequate for the resolution of the highly basic core polypeptides (See Results), and the presence of the viral capsid protein VP1 and actin on these gels, leaves this data open to question. One-dimensional analysis of Adenovirus 2 infected HeLa cell hnRNP particles has revealed few changes in the polypeptide with respect to the control condition, (Gattoni et al, 1980).

The effects of the drugs α-amanitin, D-galactosamine, and actinomycin D have been compared with respect to their effects on rat liver hnRNP particles. The RNA synthesis inhibitors α-amanitin and actinomycin D both decreased the incorporation of labelled precursor into hnRNA by 15-40% after two hours, with a similar reduction in the yield of hnRNP particles. A slight increase in the buoyant density of hnRNP particles after administration of these drugs may indicate a relative depletion of the protein component (Louis & Sekeris, 1976).

Extension of these studies to include a third inhibitor D-galactosamine, gave somewhat different results, (Gross et al, 1977; Stunnenberg et al, 1978). Both D-galactosamine and actinomycin D

reduced nuclear RNA synthesis to 20% of control levels three hours after administration. However, while Actinomycin D reduced the yield of hnRNP particles to 78% of control levels, D-galactosamine administration decreased the yield to 41% (Gross et al, 1977). It is not clear whether this difference is caused by an increased loss of hnRNP particles due to stimulated transport, or effects on the nuclear envelope.

All three inhibitors appear to deplete the nucleus of the polypeptides normally associated with hnRNP particles (Stunnenberg et al, 1978) The possibility that these proteins enter the nucleus from a cytoplasmic store has been raised on the basis of these results.

The administration of cyclohexamide to whole animals leads to a stimulated transport of nuclear ribonucleoprotein particles (Chih et al, 1979). Similar experiments by other workers, however, suggest that by two hours after administration, the inhibitor causes a specific depletion of two hnRNP particle proteins of mol. wt. 103,000 and 110,000 (Kostka & Schweiger, 1980). The same two polypeptides are also found associated with mRNP particles. It is thus possible that the depletion effect is due to the transport of these proteins into the cytoplasm with mRNA sequences.

Finally, the induction of carcinogenesis in rat liver by the administration of 3-methyl-4-diethylaminoazobenzene, has been found to alter specifically the polypeptide profile of hnRNP particles (Yoshida & Holoubeck, 1976; Patel & Holoubeck, 1976). One protein in particular is absent in the hepatoma hnRNP particles. Similar results had previously been obtained in the comparison of normal liver and hepatoma hnRNP particles (Albrecht & Van Zyl, 1973). In this case a polypeptide of mol. wt. 125,000 became depleted in the hepatoma particles.

5. MESSENGER RIBONUCLEOPROTEIN PARTICLES.

Messenger ribonucleoprotein particles (mRNP) were first characterised from fish embryo cytoplasm by Spirin and co-workers (Belitsina et al, 1964) and have since proved to be a universal component of actively metabolising cells. Two main mRNP populations may be defined. In Hela cells, for example, between 40 and 60% of labelled mRNA is associated with the polysomes (i.e. being actively translated), the remainder is found as "free" mRNP particles (Spohr et al, 1976). Only a small percentage of the mRNA component of free mRNP particles can be chased into polysomes (Mauron & Spohr, 1978). In avian erythroblasts, where globin mRNA is the most abundant species, measurement of the globin mRNA content of "free" and polysomal mRNP particles indicates that free mRNP contains little globin message despite a continual expression of globin on the polysomes. Thus, the bulk of free mRNP appear to contain non-expressed mRNA species.

Scherrer and co-workers (Maundrell et al, 1979) have extended this two compartment model by incorporating a "transfer pool" of mRNP, present as a subset of the free mRNP, but destined to become translated. Unpublished data referred to in the above article, compares both sequence complexity and cell free translation of the mRNA sequences in both particle types, and appears to agree with such a model.

Each type of mRNP appears to contain a characteristic subset of polypeptides with very little overlap between those associated with free mRNP, polysomal mRNP and hnRNP particles (Liautard et al, 1976; Ochinnikov et al, 1978). In the well characterised avian erythroblast system, the polysomal globin mRNP particle (15S) contains 9-10 polypeptides of molecular weight 40,000-120,000 (Morel et al, 1973). The free globin mRNP particle (20S) contains about 12 major species of mol. wt. 20,000-120,000, none of which are found on the 15S polysomal

mRNP particle (Vincent et al, 1977). The 20S particle can be further fractionated on high salt sucrose gradients into four peaks of 19S, 16S, 13S and 4S, each containing a characteristic subset of the proteins of the 20S complex (Vincent et al, cited in Maundrell et al, 1979).

One possibly significant observation in the comparison of the polypeptide profiles of the various mRNP particles of avian erythroblasts, is the presence of a 73,000 mol.wt. "poly-(A) binding" polypeptide in both hnRNP (Maundrell et al, 1979) and polysomal mRNP (Morel et al, 1973). The free mRNP, however, which are polyadenylated, lack the 73,000 polypeptide. Nuclease digestion of the 20S mRNP particle has suggested that a more heterogeneous group of polypeptides is associated with the poly-(A) tract (Maundrell et al, 1979).

It has been proposed (Spirin, 1978), that the protein components of the mRNP particles are important mediators of translation efficiency. Thus, these proteins may be involved in the post-transcriptional phase of the control of gene expression. Corroborative evidence for this possibility has come from the observation that some translational initiation factors and elongation factors have a considerable RNA binding capacity (Ochinnikov et al,1978), and a protein kinase activity, which may have a regulatory role, has been found in association with both free and polysomal mRNP particles. (Egly et al, 1976). This evidence is, however, largely circumstantial.

MATERIALS AND METHODS

1. MATERIALS.

1.1 Radio-isotopes and materials for liquid scintillation counting:

A. Radiochemical Centre, Amersham, Bucks.

L - [U - ^{14}C] Leucine	(324mCi.mMol.$^{-1}$)
[5,6 - ^{3}H] Uridine	(47Ci.mMol.$^{-1}$)
[8 - ^{3}H] ATP	(20 - 30Ci.mMol.$^{-1}$)
[γ- ^{32}P] ATP	(20Ci.mMol.$^{-1}$)
"	(2,000Ci.mMol.$^{-1}$)
"	(5,000Ci.mMol.$^{-1}$)
[γ- ^{32}P] GTP	(20Ci.mMol.$^{-1}$)
^{32}P (Orthophosphate)	(10mCi.ml.$^{-1}$)

B. Regional Radio-isotope Dispensary, Western Infirmary, Glasgow Scotland.

^{125}I (IMS 30) Carrier free (250mCi.ml.$^{-1}$)

C. New England Nuclear,

[γ- ^{32}P] ATP (2,000Ci.mMol.$^{-1}$)

D. International Enzymes, Windsor, England.

2,5 Diphenyloxazole (PPO) - Scintillation Grade.

E. Fisons Scientific Apparatus, Loughborough, England.

Hyamine hydroxide - 1 M solution in Methanol.

1.2 Reagents for Electrophoresis.

A. British Drug House Chemicals Ltd., Poole, Dorset, England.

Acrylamide - Electrophoresis Grade.

Methylene-bis-Acrylamide - Electrophoresis Grade.

Coomassie Blue R250.

Urea - AristaR Grade.

SDS - Electrophoresis Grade.

Ammonium persulphate - Analar Grade.

B. Koch-Light Laboratories, Colnbrook, Bucks, England.

Bromophenol Blue

Toluidene Blue.

Methylene Blue.

NNN'N' Tetramethylethylenediamine (TEMED).

β-Mercaptoethanol.

C. Pharmacia, Uppsala, Sweden.

Ampholines-40% solution :- pH Ranges 3 5-10, 5-7, 9-11.

D. Fluka, Busch, Switzerland.

Formamide (puriss).

1.3 Hormones and Metabolites.

A. Sigma Chemical Co., London, England.

ATP, Sodium Salt.

Creatine phosphate.

cyclic 3'5' AMP.

cyclic 3'5' GMP.

Dibutyryl cyclic 3'5' AMP.

Hydrocortisone.

Spermine.

Spermidine.

Putrescene.

B. Merck, Sharp and Dohme Ltd., Hoddesdon, Herts.

Dexamethasone Sodium Phosphate.

C. P-L Chemicals Inc., Wisconsin, U.S.A.

ATP, Sodium Salt.

1.4 Miscellaneous.

All other reagents were AnalaR grade , and purchased from B.D.H, England.

2. METHODS.

2.1. Isolation of Sub-cellular Organelles.

2.1.1 Preparation of Nuclei.

2.1.1.1 Preparation of Rat Liver Nuclei.

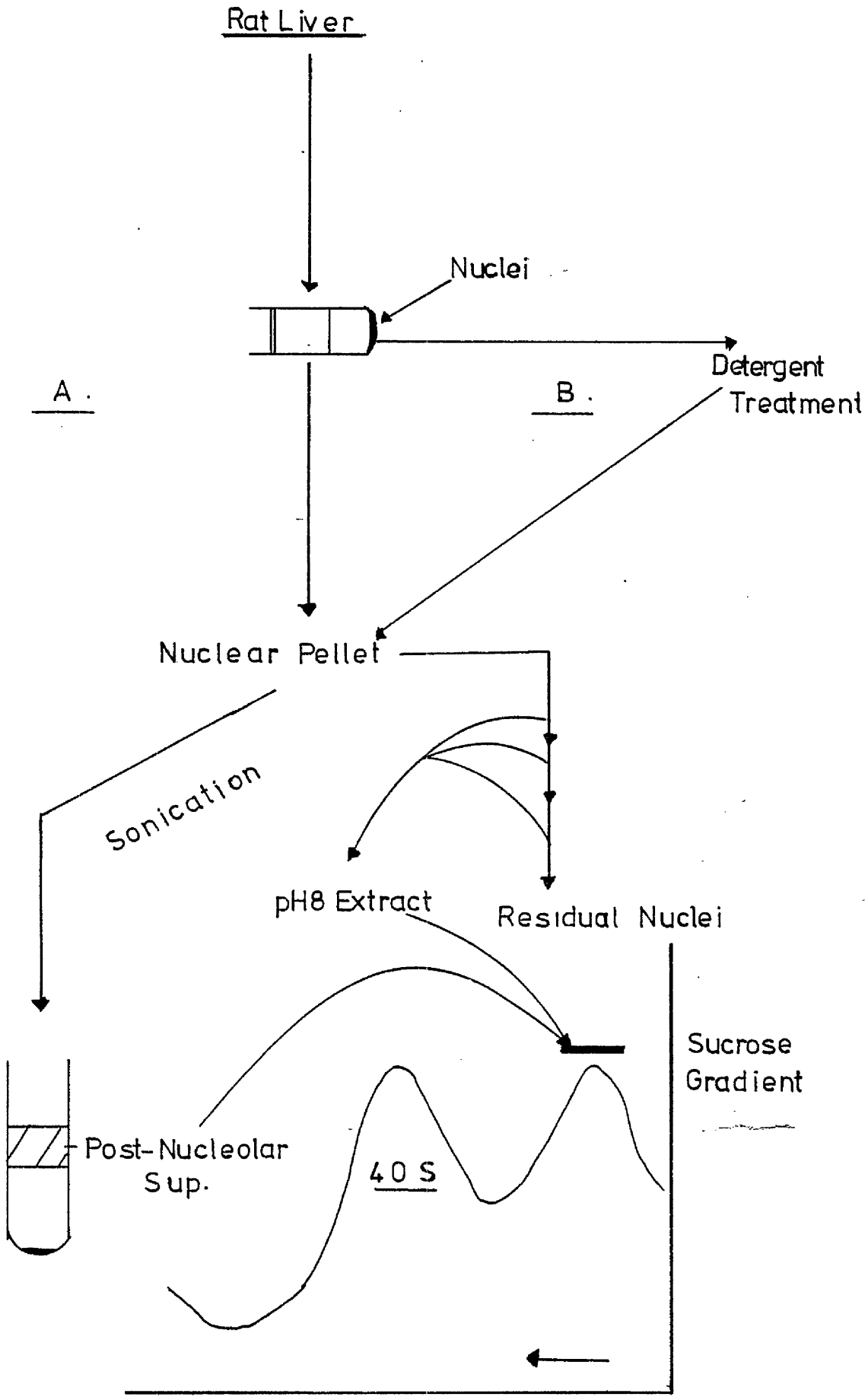
Rat liver nuclei were prepared by a modification of the method of Chauveau et al (1956). The livers of recently killed rats were finely chopped with dissecting scissors and then homogenised at 30 ml per liver in 2·3M sucrose, 10mM $MgCl_2$ using 3 strokes of a Potter-Elvehjem glass/teflon homogeniser. The resulting suspension was filtered through four layers of cotton muslin and the filtrate layered over 7 ml cushions of 2·3M sucrose, 10mM $MgCl_2$ in cellulose nitrate SW 27 centrifuge tubes. The homogenates were then centrifuged at 40,000 g for one hour in a Beckman SW 27 centrifuge.

The supernatent was aspirated and the purified nuclear pellet resuspended in a buffer appropriate for the next stage of the experiment (See Methods 2.1.2.1 and 2.1.2.2). In some experiments, a further purification step was employed. The nuclear pellet was resuspended in 0·25M sucrose, 25mM KCl, 10mM Tris.HCl pH 7·4, containing 1% $^{w}/_{v}$ Triton X-100, as described by Blobel and Potter (1966). Membrane-free nuclei were recovered from suspension by a brief centrifugation at 3,000 g in a Sorval HB 4 rotor, and employed in the preparation of hnRNP particles as described in Section 2.1.2.1 and 2.1.2.2. All steps were performed at $0^{\circ}C$ - $4^{\circ}C$.

2.1.1.2 Preparation of Rat Brain Nuclei.

Rat brain nuclei were prepared after the method of Stevenin and Jacob (1972). The brains of 40 adult rats (both male and female) were removed and stored at $-20^{\circ}C$ before use. After thawing at 0°- $4^{\circ}C$ for 3 hours , the brains were finely chopped, and then homogenised in

Fig. 3 PREPARATION OF RAT LIVER HnRNP PARTICLES.



a Potter-Elvehjem glass/teflon homogeniser in 0·3M sucrose, 10mM Tris.HCl pH 7·0, 25mM KCl, 2·5mM $MgCl_2$. The homogenate was filtered through two layers of muslin gauze and made 1·3M with respect to sucrose, by the addition of an equal volume of 2·0M sucrose, in the above buffer. Sodium deoxycholate was added to 0·1% $^{w}/_{v}$ and the crude nuclear fraction recovered by a low speed (1650 g) centrifugation for 10 minutes.

The pellet was resuspended in 2·0M sucrose, 10mM Tris.HCl pH 7·0, 25mM KCl, 2·5mM $MgCl_2$ layered over a 10 ml cushion of the same medium and centrifuged at 63,800 g in a Beckman SW 27 for 45 minutes. The nuclear pellet from this step was used in the preparation of rat brain hnRNP particles by sonication technique. (See 2.1.2.2.). To minimise degradation of nuclear components, all stages were performed at 0°- 4°C, and RNAse free technique was employed throughout.(See 2.5.2).

2.1.1.3 Preparation of HTC Cell Nuclei.

Pelleted HTC cells were resuspended at 5 ml per burler in 1% $^{w}/_{v}$ Triton X-100 in H_2O, by gentle homogenisation in a Potter-Elvejhem glass/teflon homogeniser. After standing on ice for 10 minutes, nuclei were recovered by centrifugation at 800 g for 5 minutes. The lysis procedure was repeated, and the pellet finally washed thrice in RSB (10mM Tris.HCl pH 7·4, 10mM NaCl, 1·5mM $MgCl_2$). The pellet obtained from the final wash was used for further experimentation as described in Fig. 11 .

2.1.2 Preparation of HnRNP Particles.

2.1.2.1 Extraction Technique. (Samarina et al, 1968).

Purified nuclear pellets, prepared by a modification of the method of Chauveau et al, (1956), (See Methods 2.1.1), were resuspended

in STM 7 buffer (0·1M NaCl, 1·0 mM $MgCl_2$, 10mM Tris.HCl pH 7·0), (3-5 ml per liver) and extracted with gentle stirring at 0°C for 20 minutes. Nuclei were recovered from suspension by a 10 minute centrifugation at 6,000 g in a Sorval HB 4 rotor.

The "pH 7" extract obtained in this way contains no, or only very low levels of, hnRNP particles and was routinely discarded. Three further 30-45 minute extractions of the residual nuclei were then performed at 0°C with the same buffer, adjusted to pH 8·0 (STM 8). The pH 8·0 extracts were pooled and applied to the top of a 12 ml 15% - 30% $^{w}/_{v}$ sucrose density gradient at 1·5 ml of STM 8 buffer per gradient. Gradients were centrifuged at 78,000 g in a Beckman SW 40 rotor for 15 - 17 hours. The completed gradients were harvested via a narrow bore tube introduced from the top of the gradient using a Sigma motor pump, and the OD_{260nm} was continuously monitored by passage through flow cell of a Gilford 240 spectrophometer. Portions of the gradient containing hnRNP particles and, in some experiments, other fractions of the gradient were collected for further analysis.

2.1.2.2 Sonication Technique. (Pederson, 1974b).

Purified nuclei (See Methods 2.1.2 and 2.1.3), purified by the method described in 2.1.1.1 (Chauveau et al, 1956), were resuspended in 0·88M sucrose, 25mM NaCl, 5mM $MgCl_2$, 50mM Tris.HCl pH 7·0 and recovered from suspension by centrifugation at 5,000 g for 5 minutes, in the Sorval HB 4 rotor. The purified nuclei were washed twice in 10mM NaCl, 1·5mM $MgCl_2$, 10mM Tris.HCl pH 7·0 (RSB) and resuspended in the same buffer at between 10^7 - 10^8 nuclei per ml for sonic disruption (2 x 10 seconds at 1·5A in an MSE ultrasonic power unit). Nuclear lysis was monitored by phase contrast microscopy. The sonicate was layered onto 25 ml of 30% $^{w}/_{v}$ sucrose in RSB , and centrifuged at

4,500 g for 15 minutes, in a Sorval HB 4 rotor. The material remaining above the sucrose layer was used for the preparation of hnRNP particles by further fractionation on 15% - 30% $^{w}/_{v}$ sucrose density gradients, as described in section 2.2.1. Gradients were harvested and simultaneously monitored at 260nm by passage through a Gilford 240 spectrophotometer flow cell.

2.1.3 Preparation of Ribosomal Subunits.

Rat livers were finely chopped with dissecting scissors and washed twice with solution A (50mM Tris.HCl, pH 7·0, 80mM KCl, 12·5mM $MgCl_2$, 0·25M sucrose). The liver was then homogenised in three volumes of solution A with ten strokes of a Potter-Elvejhem glass/teflon homogeniser.

The homogenate was centrifuged at 20,000 g in a Sorval SS 34 rotor for 20 minutes, and the supernatent from this step was submitted to a further centrifugation (20,000 g for 20 minutes). This final supernatent was filtered through glass fibre wool and the filtrate was further centrifuged in polyallomer tubes, at 176,000 g for 85 minutes, in a Beckman 60 Ti rotor, in order to pellet the polysomes.

Polysomal pellets were resuspended in solution A at 6 ml per pellet, homogenised in a Potter-Elvejhem glass/teflon homogeniser, and the homogenate brought to a final concentration of 0·5% $^{w}/_{v}$ sodium deoxycholate. After 1 minute on ice, 7 ml of homogenate was layered over 5 ml of 50mM Tris.HCl, pH 7·6, 80mM KCl, 12·5mM $MgCl_2$, 0·5M sucrose. Ribosomes were then pelleted by centrifugation at 165,000 g for 95 minutes in a Beckman Ti rotor. Ribosomal pellets were washed in 50mM Tris.HCl, pH 7·6, 80mM KCl, 12·5mM $MgCl_2$ and were finally stored at -20°C before use.

Ribosomal subunits were prepared by EDTA dissociation of

ribosomes. Twenty five OD_{260nm} units of rat liver ribosomes were brought to 10mM with respect to EDTA by the addition of 0·1 volumes of 0·1m EDTA. After 10 minutes on ice, the dissociated subunits were layered onto 15% - 30% $^{w}/_{v}$ sucrose density gradients in 10mM NaCl, 10mM Tris.HCl, pH 7·5, 10mM EDTA, and were centrifuged at 80,000 g for 15 hours in a Beckman SW 40 rotor. When EDTA dissociated ribosomes were used as markers to calculate the sedimentation characteristics of hnRNP particles, they were run on gradients in parallel with hnRNP particles (Section 2.1.2.1) and their OD_{260nm} profile monitored as described for the particles.

2.2. Techniques used in the Analysis of HnRNP Particles.

2.2.1 CsCl Buoyant Density Gradient Centrifugation.

HnRNP particles were prepared, as described previously using 10mM triethanolamine.HCl, pH 8·0 as the buffer in place of 10mM Tris. HCl, pH 8·0, from the livers of two rats which had received 250µCi of ^{32}P phosphate intraperitoneally 90 minutes before death. Pooled gradient fractions were made 2% $^{w}/_{v}$ with respect to formaldehyde (pH 7·4), by the addition of 40% formaldehyde stock solution, and then dialysed against two changes of 50mM sodium phosphate buffer pH 7·4, 1mM $MgCl_2$ 2% formaldehyde at 4°C overnight.

The dialysate was brought to a density of 1·45 $g.cm^{-3}$, (as defined by refractometry on an Abbey refractometer), by the addition of solid CsCl. Aliquots of 3 ml were placed in SW 50·1 nitrocellulose tubes and were overlaid with liquid paraffin to within 2mm of the top of the tube. Tubes were finely balanced and centrifuged at 189,000 g for 36 hours at 20°C.

Gradients were harvested by drip fractionation (500µl per fraction), from a hole punctured in the bottom of the centrifuge tube.

Every fourth fraction was aliquoted for analysis of refractive index, in order to assess the shape of the density gradient formed. Fractions were analysed for radioactivity by aliquoting onto filter discs, followed by 5% $^{w}/_{v}$ trichloroacetic acid precipitation on ice. After washing the filter discs sequentially with 2 volumes of 5% $^{w}/_{v}$ trichloroacetic acid (10 ml per filter disc), one volume of absolute ethanol (5 ml per filter disc) and one volume of diethyl ether (5 ml per filter disc), they were dried under a heat lamp, placed in scintillation vials and treated with 1·0M hyamine hydroxide prior to scintillation counting.

2.2.2 Gel Filtration Analysis of HnRNP Particles.

HnRNP particles were subjected to gel filtration analysis on a Biogel A-0·5M (BioRad Ltd.) column. An all glass Pharmacia column, of internal diameter 3 cm, was filled to a height of 8 cm with degassed Biogel A-0·5, pre-equilibrated at 4°C with STM 8, (0·1M NaCl, 1mM $MgCl_2$, 10mM Tris. HCl, pH 8·0). The column was then arranged with the entry port within 0·5mm of the surface of the gel, and a flow speed of 10 cm per hour set on the peristaltic pump which was placed at the exit port of the column. The column was then flushed overnight (16 hours) with STM 8 at 4°C.

The void volume was estimated by the exclusion of blue dextran. HnRNP particles, suspended in STM 8 at a concentration of approximately 1 mg. ml^{-1} (with respect to protein), were applied to the surface of the column in a total volume of 1 ml, and the flow rate of 10 cm per hour maintained. Halfmillilitre fractions were collected in a Gilford automatic fraction collector and each fraction was analysed at 260nm for the presence of RNA, and for the presence of protein by the Bradford commassie blue staining procedure (See Methods 2.4.3).

The elution of hnRNP particles in the void volume was further demonstrated by gel electrophoresis. (See Section 2.3).

2.2.3 Crosslinking of HnRNP Particles using Bis-imido Reagents.

HnRNP particles, prepared as described previously, were dialysed against two changes of 0·01M Triethanolamine HCl, pH 8·0, 1·0mM $MgCl_2$, 0·1M NaCl, for 16 hours at 4°C. Aliquots of this preparation were assayed for protein concentration, and adjusted to between 0·5mg - 1·0mg per ml by further dialysis against Sephadex G200 or polyethylene glycol 6000.

Crosslinking was performed using dimethyl suberimidate (the kind gift of Dr. J.R. Coggins of this department), a bi-functional bis-imido ester. Because of the instability of this compound in solution, the crosslinker was dissolved immediately before use in 100mM Triethanolamine HCl, pH 8·0 at a concentration of 100mM, and the buffer re-adjusted to pH 8·0 using 0·1M NaOH. Aliquots (20-50µg) of protein were made 10mM with respect to crosslinker and allowed to react at 4°C overnight. The samples were then made 0·1% w/v with respect to SDS, and to it was added one tenth its volume of a solution containing 2·3M sucrose and 0·005% bromophenol blue. The solution was heated to 100°C in a boiling water bath for 3 minutes, and subjected to SDS polyacrylamide gel electrophoresis. (See Methods 2.3.1).

2.2.4 Estimation of Enzymic Activities in HnRNP Particles.

2.2.4.1 Estimation of Protein Kinase Activity.

Isolated hnRNP particles were dialysed overnight at 4°C against two changes of 500-1000 volumes of phosphorylation buffer (0·1M sodium acetate pH 6·5, 10mM $MgCl_2$). The hnRNP particle preparation was concentrated to 0.5mg protein per ml by dialysis against dry Sephadex

G25 or polyethylene glycol 6000 and aliquots were removed for phosphorylation (usually 25 µg protein in 50 µl phosphorylation buffer). Incubations were performed in a thermostated water bath at 30°C for 10 minutes, in the presence of 1 µCi [γ^{32}P]-ATP (0.1mM).

Protein phosphorylation was determined by spotting the contents of each reaction vessel onto Whatman 3mm filter discs. The discs were then dried and washed with ice cold 5% $^{W}/_{v}$ trichloroacetic acid before being digested for 10 minutes with 5% $^{W}/_{v}$ trichloroacetic acid held at 100°C. The drained filters were successively washed with 5% $^{W}/_{v}$ trichloroacetic acid at 4°C, absolute alcohol and diethyl ether and then dried under a heat lamp. They were then placed in scintillation vials and treated with 0.5 ml of 1.0M hyamine hydroxide for 20 minutes at 60°C. ^{32}P radioactivity was then determined by scintillation counting in a scintillation cocktail composed of 5g P.P.O. per litre of toluene, which was added at 10 ml per vial.

When the investigation of the effects of various parameters on in vitro phosphorylation of hnRNP particles was performed, hnRNP particles were dialysed against distilled water or 0.1M sodium acetate pH 6.5. Additional factors (e.g. NaCl, Mn^{++}, cAMP) were added to the appropriate concentration from stock solution, as defined in the appropriate figure legends. (Figs 30-33)

2.2.4.2. Estimation of Poly A-Polymerase Activity.

Fractions of sucrose density gradients upon which Poly-(A) polymerase assays were to be performed were either used as isolated from the gradient, or microdialysed (See Section 2.5.1.) against 0.1M NaCl, 0.001M $MgCl_2$, 0.01M Tris. pH 8.0. Aliquots of 100 µl of the fraction in question were placed in sterile Eppendorf microvials and $MnCl_2$, creatine phosphate, creatine kinase and poly-(A)

(where required) were added to concentrations of 5mM, 5mM, 5μg per assay and 50μg per assay respectively. Incubation in the presence of 1μCi [^{3}H]-ATP (20 Ci.mMol.) was for 45 minutes at 30°C. The reaction was stopped by rapid cooling to 0°C.

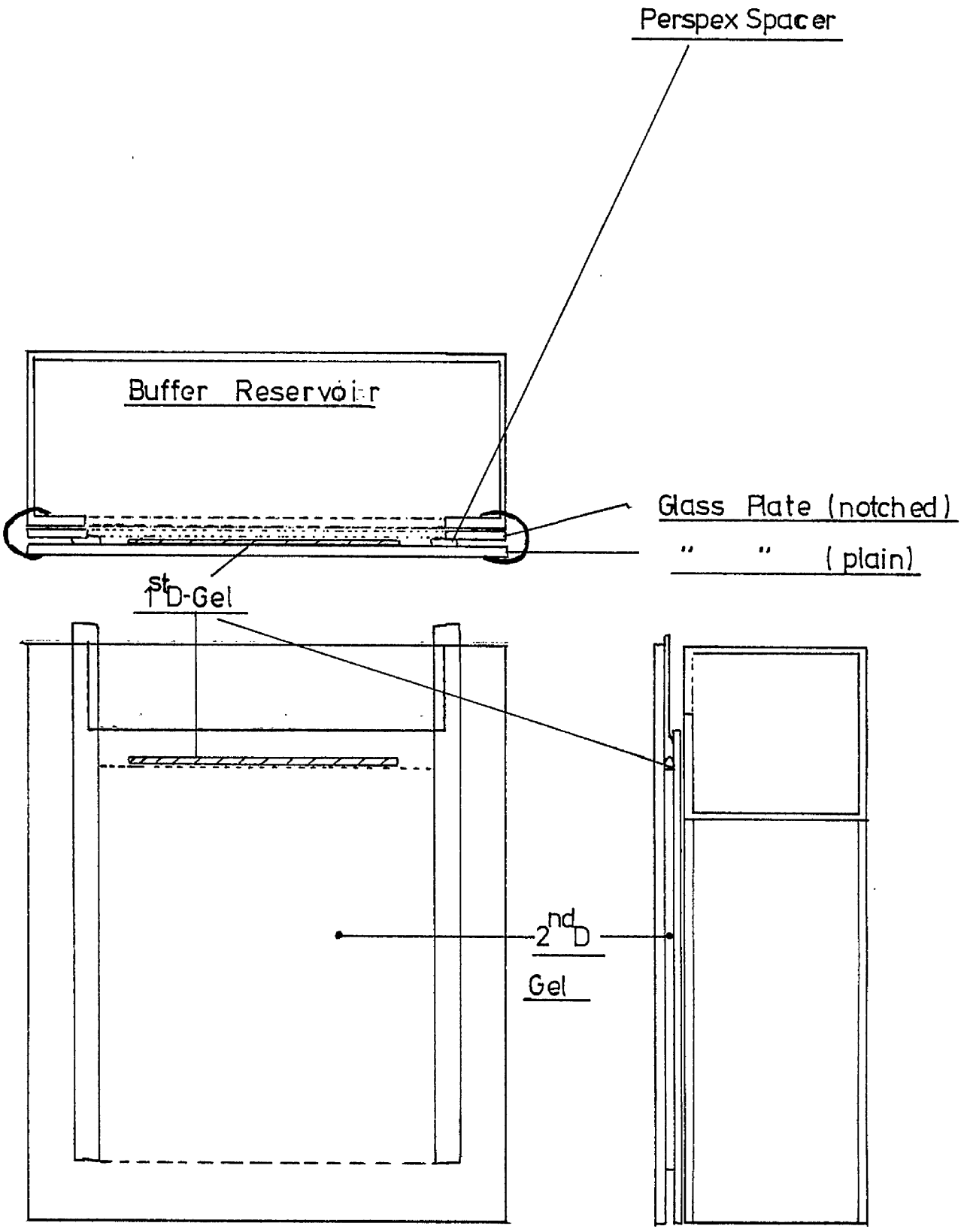
Incorporation of [^{3}H]-ATP into poly-(A) was estimated by a filter disc assay. The reaction mixture was pipetted onto Whatman 3mm filter discs, dried under a heat lamp, and placed into 5% ^{W}/v trichloroacetic acid at 4°C (10 ml 5% ^{W}/v trichloroacetic acid per disc). The discs were agitated periodically in this solution for 15 minutes, drained, and the washing repeated. The discs were finally washed sequentially with absolute ethanol and diethyl ether before being dried under a heat lamp. The filter discs were then placed in scintillation vials, treated with 0·5 ml 1·0M hyamine hydroxide at 60°C for 20 minutes and finally 10 ml of a scintillation cocktail consisting of 5g. per litre P.P.O. in toluene was placed in each vial, the vials capped and radioactivity measured by liquid scintillation counting on a Beckman liquid scintillation counter.

2.3 Techniques Employed in the Analysis of the Protein Components of HnRNP Particles.

2.3.1 SDS-Polyacrylamide Gradient Gel Electrophoresis.

SDS-polyacrylamide gradient gel electrophoresis was performed by a modification of the method of Jeppeson (1974). An all glass slab gel apparatus was set up as in Fig. 4, with the additional insertion of a second rubber tube inside the sealing tube, through which the polymerising mixture was poured. 5 - 15% polyacrylamide gradients were poured from a perspex gradient pourer. The light component contained 5% ^{W}/v acrylamide:bis-acrylamide in a ratio of 125:1 in a buffer containing 0·375M Tris. HCl, pH 8·8, 20% sucrose and 12.5% ^{W}/v SDS. The heavy component contained 15% ^{W}/v acrylamide

Fig.4. Assembly of the Two-Dimensional Gel Electrophoresis Apparatus.



bis-acrylamide in a ratio of 200:1 in the same buffer. Seventeen millilitres of each degassed solution were placed in the chambers of the gradient pourer, (the light component being nearest the exit port), and 20µl of 10% ammonium persulphate was added to each chamber. polymerisation was initiated by the addition of 50µl of 10% TEMED to the light component and 10µl of 10% TEMED to the heavy component. Gels were poured via the tube leading to the apparatus.

After pouring, the gels were overlayed with isobutanol and left to polymerise for 2 hours. A stacking gel consisting of 4 ml of 0·124M Tris. HCl, pH 6·8 containing 3% $^{W}/_{v}$ acrylamide and 0·1% SDS was then poured on top of the set gel, and allowed to set around a teflon well-forming comb. Electrophoresis of protein samples on these gels was at 35 mA for 6 hours.

2.3.2 Two-Dimensional Gel Electrophoresis of Proteins.

Two-dimensional fractionation of proteins was performed essentially as described by O'Farrell (1975) and O'Farrell et al, (1977), but including various modifications, as outlined by Garrels (1979) and Lestourgeon and Beyer (1978).

Protein preparations, (either lyophilisates or acetone, ethanol or trichloroacetic acid precipitates) were prepared for electrophoresis by dissolving them at 5-15 mg per ml in lysis buffer (9·5M urea, 5% $^{W}/_{v}$ mercaptoethanol, 2% $^{W}/_{v}$ Nonidet-P40, 1·6% $^{W}/_{v}$ pH 5-7 ampholines, 0·4% $^{W}/_{v}$ pH 3·5-10 ampholines. Depending on the expected complexity of the sample, between 100µg and 500µg of protein was applied to the first dimension gel.

Protein fractionation in the first dimension gel depended on charge and was performed using either isoelectric focussing (IEF), (O'Farrell, 1975) or, most frequently, non-equilibrated pH gradient electrophoresis (NEPHGE), (O'Farrell et al, 1977).

2.3.2.1 Non-Equilibrated pH Gradient Electrophoresis.

NEPHGE gels were prepared essentially as described by O'Farrell et al, (1977). Pyrex glass tubing, of internal diameter 2·5mm and length 12 cm, was prepared for electrophoresis by washing in Decon or chromic acid, followed by thorough rinsing in water and air-drying. One end of each tube was sealed with the rubber cap of a teflon cannula, and the tubes were arranged in a vertical position for pouring by placing them in corrugated cardboard,held to the side of a large bottle with rubber bands.

The gel solution was prepared by mixing 5·5g AristaR urea, 1·33 ml of a mixture of 28·38% $^{W}/_{v}$ acrylamide and 1.62% $^{W}/_{v}$ N N methylene-bis acrylamide, 2 ml of 10% $^{W}/_{v}$ Nonidet-P40, 2 ml of water and 0·5 ml of 40% $^{W}/_{v}$ ampholines (pH range 3·5 - 10). The gel mix was degassed and 15µl of 10% ammonium persulphate and 10µl of TEMED were added. The polymerising mixture was then poured into the previously prepared gel tubes to a height of 10 cm, via a teflon cannula. The gels were overlayed with 20µl of distilled water and left for 2 hours to polymerise. The above mixture was sufficient to prepare 10 gels.

NEPHGE gels were not pre-run and were loaded anodically with the appropriate protein sample. Electrophoresis was performed using a disc gel electrophoresis apparatus with 10mM phosphoric acid as the upper reservoir solution, and 20mM sodium hydroxide as the lower reservoir solution. Proteins were electrophoresed for 4 hours at 500 volts (2000 v.hr.) unless otherwise stated.

Before application of the completed first dimension gel to the second dimension, a brief incubation (5 - 10 minutes) in SDS sample buffer (2·3% $^{W}/_{v}$ SDS, 5% $^{W}/_{v}$ mercaptoethanol, 10% $^{V}/_{v}$ glycerol, 62·5mM Tris. HCl pH 6·8, 0·01% $^{W}/_{v}$ bromophenol blue) was carried out. Gels were frequently stored frozen at -20°C in SDS sample buffer

*

The measurement of pH in 8M urea may result in the overestimation of the correct pH by as much as 1 pH unit. (Bull et al, Arch. Biochem. Biophys. 104, 297 (1960). This is believed to be due to the interaction of urea with the glass electrode, although alteration of the pI of the ampholytes has also been suggested (Josephson et al, Anal. Biochem. 40, 476 (1971). Similarly, the pI's of proteins may be altered by high urea concentration (Ui, Biochem. Biophys. Acta. 229, 567 (1971).). Although no precise pI values are assigned to proteins in this study, it is appreciated that some proteins present in the mixtures analysed, may be anomalously placed in the charge dimension.

before use, with no detrimental effects on resolution.

2.3.2.2 Isoelectric Focussing. (IEF).

IEF gels were prepared exactly as described above for NEPHGE gels, with the exception that the ampholine ranges employed included 1·6% $^{w}/_{v}$ ampholines pH 5-7 and 0·4% $^{w}/_{v}$ ampholines pH 3·5 - 10.

In contrast to the NEPHGE gels, IEF gels were overlaid with 20µl lysis buffer and pre-electrophoresed at 200 v for 10 minutes, 300 v for 20 minutes and 400 v for 30 minutes before being loaded cathodically, and electrophoresed for a final total of 7,000 v.hr. (i.e. 5 hr. at 400 v and 2 hr. at 500 v). IEF gels were prepared for second dimensional electrophoresis as described for NEPHGE gels.

Where appropriate, pH gradients of gels were measured by dissolving 2mm gel slices in 1 ml degassed 8M urea. The pH of the resulting solution was measured on a Beckman pH meter.

2.3.2.3 Second Dimensional Fractionation.

SDS polyacrylamide gel electrophoresis was performed essentially as described by Lestourgeon and Beyer (1978). An all glass slab gel apparatus, built according to the dimensions outlined by O'Farrell (1975), was set up as shown in Fig. 3. (pg 50).

The apparatus consisted of two glass plates (one notched, the other plain), two 1mm thick perspex spacers, one piece of compressible silicon tubing and four large bulldog clips, which were arranged as shown in Fig. 3.

For an 8·75% $^{w}/_{v}$ polyacrylamide gel, the second dimension gel solution was prepared by mixing 18 ml of distilled water, 10 ml of resolving gel buffer (1·48M Tris. HCl pH 8·8, 0·25% $^{w}/_{v}$ SDS), and

11·7 ml stock acrylamide solution (30% $^{w}/_{v}$ acrylamide, 0·8% $^{w}/_{v}$ bis-acrylamide). The mixture was degassed and polymerisation initiated by the addition of 150µl 10% $^{w}/_{v}$ ammonium persulphate and 10µl TEMED. The mixture was then rapidly pipetted into the assembled apparatus. The above mix gave the 8·75% $^{w}/_{v}$ acrylamide gel employed in most experiments,but occasionally the quantity of acrylamide stock solution included in the mix was varied to give different final concentrations. Polymerising gels were overlayed with iso-butanol to provide a level gel surface.

Pre-incubated first dimension gels were applied to the polymerised second dimension gels by squeezing them into direct contact with the gel surface, (Garrels, 1979). No agarose stacker was employed, and once the completed assembly had been attached to the electrophoresis apparatus (See Fig. 3), SDS gel running buffer (25mM Tris, 192mM glycine pH 8·8, 10% $^{w}/_{v}$ SDS) was poured into the gel notch such that contact was made between the upper gel reservoir and the second dimension gel. Electrophoresis was performed at 35 mA per gel until the bromophenol blue marker was 1 cm from the bottom of the gel.

2.3.2.4 Fixing, Staining and Autoradiography of Gels.

Completed gels were placed in 50% $^{v}/_{v}$ methanol, 10% $^{v}/_{v}$ acetic acid containing 0·25% $^{w}/_{v}$ coomassie brilliant blue R250, and simultaneously fixed and stained overnight at room temperature. Destaining was performed using several washes of 50% $^{v}/_{v}$ methanol, 10% $^{v}/_{v}$ acetic acid, until the gel background was suitably clear. The gels were then dried onto 3mm filter paper under vacuum, on a BioRad gel drier, and photographed dry. Where necessary, autoradiography was performed using Kodak X-Omat H film.

2.3.3. Tryptic Peptide and Amino Acid Mapping.

2.3.3.1 Tryptic Peptide Mapping.

Tryptic peptide mapping of individual proteins was performed by the method of Elder et al (1977). Two-dimensional gels of hnRNP particles (performed as previously described) were fixed, stained, destained and dried. The dried gel was photographed, and the stained polypeptide spots of interest were excised from the gel by means of a fine scalpel blade. The backing paper was peeled from the gel discs which were then placed individually in the wells of a microwell dish. The top of the microwell dish was replaced with nylon gauze, and the whole arrangement submerged in 10% $^{v}/_{v}$ methanol in order to remove SDS and other contaminents.

After extensive washing in 10% $^{v}/_{v}$ methanol, the discs were individually dried under a heat lamp and placed in a second microwell dish. The proteins in the gel discs were then iodinated by the sequential addition to each of 20µl (0·5M) sodium phosphate buffer pH 7·6, 150µCi ^{125}I (carrier free) (5µl) and 5µl of chloramine T (1 mg. per ml). After 3 hours at room temperature, the reaction was stopped by the addition of 0·5 ml of sodium metabisulphite (1 mg. per ml). The nylon gauze was replaced on the microwell dish and the whole arrangement submerged in 2 litres of 10% $^{v}/_{v}$ methanol overnight. Two further changes of 10% $^{v}/_{v}$ methanol (two litres per change) were sufficient to bring the background radioactivity down to acceptable levels.

Each gel disc was then individually dried under a heat lamp, placed in a 1·5 ml Eppendorf tube, and 0·5 ml of 50µg. per ml Trypsin was added in 0·05M NH_4HCO_3 buffer, pH 8·0. The samples were incubated at 37°C overnight, the gel slice removed and the extracted protein digest in the tube lyophilised to dryness.

Each sample was then dissolved in 20µl of solution I, acetic acid, formic acid, water (15:5:80:), and 3 - 5,000 cpm of each was spotted onto a 10 cm x 10 cm tlc plate.Electrophoresis was performed in acetic acid, formic acid, water (15:5:80) at room temperature for 45 minutes at 7mA per plate in a Cohn electrophoresis tank.

The electrophoresed plate was thoroughly dried and the peptides were then chromatographed through a second dimension in butan-1-ol, acetic acid, pyridine, water (32·5:5:25:20) until the solvent front was within one cm of the top of the plate. The plates were thoroughly dried in a fume hood and analysed by autoradiography using X-Omat H film and a Kodak X-ray intensifying screen.

2.3.3.2 Amino Acid Mapping.

Identification of amino acids from hnRNP particles phosphorylated *in vitro* was performed essentially as described by Eckhart *et al*, (1979). Ethanol precipitated hnRNP particles were dissolved in 1 ml 6M HCl. by gentle warming in a 60°C water bath, and the dissolved hnRNP particles transferred to a "cold finger". The cold finger was then evacuated and sealed, and the proteins hydrolised by heating to 110°C for 2 hours.

The hydrolysate was then evaporated under vacuum, the residue redissolved in 20µl 10mM HCl. The amino acids were fractionated using two-dimensional electrophoresis/chromatography on cellulose backed tlc plates, as described for the tryptic peptide mapping, except that the second dimension medium was butan-1-ol, acetic acid, pyridine, water (15:3:10:12). Ten microlitres of phosphothreonine and phosphoserine markers (1 mg per ml) were run on each chromatogram in order to provide ninhydrin stainable standards. The position of

these markers was revealed by spraying the dried, completed chromatogram with a 1% $^{w}/_{v}$ ninhydrin spray in acetone, and subsequently incubating the plates at 100°C for two minutes. Comparison of the autoradiograph and stained spectrum allowed identification of the ^{32}P-labelled amino acids.

2.4 Techniques Employed in the Analysis of the RNA of HnRNP Particles.

2.4.1 Extraction of RNA from HnRNP Particles.

Ethanol precipitated hnRNP particles were dried under a gentle stream of nitrogen and resuspended in 1 ml 0·1M NETS buffer (0·1M NaCl, 0·001M EDTA, 0·1M Tris. HCl pH 7·5, 0·2% $^{w}/_{v}$ SDS). The resulting solution was clarified by a brief centrifugation at 5,000 rpm in a Sorvall HB4 rotor. An equal volume of phenol, chloroform, isoamyl alcohol (1:1:0·1) was added to the suspension and proteins were extracted from the aqueous phase by mechanical shaking at room temperature for 10 minutes. The organic and aqueous phases were separated by centrifugation at 5,000 g in a Sorvall HB4 rotor for 5 minutes at 4°C. The upper, aqueous phase was removed and further extracted with 1 ml redistilled phenol, chloroform, isoamyl alcohol (1:1:0·1). The aqueous phase was retained and the combined phenolic phases were subjected to two further extractions with 0·1M Tris. HCl pH 9·0, 1% $^{w}/_{v}$ SDS. The aqueous extracts were ultimately pooled and RNA was precipitated overnight at -20°C, with 2·5 volumes of ethanol. The RNA was recovered by a 10 minute centrifugation at 8,000 g in a Sorvall HB4 rotor, washed with ethanol and dried under a gentle stream of nitrogen. The virtual absence of DNA in hnRNP particles precluded the requirement for a DNAse digestion step.

2.4.2 Fractionation of RNA on 99% Formamide-Polyacrylamide Gels.

RNA isolated from hnRNP particles, as described above, was

subjected to polyacrylamide gel electrophoresis in the presence of 99% formamide. Formamide was deionised by the method of Pinder et al, (1974) by stirring 100 ml with 4 g of BioRad mixed-bed resin (AG 501-X8, 20-50 mesh) for 3 hours. Deionised formamide was filtered through a sintered glass funnel and stored at -20°C prior to use.

Gels were prepared by the addition of 0·82g acrylamide and 0·18 bis-acrylamide to 10 ml of deionised formamide, buffered with 0·01M $Na_2 HPO_4$ - 0·01M $Na H_2PO_4$. Polymerisation was initiated by the addition of 24µl of TEMED and 75µl of 18% ammonium persulphate, and the mixture rapidly transferred to 13cm x 0·5cm plexiglass gel tubes, previously sealed at the bottom with parafilm. Fifty micro-litres of 70% formamide was layered over each of the gels and poly-merisation allowed to proceed overnight.

RNA samples were dissolved in buffered formamide, heated to 65°C for 20 seconds, fast cooled to room temperature and mixed with 0·2 volume glycerol. Approximately 20µg of RNA (50µl) was carefully applied to the top of each gel, and electrophoresis performed for 3½ hours at 3mA per gel. Gels were ultimately stained in 10µg per ml ethidium bromide in 0·1M ammonium acetate, and photographed under short wave U.V. light with a Polaroid MP 3 land camera with a yellow filter using Polaroid '665' film.

2.5. Miscellaneous.

2.5.1 Microdialysis.

For the dialysis of very small volumes (<200µl) the use of conventional dialysis techniques proved inadequate. Under these circumstances the volume to be dialysed was placed in an Eppendorf reaction tube, which had previously had the centre of its cap removed. The tube was then re-capped with a piece of dialysis tube

trapped such that the semi-permeable membrane effectively replaced the cut out segment of the Eppendorf cap. The dialysate was then brought into contact with the dialysis membrane by a brief centrifugation in an inverted position. The inverted tube was then bound to a piece of polystyrene by elastic bands such that, when the polystyrene was allowed to float on the dialysis buffer, the dialysing surface was immersed. Any air bubbles trapped between the dialysis tubing and the buffer surface were removed by flushing. Dialysis was rapid under these conditions and was complete in 1-4 hours.

2.5.2 Sterility Precautions.

To eliminate problems with endoribonuclease digestion during the preparation of hnRNP particles, several precautions were employed. All glassware was oven baked at 200 °C for at least four hours. Solutions were autoclaved at 5lb. per $in.^{-2}$ for 40 minutes. Dialysis tubing was boiled with 1% EDTA and 1% SDS, followed by copious washing with sterile distilled water and autoclaving at 5lb. per $in.^{-2}$ for 40 minutes. Disposable rubber gloves were used for all manipulations where RNAse activity was likely to be a problem.

2.5.3 Protein Estimation.

Protein concentrations were estimated by the method of Bradford (1976). The assay is based upon the binding properties of coomassie brilliant blue G-250 with protein, and estimates the resultant spectral shift of the dye-protein complex. The assay solution was made by dissolving 100 mg. of coomassie brilliant blue G-250 in 50 ml of 95% $^{w}/v$ ethanol. One hundred millilitres of 85% $^{w}/v$ phosphoric acid was added. The solution was filtered to remove insoluble material, and finally made up to one litre. This assay solution remained stable

if stored at 4°C for 5-10 weeks.

The protein assay consisted of the addition of 1·0ml of assay solution to the appropriate volume of unknown protein solution, (usually 5µl or 10µl). For direct calibration of such solutions, a simultaneous standard curve using appropriate amounts of a standardised solution of bovine serum albumin (1 mg. per ml) was performed. The optical density of the solution was read at 595nm, using 1 ml of the assay solution and 10µl of the solution in which the unknown was dissolved as a blank. Protein concentrations were read directly from the standard curve.

RESULTS

Since the later stages of this investigation rely for their interpretation upon an understanding of the nature of the isolated hnRNP particle and its relationship with other components of the eukaryotic nucleus, initial attention was directed towards the effective characterisation of these particles. Particular emphasis was placed on analysis of the protein components.

1. ISOLATION AND CHARACTERISATION OF RAT LIVER HnRNP PARTICLES.

1.1 Physical Properties of HnRNP Particles.

Fig. 4.* shows the fractionation of rat liver nuclei by the two most frequently employed techniques for the preparation of hnRNP particles.

Nuclei were isolated by a slight modification of the method of Chauveau et al, (1956) which incorporated features of the method of Blobel and Potter (1966), (See Methods Section 2.1.1.1). These nuclei appeared free of cytoplasmic contamination when examined by phase contrast microscopy, but some cytoplasmic tags would be expected to be associated with the nuclear membranes. Most of the major findings to be presented, however, have been repeated with nuclei which were subjected to a further purification employing Triton X100 to remove the nuclear membrane (Blobel & Potter, 1966). This treatment has had no adverse effect on any of the reproducibility of any of the data to be presented but was, nevertheless, not routinely used as we remain concerned that the detergent may have some effects on the components of hnRNP.

Although both techniques for hnRNP isolation were employed in this study, the majority of the work described was performed with material derived by route B, that is, hnRNP particles were extracted from purified nuclei by the method of Samarina et al, (1968).

* See p. 42 .

FRACTION	C.P.M.	% of Total.
Total Nuclei	1.108×10^6	100%
pH7.0 Wash	3.39×10^5	30.67%
pH8.0 Wash I	7.28×10^4	6.57%
pH8.0 Wash II	1.21×10^5	10.94%
pH8.0 Wash III	1.36×10^5	12.33%
Post-Nucleolar Supernatent.	3.56×10^5	32.12%

Table 3

Location of Cold Trichloroacetic Acid Insoluable Radioactivity During Extraction of hnRNP Particles.

Rat liver nuclei, isolated from adult animals previously injected intraperitoneally* with 250µCi. of (^{3}H)-uridine, were sequentially washed with low salt buffers as described in Methods 2.1.2.1. p . 42. The residual nuclei were sonicated at between 10^7-10^8 nuclei per ml in an MSE ultrasonic power unit (2x10 sec. at 1.5A). The sonicated nuclei were layered over a 25ml 30% sucrose cushion containing 10mM Tris. HCl pH 7.4, 10mM NaCl 1.5mM $MgCl_2$, and a post-nucleolar supernatent fraction obtained by centrifugation at 4,500g for 15 minutes (See Methods 2.1.2.2. p . 43). Estimation of cold 5% $^w/_v$ trichloroacetic acid insoluble radioactivity (RNA) in each of the subnuclear fractions generated was performed by the filter disc assay described in Methods 2.2.4.2. p . 48.

* 1 hr prior to death

Table 3 shows a quantitation of the material extracted from nuclei, isolated from animals injected interperitoneally with (^{3}H)-uridine, at all stages in the preparation of hnRNP particles. The pH 7 wash appears to remove approximately 30% of cold trichloroacetic acid insoluble radiolabel (i.e. RNA). The identity of the RNA has not been investigated but probably includes RNA from mRNP particles, ribosomes attached to the nuclear membrane and tRNA. The pH 7·0 extract contains very little of the hnRNP particle proteins, and it is, therefore, assumed that little of the RNA derives from the particles.

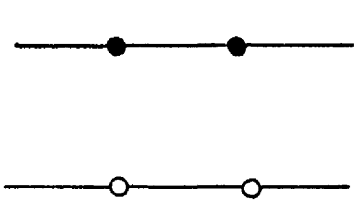
The further extraction of these nuclei with pH 8·0 buffer releases an additional 30% of RNA associated (^{3}H)-uridine. The majority of this RNA is associated with the peak of hnRNP particles, which can be collected from sucrose density gradients. Sonication of the extracted nuclei, as described by Pederson (1974a), releases an additional 30% of the total nuclear RNA, presumably from chromatin associated hnRNP particles, although contamination of this fraction with pre-ribosomal particles has not been ruled out. The remaining 10% of the RNA appears to be retained in the chromatin/nucleolar pellet, and probably represents trapped hnRNP-RNA, as suggested by Stevenin et al, (1970), and/or pre-ribosomal particles, located in the the nucleolus.

Fig. 5 shows an analysis of the combined pH 8·0 extracts, from rats injected with ^{32}P orthophosphate, on 15% - 30% sucrose density gradients. Resistance of incorporated ^{32}P to cold 5% $^{w}/_{v}$ trichloroacetic acid and hot 5% $^{w}/_{v}$ trichloroacetic acid digestion was used to estimate incorporation into RNA plus protein, and protein respectively. While a significant quantity of RNA associated radiolabel sedimented at values of less than 20S, the major RNA component appeared to sediment at a value of 40S, as defined by comparison with EDTA-dissociated

Fig. 5.

Sucrose Density Gradient Centrifugation of a Nuclear Extract Containing HnRNP Particles.

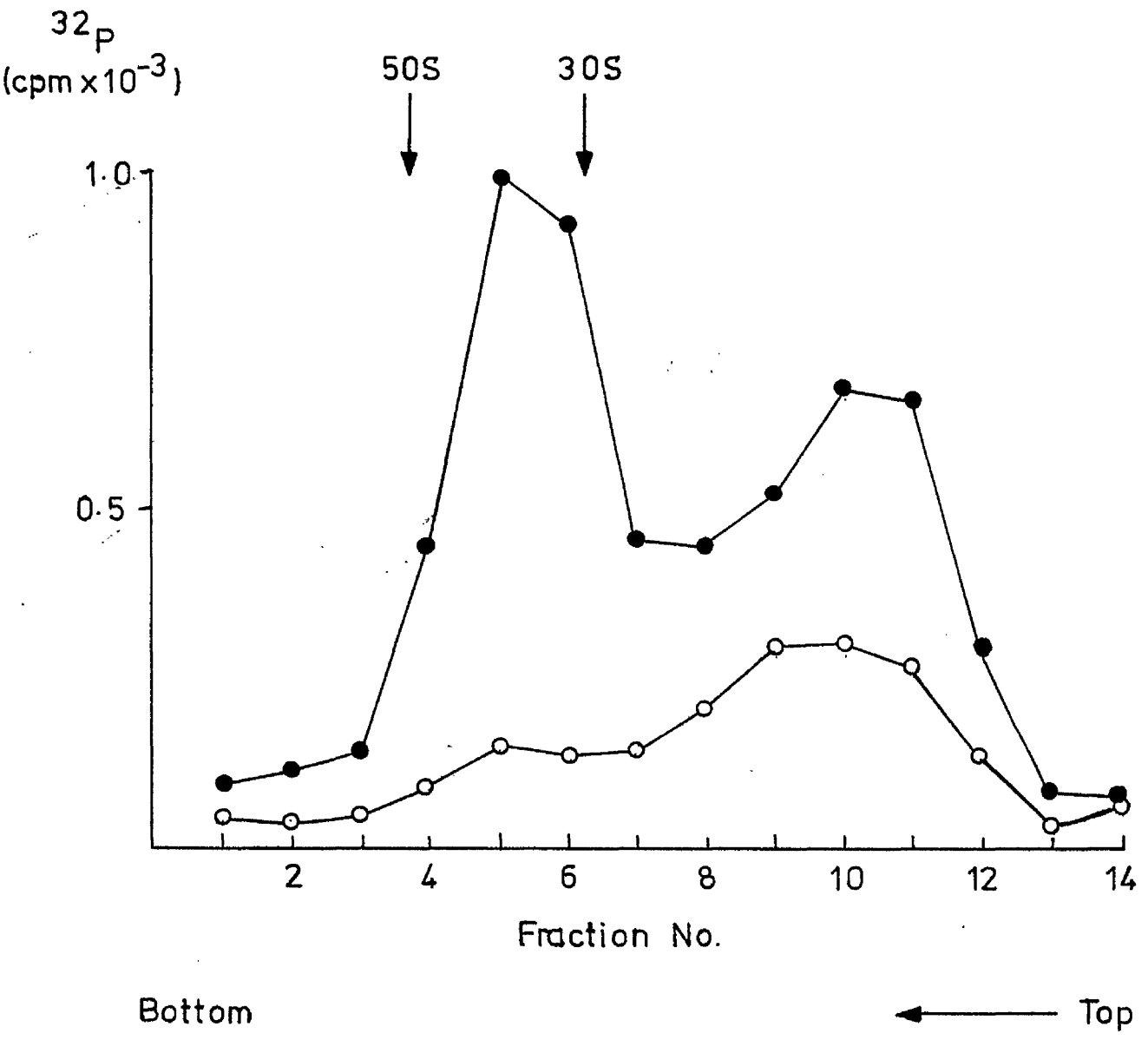
One millilitre of pH 8·0 nuclear extract (Methods 2.1.2.1) prepared from nuclei isolated from animals injected with 2·0mCi. of ^{32}P-ortho-phosphate 2hours before death, was layered onto 13 ml. of a 15%-30% W/v sucrose density gradient, containing 0·1M NaCl, 10mM Tris. HCL, pH 8·0, 10mM $MgCl_2$. The gradient was centrifuged at 78,000 g for 16 hours in a Beckman SW 40 rotor. They were then scanned at 260nm by passage through a flow cell attachment of a Gilford 260 spectrophotometer, the effluent was fractionated and each fraction was assayed for cold (4°C) 5%W/v trichloroacetic acid and hot (95°C) 5% W/v trichloroacetic acid insoluble material. 50S and 30S ribosomal subunits were employed as sedimentation coefficient markers and were run on parallel gradients, containing 0·1M EDTA.



cold trichloroacetic acid insoluble ^{32}P

hot trichloroacetic acid insoluble ^{32}P

Each point on the figure represents the amount of radioactivity present in one-tenth aliquots (100µl) of each fraction.


32P
(cpm x10-3)
50S
30S
1·0
0·5
2
4
6
8
10
12
14
Fraction No.
Bottom
Top

ribosomes, run on parallel gradients. This sedimentation coefficient is typical of the hnRNP monoparticle preparations isolated by many workers in this field, (e.g. Samarina et al, 1978; Karn et al, 1977 inter alia.). The characteristic radioactivity profile shown in Fig. 5 was mirrored by an OD_{260} profile (e.g. Fig. 7A), and in subsequent experiments the optical density trace was employed in the determination of the appropriate fractions for pooling and further analysis. EDTA (10mM) made no difference to the gradient profile, in agreement with the original observation of Pederson (1974) for HeLa cell hnRNP particles.

A consideration of the protein-associated ^{32}P, fractionated in Fig. 5 , reveals that the majority of the phosphoproteins were resident in the non-particulate region (20S) of the gradient. The inability to recover adequate radiolabelled protein from the 40S region of the gradient, even with increased isotope administration, precluded the analysis on polyacrylamide gels of in vivo labelled hnRNP particle protein. The low levels of phosphoprotein associated with the 40S hnRNP particles in comparison with polyparticle hnRNP particles has previously been noted in HeLa cells (Lelay & Brunel, 1979), but the significance of the observation if not understood.

One particularly characteristic property of isolated hnRNP particles is a low buoyant density. This is consistently in the range 1·39 - 1·41 $g.cm^{-3}$ and is regardless of the source, or the sedimentation coefficient of the hnRNP particles employed (See Introduction 4.2.2 and Table 1). Examination of the 40S peak obtained in experiments similar to that described in Fig. 5 has revealed a similar buoyant density for this component.

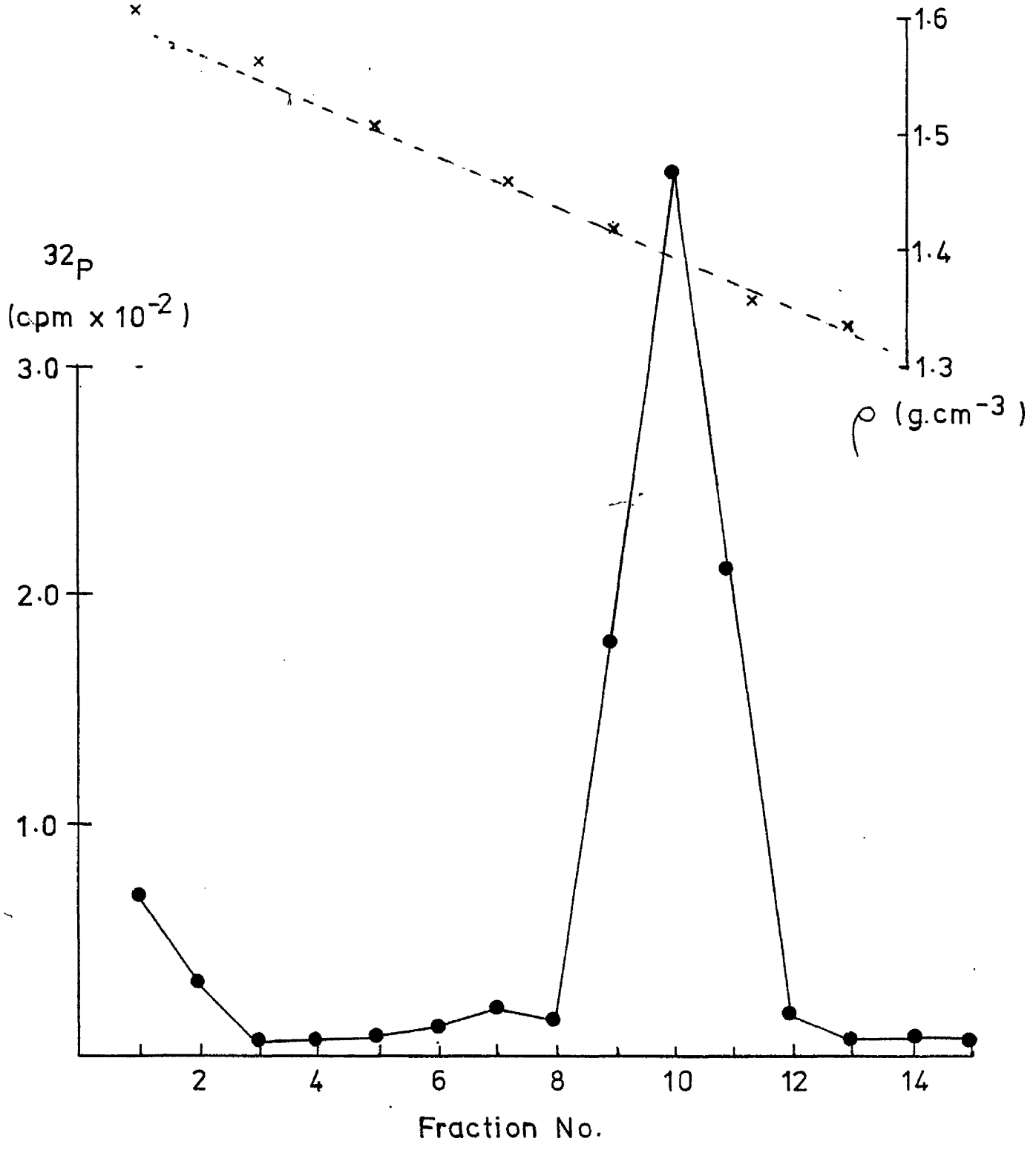
Fig. 6 shows CsCl buoyant density gradient analysis of formaldehyde fixed 40S hnRNP particles previously labelled with

Fig. 6

CsCl Buoyant Density Gradient Centrifugation of Formaldehyde-Fixed HnRNP Particles.

Rat liver 40s hnRNP particles, labelled *in vivo* with ^{32}P orthophosphate, as described in the legend to Fig. 5, were isolated from a sucrose density gradient. The pooled gradient fractions were made 2% with respect to formaldehyde, by the addition of 40% formaldehyde stock solution and subsequently dialysed against two changes of 500 volumes of 50mM sodium phosphate, pH 7.4, 1mM $MgCl_2$, 2% formaldehyde at 4°C.

CsCl buoyant density gradient centrifugation was performed as outlined in Methods 2.2.1 (p . 45) in an SW 50.1 (Beckman) rotor. Fractions of approximately 0.5ml. were taken from the completed gradient and assayed for ^{32}P radioactivity by the filter disc assay described in Methods 2.2.1 (p . 46). The shape of the gradient was determined by refractometry of an aliquot of every fourth fraction. Each point in the figure represents the quantity of radioactivity in a 200 µl aliquot of each fraction.


32P
(cpm x 10-2)
3·0
2·0
1·0
1·6
1·5
1·4
1·3
ρ (g.cm-3)
2
4
6
8
10
12
14
Fraction No.

^{32}P *in vivo*. The predominant feature of the radioactivity profile of this gradient is a peak corresponding to a buoyant density of 1·39g.cm^{-3}. A minor radioactive component of buoyant density 1·57 - 1·59 g.cm^{-3} can be seen close to the bottom of the gradient. It is possible that this component is a ribosomal subunit, (presumably 40S). However, several features are inconsistent with this possibility. Firstly, the accepted figure for the buoyant density of the 40S ribosomal subunit is around 1·53g.cm^{-3} (Kumar & Pederson, 1975; Knowler, 1976), and secondly, the analysis of the protein components of the 40S hnRNP particle by SDS polyacrylamide gel electrophoresis (Fig.8) is devoid of the low molecular weight polypeptides characteristic of ribosomes. It is probable that this material is simply pelleted, uncrosslinked RNA, which has been disturbed during the puncturing and subsequent drip fractionation of the gradient tube.

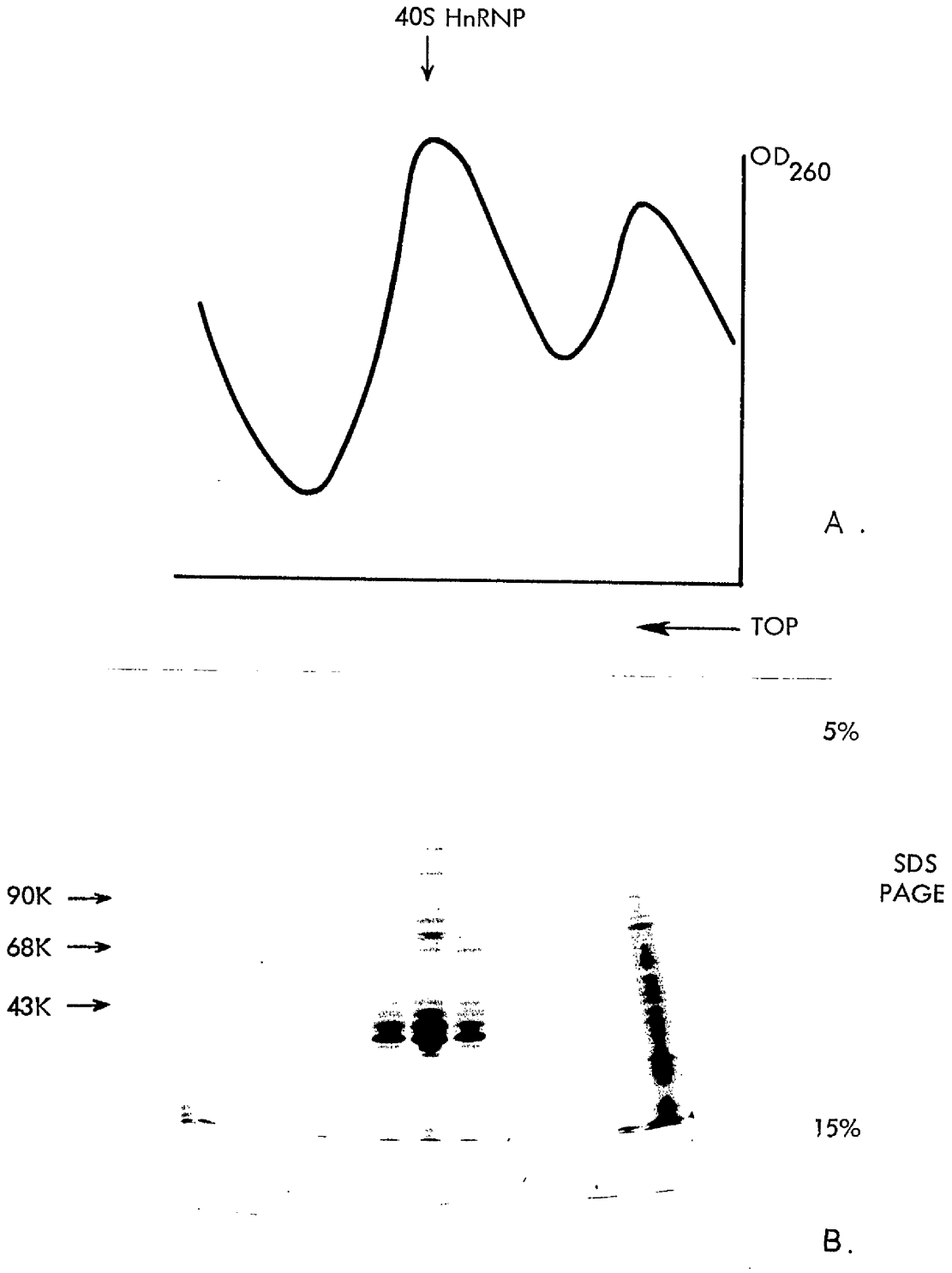
The buoyant density of the particles thus serves to distinguish hnRNP particles from other cellular ribonucleoproteins such as pre-ribosomal particles and ribosomes. However, cytoplasmic mRNP particles (See Introduction Section 5), both in their "free" form (informosomes), or in association with polysomes, are also characterised by a buoyant density of ~1·4g.cm^{-3} (Kumar & Pederson, 1975). Although the absence of contaminating mRNP particles is difficult to prove, none of a number of characteristic mRNP proteins are detected in these preparations (J. Beaumont, personal communication of unpublished data).

In accordance with the empirical formula of Spirin (1969), a buoyant density in CsCl gradients of 1·39g.cm^{-3} corresponds to a protein:RNA ratio of 5:1. A similar conclusion is obtained by direct chemical measurement of the individual components (data not shown).

Fig. 7.

Analysis of the Protein Components of Each Fraction of a Sucrose Density Gradient Containing HnRNP Particles.

One millilitre fractions of the sucrose density gradient were dialysed against distilled water at 0°C overnight, lyophilysed and analysed on a 5-15% polyacrylamide gel as described in the Methods section 2.3.1. The stained gel pattern (B) is arranged below the $OD_{260\ nm}$ trace of the gradient (A), so that their relative positions correspond. The positions of standard molecular weight markers are indicated.


40S HnRNP
OD260
TOP
5%
A .
90K
68K
43K
SDS
PAGE
15%
B.

In summary, 40S hnRNP particles have been isolated from purified rat liver nuclei by the extraction method of Samarina et al, (1968). These particles exhibited a number of properties comparable with those exhibited by hnRNP particles obtained and characterised by other workers (for review see Heinrich et al, 1978, and Table 1). They include rapid (i.e. 1hr.) labelling of the RNA component, sedimentation coefficient in sucrose density gradients of around 40S, and a buoyant density of 1·39 $g.cm^{-3}$.

1.2 Characterisation of the Protein Component of HnRNP Particles by SDS-Polyacrylamide Gel Electrophoresis.

Early investigations of the protein components of hnRNP particles employed urea-polyacrylamide gel electrophoresis. Under these conditions a single polypeptide of molecular weight 40,000 was resolved, and designated "Informatin" (Samarina et al, 1968). Subsequent analyses, usually employing SDS-polyacrylamide gels, succeeded in delineating considerably more polypeptide components, (between 14, (Neissing & Sekeris, 1971a) and 45, (Gallinaro-Matringe et al, 1975)). Numerous workers, (Neissing & Sekeris, 1971b; Gallinaro-Matringe & Jacob, 1974; Northemann et al, 1978; Suria & Liew, 1979) have shown that the early observations of a single polypeptide species was an unfortunate artefact of the urea-based gel fractionation system employed, and that SDS-polyacrylamide gel electrophoresis is a superior technique for the analysis of these proteins.

Fig. 7 outlines several important and interesting points with regard to the protein components of hnRNP particles. The upper panel of Fig. 7 corresponds to a typical OD_{260} profile of a sucrose gradient, upon which a pH 8·0 nuclear extract has been fractionated. The lower panel represents the analysis of each fraction of this gradient by SDS-polyacrylamide gradient gel electrophoresis. The proteins

Fig. 8.

Comparison of HnRNP Particle Proteins with other Sub-Cellular Extracts.

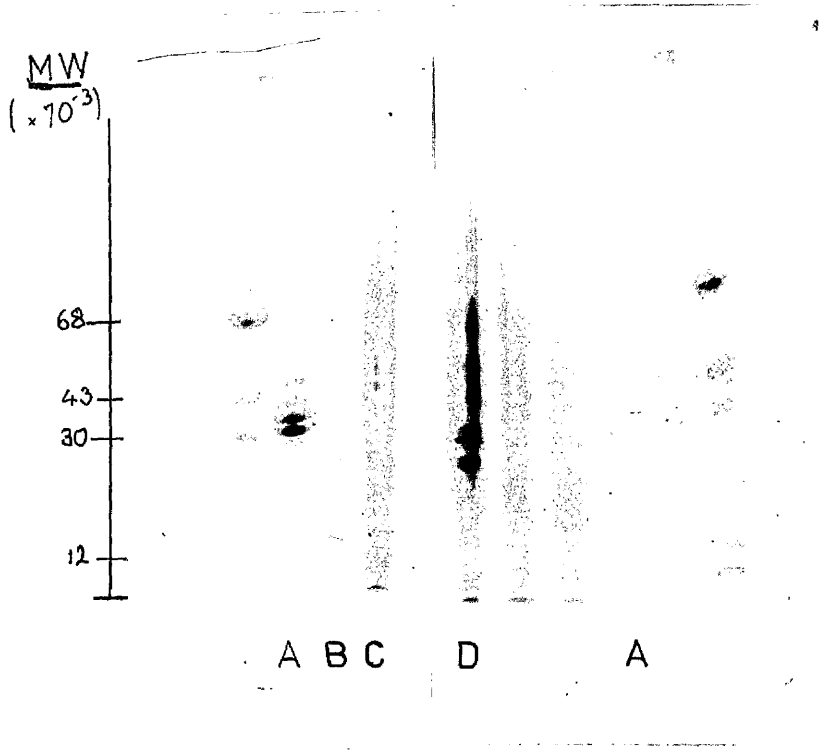
One dimensional SDS- polyacrylamide gel electrophoresis of hnRNP particles, ribosomes (80S), the pH 7.0 nuclear extract and the pH 8.0 nuclear extract was performed on an 8.75% polyacrylamide gel (Methods 2.3.2.3), for 3 hours at 35 mA.

A - HnRNP particles. (80 µg).

B - 80S ribosomes (50 µg).

C - pH 7.0 extract (100 µg).

D - pH 8.0 extract (110 µg).


MW
(×10⁻³)
68
43
30
12
A B C D A

corresponding to the non-particulate region of the gradient ($\leq$20S) exhibited considerable complexity. The major point to observe, however, is the exact comigration of the OD_{260} peak with a protein profile characteristic of hnRNP particles. This profile was dominated by a group of polypeptides of mol.wt. 25,000 - 45,000, which on this gel were resolved into 7 bands, and have come to be known as "core proteins", (See Introduction, Section 4.3.3.2). Several polypeptides of molecular weight greater than 45,000 were also present and included components at 120,000, 100,000, 78,000, 70,000 (doublet), and 68,000, all of which have been noted previously by other workers (e.g. Karn et al, 1977; Beyer et al, 1978, inter alia).

Histones, ribosomal proteins, and other low molecular weight polypeptides were notably absent from the protein profile. This is made clearer in Fig. 8 , where a direct comparison of hnRNP particles and total ribosomal proteins is made.

Fig. 7 is somewhat contrary to the data of Beyer et al, (1977) who also examined each of the fractions of an analytical sucrose gradient of 40S hnRNP particles (in this case from HeLa cells) by SDS-polyacrylamide gel electrophoresis. These workers observed the apparent presence of certain protein components throughout the gradient, creating a general protein contaminant "background" upon which the hnRNP particle peak proteins were superimposed. Some of the polypeptides characteristic of hnRNP can be detected in gels which correspond to a sucrose density greater than that to which 40S particles had migrated. However, these probably represented low levels of hnRNP polyparticles (Samarina et al, 1968; Pederson, 1974a). The rather severe conditions of extraction of hnRNP particles employed by Beyer et al, (1977), (i.e. extraction at 20°C and finally at 37°C) may result in the lysis of some of the nuclei, and the subsequent co-extraction of large heterodispersely sedimenting components, which

consequently contaminate the 40S hnRNP particles.

1.3 The Association of Proteins with HnRNP Particles.

Questions of contamination and artefactual rearrangement have always dogged the investigation of isolated sub-nuclear organelles. An assessment of the extent of either, or both, of these possibilities is an absolute requirement for the interpretation of subsequent data. With regard to hnRNP particles, this problem is a particularly profound one, and it would be unrealistic to suggest that definitive experiments ruling out either possibility have been published. In this study, two approaches have been taken, and although neither approach can be said to eliminate the likelihood of contamination or rearrangement, the results were reassuring.

Several workers have employed crosslinking reagents to estimate the integrity of the isolated hnRNP particle (Karn et al, 1977; Samarina et al, 1979). A similar approach has been successfully employed with ribosomes by Traut and co-workers (Traut et al, 1973) . The rationale behind the use of protein crosslinking reagents is straight forward. Any two polypeptides, each with a suitable free amino group, may be crosslinked if the amino groups are within a distance of 6 carbon atoms of each other. In a ribonucleoprotein complex, such a situation is likely to occur frequently, each protein becoming crosslinked to one, or frequently a number, of its neighbours. Here crosslinking would be a concentration independent event. In free solution, however, the proximity of crosslinkable groups will be an infrequent, concentration dependent, event. Thus, in a given mixture of ribonucleoprotein complexes, and free proteins, all the ribonucleoproteins would crosslink, resulting in stable complexes, while free proteins would remain in solution. It is then a relatively simple matter to fractionate these two components, for example by SDS-polyacrylamide gel electrophoresis.

Fig. 9.

Chemical Crosslinking of HnRNP Particles Using dimethyl suberimidate.

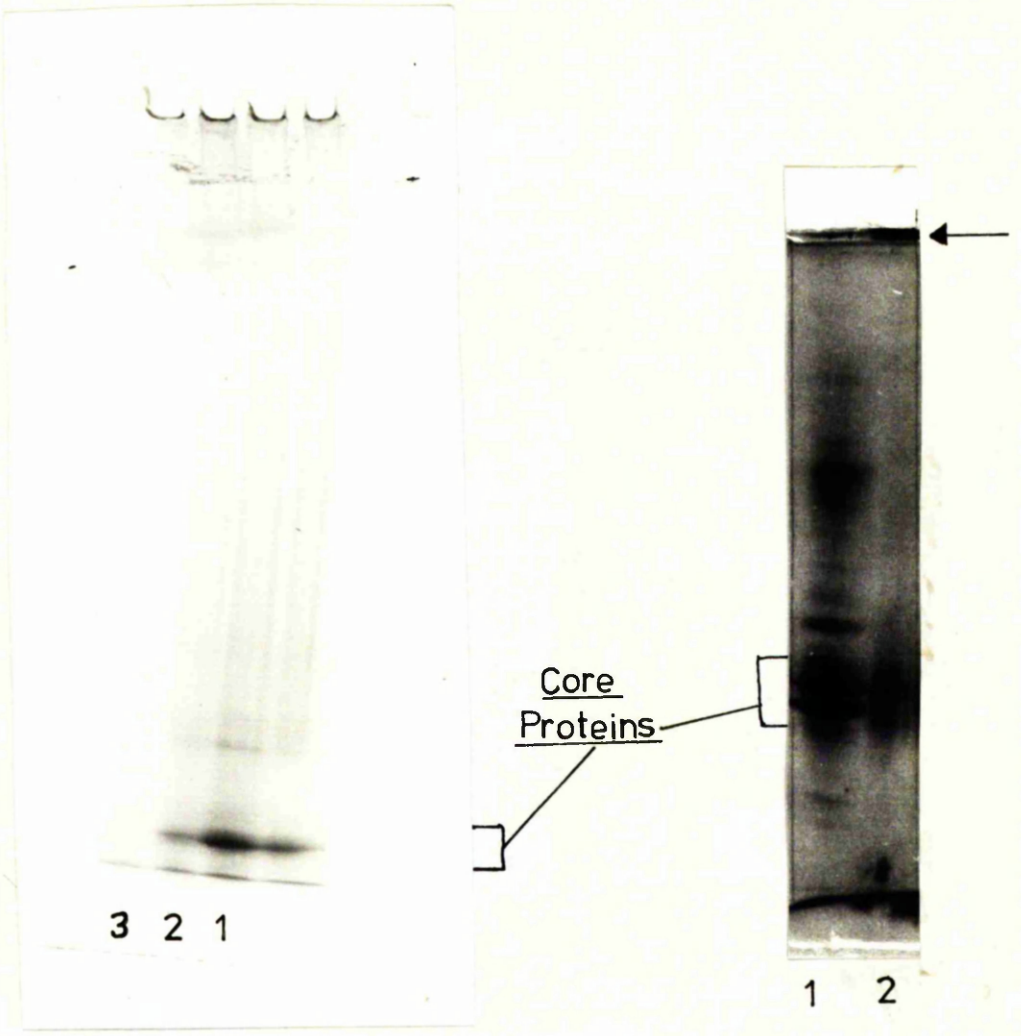
HnRNP particles, isolated from 15%–30% $^{w}/_{v}$ sucrose density gradients containing 0·1M NaCl, 0·01M Triethanolamine, pH 8·0, 0·001M $MgCl_2$, were dialysed against two changes of 0·01M Triethanolamine. HCl, pH 8·0, 0·1M NaCl, 0·001M $MgCl_2$, for 16 hours at 4°C. Aliquots of 50µg of protein were removed and made 10mM with respect to dimethyl suberimidate. After incubation overnight at 4°C, the crosslinked material was analysed by SDS-polyacrylamide gel electrophoresis using a 3%–10% polyacrylamide gel (A) or an 8·75% polyacrylamide gel with a 1% agarose stacking gel (B).

Gels were stained and destained as described elsewhere (2.3.2.4).

A. 3%–10% polyacrylamide gradient gel electrophoresis.
   1. No crosslinker.
   2. 5mM dimethyl suberimidate.
   3. 10mM dimethyl suberimidate.

B. 8·75% polyacrylamide gel (with 1% agarose stacker).
   1. No crosslinker.
   2. 10mM dimethyl suberimidate.

The arrow in gel B2 shows the crosslinked ribonucleoprotein complex not present in B1.


3 2 1
Core
Proteins
1 2
A.
B.

Such an experiment is described in Fig. 9 . Rat liver hnRNP particles were crosslinked with dimethyl suberimidate, and subjected to SDS-polyacrylamide gel electrophoresis. Fig. 9a shows a 3% - 10% polyacrylamide gradient gel, employed for the fractionation of hnRNP particles which were crosslinked with increasing concentrations of dimethyl suberimidate. The gradual loss of bands corresponding to hnRNP particle proteins, with increasing concentrations of crosslinker, indicates the increasing extent of particle crosslinking. The large size of the 40S hnRNP particle precludes the entry of crosslinked particles into the gel matrix, and no smaller "sub-particles" can be seen.

Fig. 9b shows an 8·75% SDS-polyacrylamide gel employing a 1% agarose stacker, through which the hnRNP particle complex is known to migrate (Karn et al, 1977). In this example, the disappearance of the hnRNP particle polypeptides due to crosslinking, is accompanied by the appearance of a stained band at the interface between the agarose stacker and polyacrylamide gel. This previously absent component is assumed to represent the crosslinked hnRNP particle.

These results confirm the data of Karn et al, (1977) and suggest that the 40S hnRNP particle is a true complex of all the proteins displayed by gel electrophoresis. In combination with the CsCl buoyant density data (Fig. 6) and the analysis of sucrose gradient fractions by SDS-polyacrylamide gel electrophoresis, the protein crosslinking data confirms the reality of the association of protein and RNA in the hnRNP particle.

It is, perhaps, a more difficult task to prove that the proteins of hnRNP particles are functional components of these complexes. The high protein to RNA ratio implies considerable protein-protein interaction, and it is in-appropriate to expect that all hnRNP particle proteins are involved in the packaging of hnRNA. The possibility, therefore,

Fig. 10.

Determination of Protein Adsorption to HnRNP Particles: Strategy of the Approach.

^{14}C-labelled nuclear proteins, prepared from HTC cells, as described in Fig. 11,were added to rat liver nuclei during, or after, their extraction for the preparation of hnRNP particles. (Methods 2.1.2.1). Fractionation of rat liver nuclei extracts prepared in this manner, on a sucrose density gradient, and subsequent estimation of the quantity of labelled protein in each fraction was used as an index of the adsorption of protein to the particles.

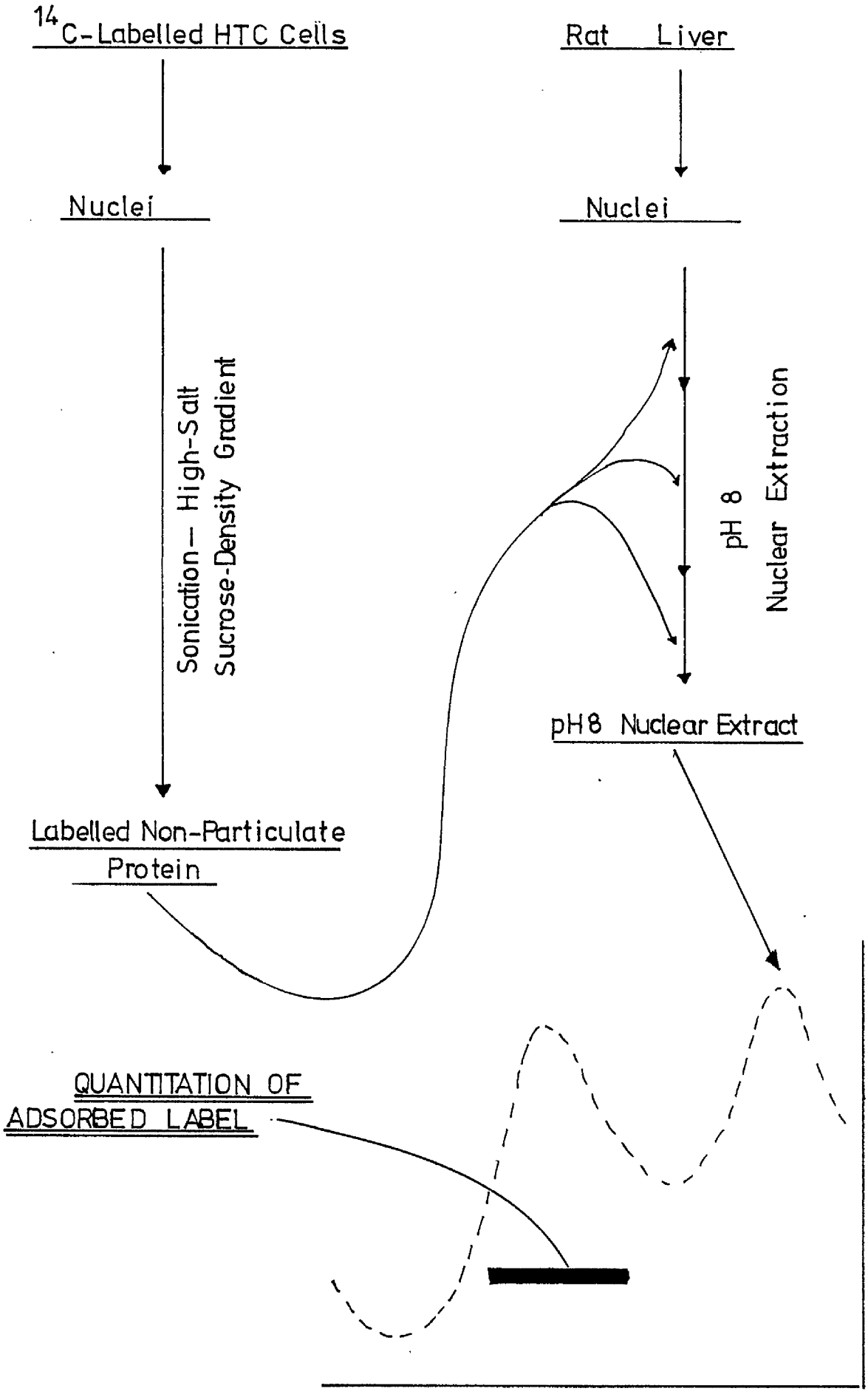
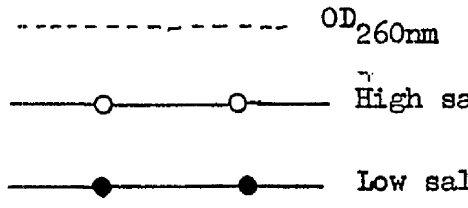
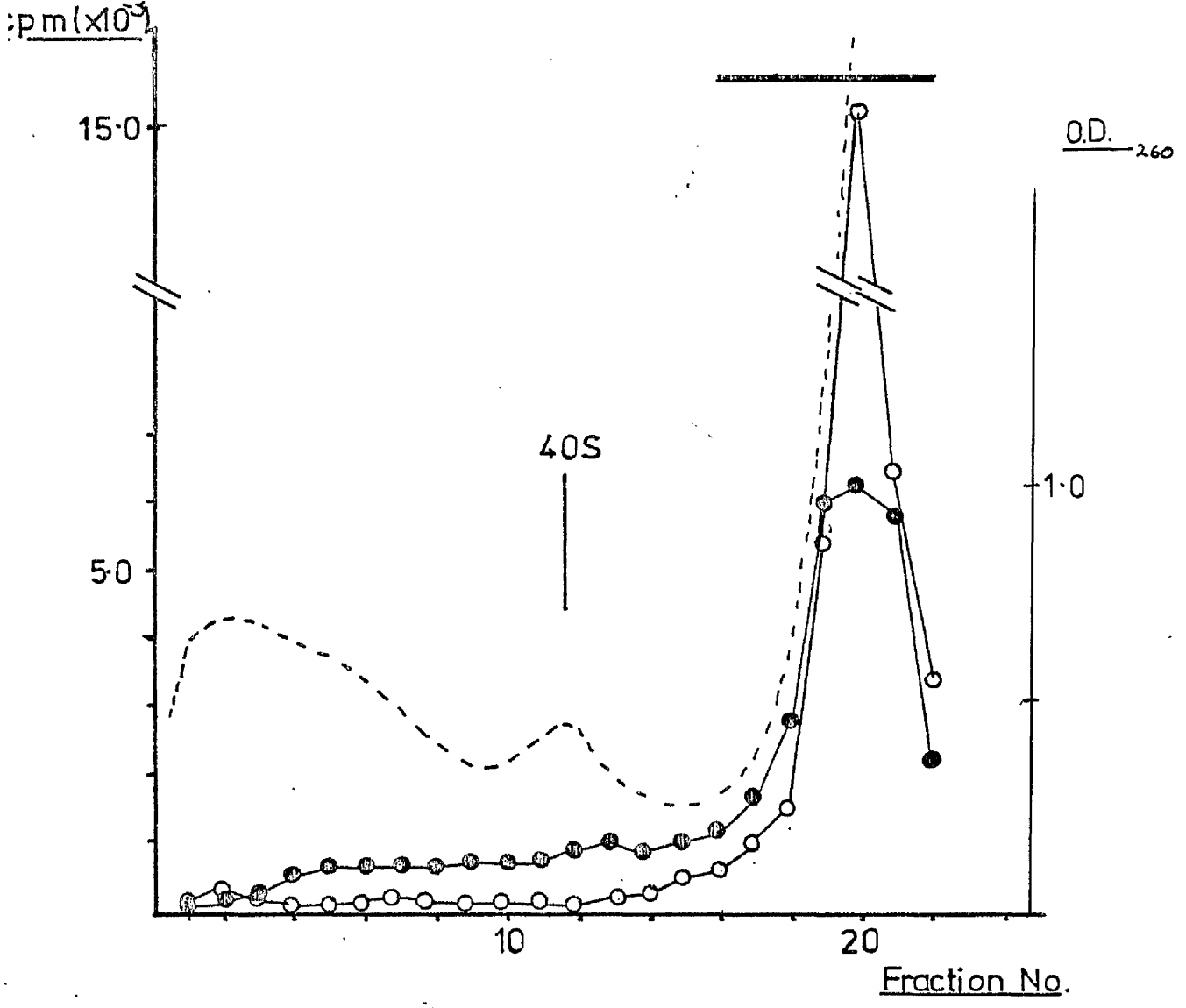

^{14}C-Labelled HTC Cells
Rat Liver
Nuclei
Nuclei
Sonication — High-Salt Sucrose-Density Gradient
pH 8 Nuclear Extraction
pH8 Nuclear Extract
Labelled Non-Particulate Protein
QUANTITATION OF ADSORBED LABEL

Fig. 11.

Preparation of ^{14}C-Labelled HTC Cell Nuclear Proteins.

The Eagles medium was removed from one burler of confluent HTC cells, and replaced with 50 ml of leucine free medium, containing 25μCi of 4[v-^{14}C]-leucine, and incubated for 20 hours at 37°C. Cells were harvested from this and 5 other unlabelled burlers of cells by means of a rubber "policeman", the cells washed twice in ice cold B.S.S. (0·116M NaCl, 5·4mM KCl, 1mM $MgSO_4$, 1mM NaH_2PO_4, 1·8mM $CaCl_2$, adjusted to pH 7·0 by the addition of 5% $NaHCO_3$) and finally resuspended in 1% Triton X100. HTC cell nuclei were then purified as described in the Methods (2.1.1.3). HTC cell nuclear extracts were prepared by a brief (30sec) sonication of the purified nuclei in 2 ml STM 7·0 (0·1M NaCl, 10mM Tris. HCl, pH 7·0, 1mM $MgCl_2$). The lysed nuclei were then layered over 5 ml of 30% sucrose, centrifuged at 5,000 g in a Sorvall HB 4 rotor, and the supernatent removed for subsequent analysis. Half of the post-nuclear supernatent was made 1·0M with respect to NaCl and fractionated on an 11 ml 15% - 30% sucrose density gradient containing 1·0M NaCl, 10mM Tris. HCl, pH 7·0, 1mM $MgCl_2$. The rest was fractionated on an 11 ml 15% - 30% sucrose density gradient containing 0·1M NaCl, 10mM Tris. HCl, pH 7·0, 1mM $MgCl_2$. Both gradients were centrifuged at 60,000 g for 12 hours in a Beckman SW 40 rotor. Each gradient was then fractionated and monitored for ^{14}C-labelled protein by liquid scintillation analysis of one-tenth aliquots, using the filter disc assay described in 2.2.4.1.





The material remaining at the top of the high salt gradient was employed in Fig. 12. The region of the gradient containing material sedementing at an S-value of greater than 40 S probably contains poly-particle hnRNP particles.

Fig. 12.

Addition of Labelled HTC Cell Nuclear Proteins to Isolated Rat Liver Nuclei During the Preparation of HnRNP Particles.

HnRNP particles were prepared from rat liver nuclei by the method of Samarina et al (1968), (Methods 2.1.2.1). Aliquots of the labelled HTC cell nuclear extracts were added as described below.

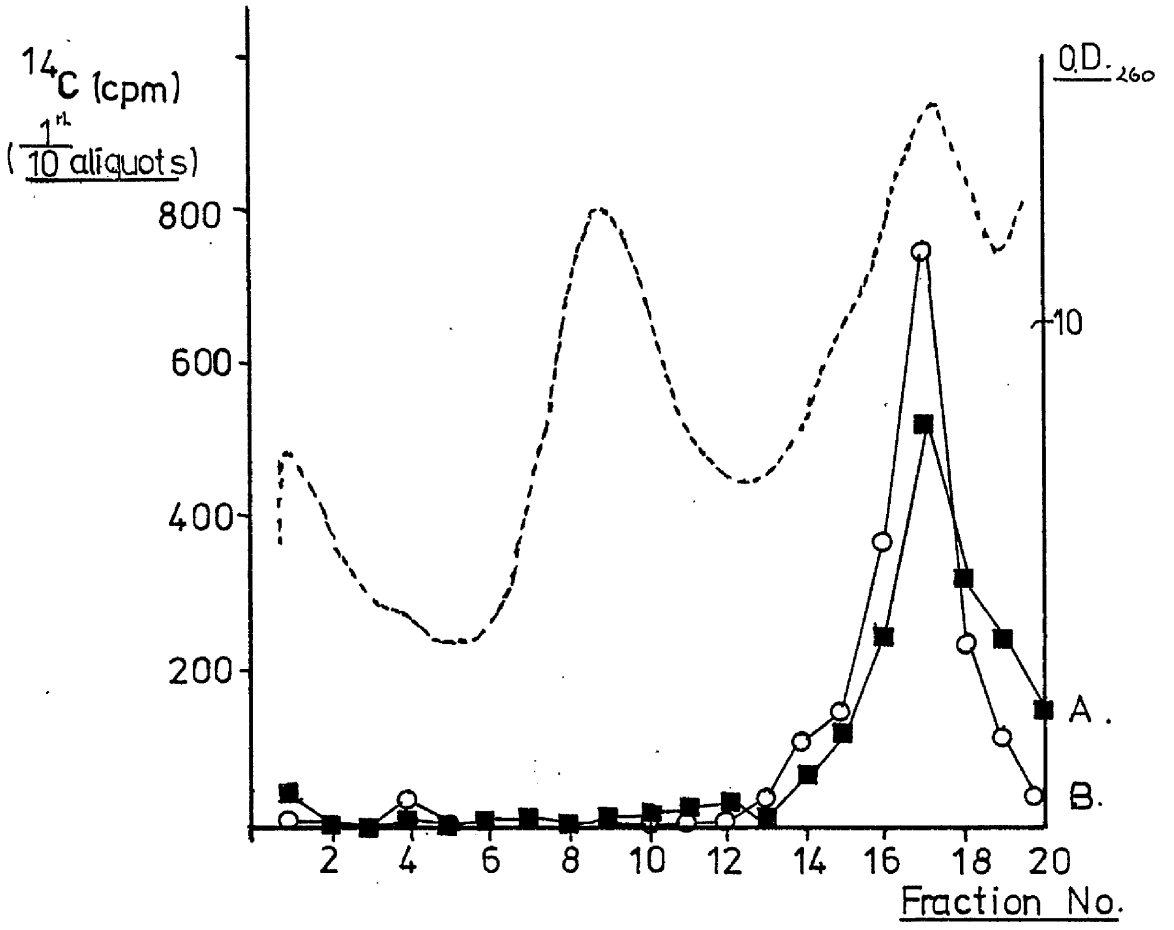
A - —■———■— 10µl (3,000 cpm) aliquots of labelled extract were added at each stage of the rat liver nuclei extraction.

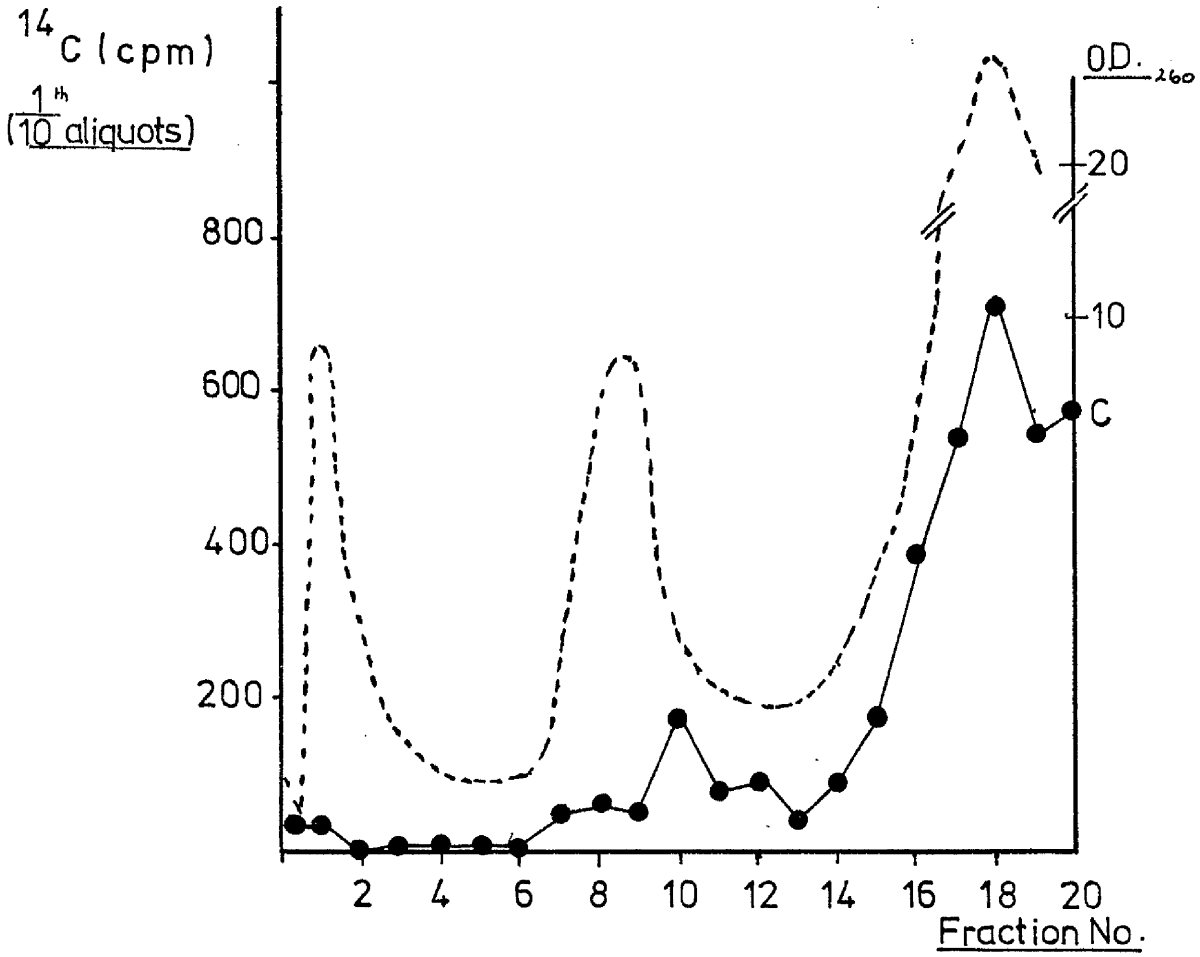
B - —○———○— 30µl (10,000 cpm) of the labelled extract was added prior to the centrifugation of the rat liver nuclei extract.

C - —●———●— As B, except that 10µg of pancreatic RNAse was added at the same time as the labelled extract, 10 minutes before centrifugation.

- - - - - - - OD_{260} trace of completed gradient.

The treated rat liver extracts were centrifuged at 78,000 g for 16 hours as described in the Methods section (2.1.2.1). Gradients were fractionated and simultaneously monitored for absorbance at 260nm. Each fraction was subsequently analysed for the presence of labelled protein by the filter disc assay described in Methods (2.2.4.1).


^{14}C (cpm)
1 ml
(10 aliquots)
800
600
400
200
O.D. 260
10
A.
B.
2
4
6
8
10
12
14
16
18
20
Fraction No.


^{14}C (cpm)
1 ml
(10 aliquots)
800
600
400
200
O.D. 260
20
10
C
2
4
6
8
10
12
14
16
18
20
Fraction No.

remains that some of the proteins associated with hnRNP particles are simply artefactually bound, either by ionic or hydrophobic interaction, during isolation. Such a possibility is exceptionally difficult to exclude.

One approach to this problem is to extract particles in the presence of added highly radioactive protein, and then look for radioactivity associated with particles as a measure of protein absorption. Similar rationale was used by Pederson (1974a) who looked for the association of protein with hot RNA and by Gallinaro et al, (1980) in examining snRNA association with hnRNP. Fig.10 outlines the strategy of the experiment. Purified HTC cell nuclei, highly labelled with ^{14}C - leucine, were fractionated by the method of Pederson (1974a) as outlined in Fig. 11 . The post-nucleolar supernatent, which contains hnRNP particles, is further fractionated on 15% - 30% sucrose gradients, either in the presence or absence of 1·0M NaCl. The low salt extract was used to confirm the identity of HTC cell hnRNP. Under the high salt conditions, all but the tight binding proteins of the hnRNP particle complex are removed (see also Fuchs & Jacob, 1980), and the region at the top of the gradient will therefore contain proteins likely to display a degree of adventitious binding to hnRNP particles, (should such a group of proteins exist). Small quantities of this high salt extract were then added to rat liver nuclei during the various stages in the preparation of hnRNP particles and the extent of adventitious binding of the added proteins was estimated from radioactivity associated with the purified particle.

As can be seen in Fig. 12 , no non-specific binding was detected. If however, the hnRNP particles were treated with RNAse in the presence of labelled proteins, there were indications that some adventitious binding occurred. This data is in agreement with that of Stevenin et al,

Fig. 13

Two Dimensional Fractionation of Rat Liver HnRNP Particle Proteins.

HnRNP particles, isolated from a sucrose density gradient similar to that shown in Fig. 5 (p . 64), were precipitated at -70°C with 2.5 volumes of ethanol and resuspended in lysis buffer (9.5M urea, 5% $^{w}/_{v}$ β-mercaptoethanol, 2% $^{w}/_{v}$ nonidet P40, 1.6% $^{w}/_{v}$ pH 5-7 ampholines, 0.4% $^{w}/_{v}$ pH 3.5-10 ampholines). Samples, at 10 mg.ml., were applied to either NEPHGE or IEF gels (See Methods 2.3.2 p .51) and electrophoresed at 500 V for either 15 hr. (IEF) or 4 hr. (NEPHGE) before being submitted to the SDS polyacrylamide gel electrophoresis second dimensional separation.

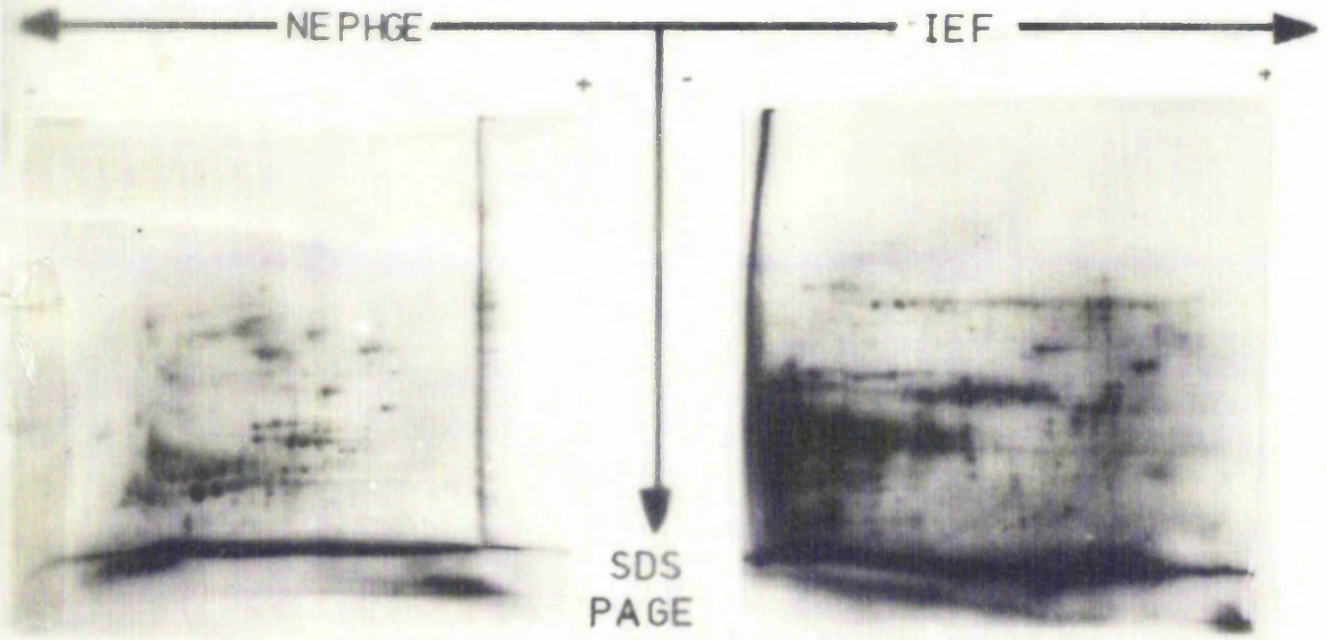
The gels in Figs. 13A and B were overloaded to reveal the minor protein species. Fig. 13C shows a gel loaded optimally for the separation of the core polypeptides.

A. NEPHGE-based two dimensional fractionation.
   (loading 300 µg: 2,000 v.hr.).

B. IEF-based two dimensional fractionation
   (loading 300 µg: 7,500 v.hr.).

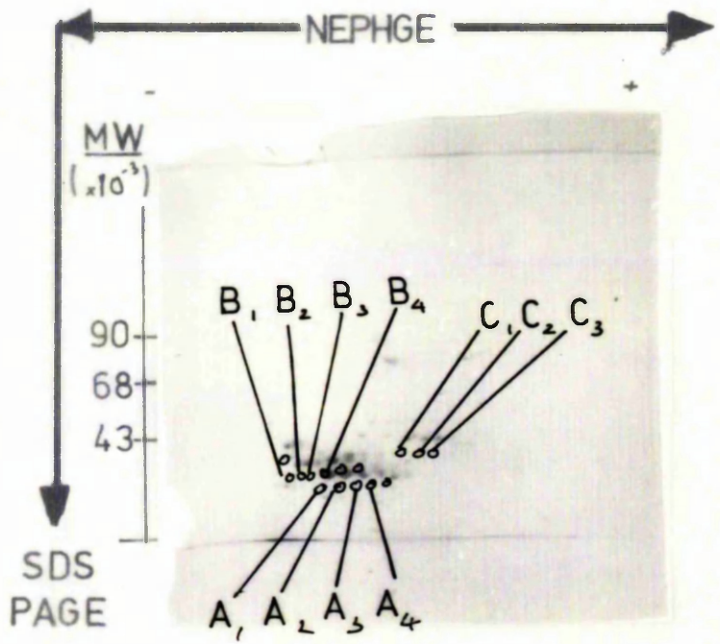
C. NEPHGE-based two dimensional fractionation
   (loading 150 µg:2,000 V.hr.).

The overlay to Fig. 13 shows the nomenclature used in this thesis to describe the main core proteins.

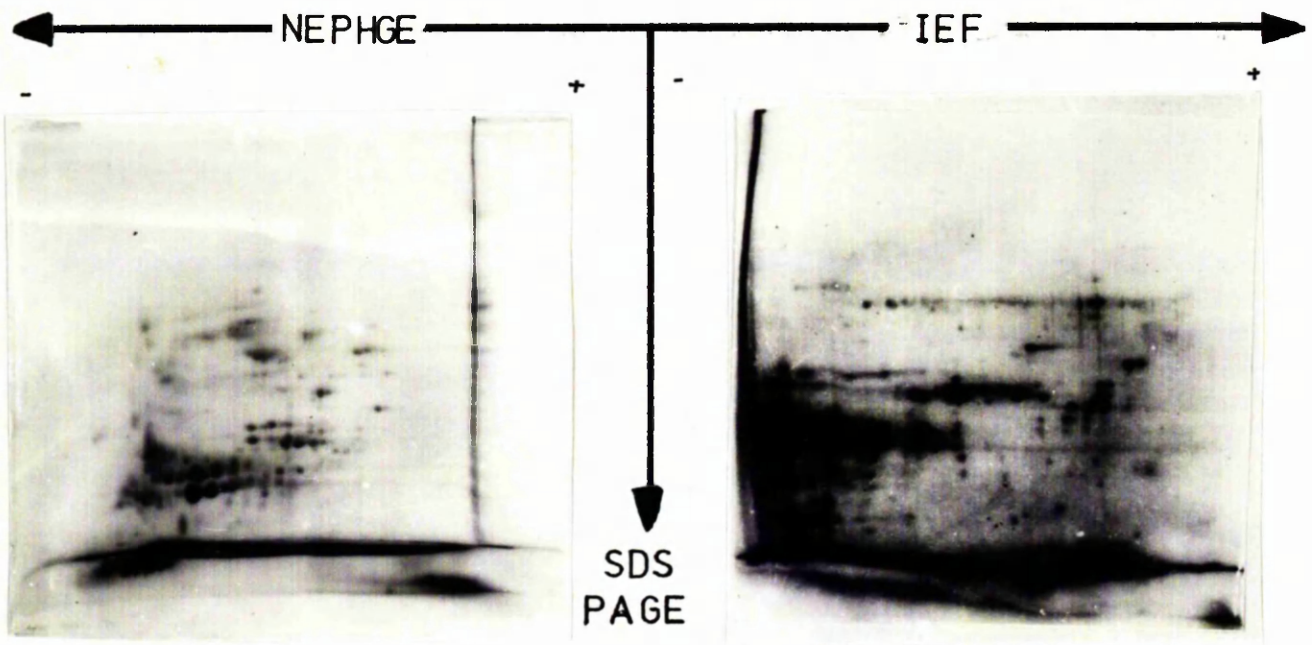

NEPHGE
IEF
SDS
PAGE

A.

B.

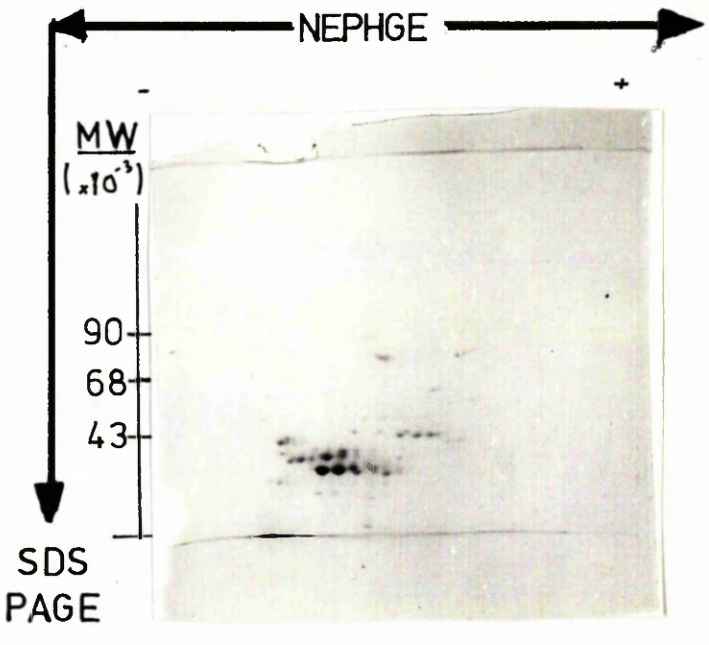

NEPHGE
MW
(x10⁻³)
90
68
43
SDS
PAGE
B1 B2 B3 B4
C1 C2 C3
A1 A2 A3 A4

C.



A.

B.



C.

(1979),who suggested that ribonuclease digestion of hnRNP particles produces rearrangement artefacts.

In summary,the two experimental approaches described have,in combination with the data in Fig.6 and Fig. 7 suggested that hnRNP particles are non-artefactual associations of RNA and a defined sub-group of nuclear proteins. The detailed investigation of the proteins associated with the hnRNP particle will now be discussed.

1.4 Two-Dimensional Analysis of HnRNP Particle Proteins.

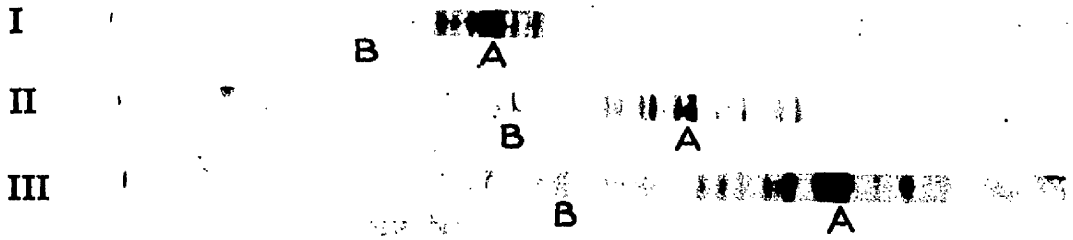
It has long been recognised that bidimensional electrophoretic fractionation is potentially a most powerful technique for the analysis of complex protein mixtures. Many systems have been employed for such analyses, the most successful and enduring being those employing an ampholine based first dimension coupled with SDS-polyacrylamide gel electrophoresis (MacGillivray & Rickwood, 1974; O'Farrell, 1975; Jackowski et al, 1976; Peterson & McConkey, 1976).

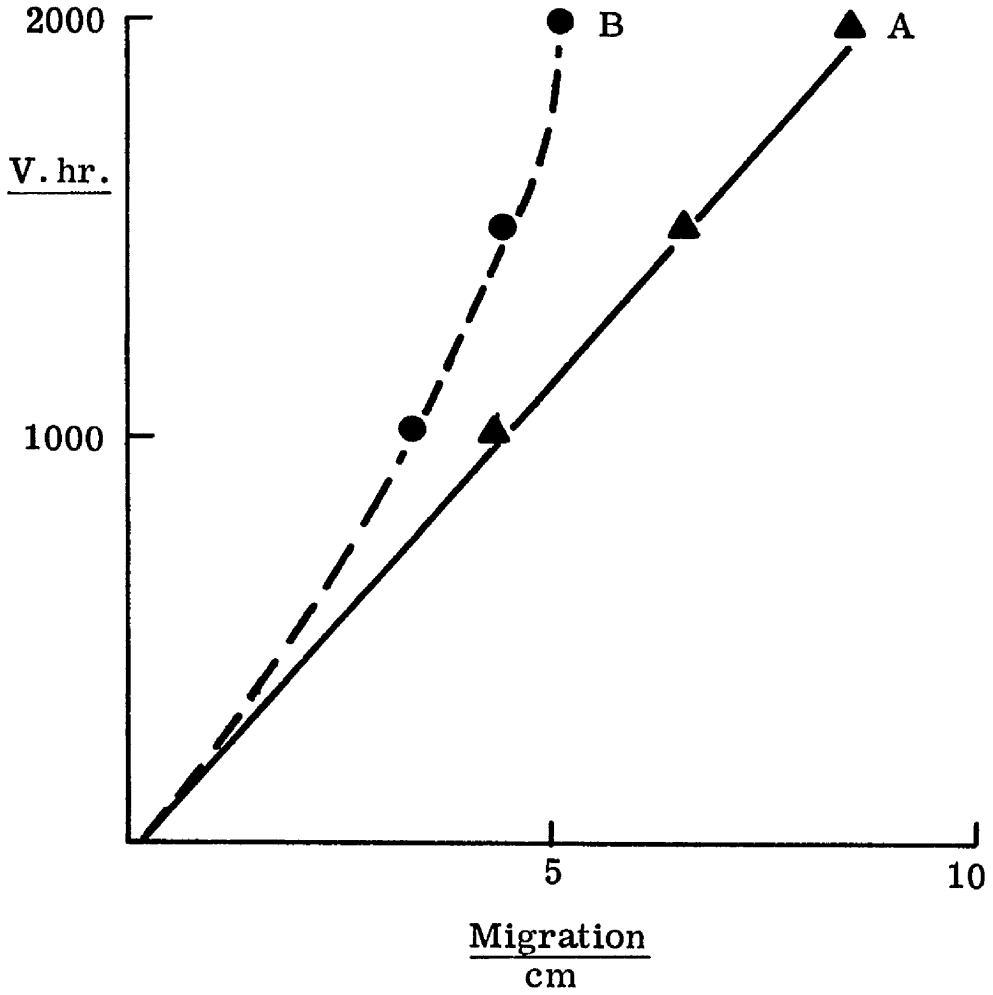
It was felt,however,that although it suffered from the drawback of limited pH range in comparison with the other systems,the system devised by O'Farrell (1975) was probably more suitable for high resolution of hnRNP particle proteins. First dimensional separation of the proteins is on the basis of intrinsic charge (pI). The fractionated proteins are then subjected to SDS-polyacrylamide gel electrophoresis,which is essentially a size dependent fractionation. The combination of these two procedures should result in the optimal separation of the components of a "typical" polypeptide mixture. In the case of the fractionation of hnRNP particle proteins,however,this technique proved inadequate. Fig. 13b shows the two-dimensional fractionation of hnRNP particles by the method of O'Farrell (1975). Although many of the minor (acidic) polypeptide species were well fractionated,it is obvious that considerable loss of the more basic "core" polypeptides has occurred. This situation

Fig. 14.

Optimisation of the Conditions of Non-Equilibrated pH Gradient Electrophoresis for the Fractionation of HnRNP Particle Proteins.

NEPHGE gels (Methods 2.3.2) were loaded with 60µg of hnRNP particle proteins in lysis buffer, and were electrophoresed at 500 v for 2 hrs. (I), 3 hrs. (II) and 4 hrs. (III). The migration of a basic "core" protein (A) and a neutral protein (B) are plotted on the graph below. After 2,000 v.hr, the neutral protein (B) is near equilibrium, while the basic protein is still migrating at a linear rate. The implications of this experiment are discussed on p82..


I
B
A
II
B
A
III
B
A


2000
V. hr.
1000
B
A
5
10
Migration
cm

is not resolved by the inclusion of basic range (9-11) ampholines in the first dimension gel, nor by the substitution of cathodic with anodic sample loading. The problems with cathode drift in the IEF separation needed to be resolved for a complete analysis of the basic range proteins of hnRNP particles.

One possible solution to this problem, and the one selected for use in this study despite the intrinsic merits of other similar systems, was also developed by O'Farrell and co-workers. A non-equilibrated first dimensional electrophoresis, which was brief enough to preclude problems with cathode drift, yet long enough to satisfactorily fractionate protein mixtures, was developed (O'Farrell et al, 1977). In order to apply this technique to the fractionation of hnRNP particle proteins, optimisation of the length of electrophoresis by non-equilibrated pH gradient electrophoresis (NEPHGE) was required, and the details of such an experiment are presented in Fig. 14. In this experiment, the migration of two polypeptides (one of approximately neutral pI, the other basic) was measured as a function of the time of electrophoresis. After 2,000 v.hr. (400v x 5hr.) of electrophoresis, the neutral polypeptide appeared to be reaching equilibrium. The basic polypeptide, however, had yet to reach equilibrium, and would be lost from the gel with prolonged electrophoresis. An optimal electrophoresis period of 2,000 v.hr. was therefore employed for the fractionation of hnRNP particle proteins.

Fig. 13a and c show the combination of a NEPHGE first dimension with SDS-polyacrylamide gel electrophoresis in the second dimension, for the fractionation of hnRNP particle proteins. Several interesting features can be seen. Firstly, the major "core" polypeptides, which dominated the one dimensional profile (See Fig. 7), also dominated the two-dimensional profile. These proteins were very basic, exhibited a considerable degree of charge heterogeneity, and could

be resolved into several separate chains of spots. To facilitate later discussion of these polypeptides, the following nomenclature will be employed, (See overlay on Fig. 13). The lower chain of spots, of mol.wt. 32,000 will be termed the A chain, each component being additionally numbered from left to right (basic to acidic; for example, A_1, A_2 etc.). Similarly, the upper chain of spots, of mol.wt. 34,000, will be termed the B chain (for example, B_1, B_2, and B_3) and the short chain of spots with an apparent mol.wt. of 40,000 will be called the C chain. The possible causes of the charge heterogeneity will be discussed in Part 2 of the Results section, however, it is of interest to note here that this phenomenon is frequently associated with the post-translational modification of proteins.

The use of both IEF based and NEPHGE based two-dimensional protein fractionation ensured that both basic and acidic proteins were resolved. Approximately 90 polypeptide species were resolved in the NEPHGE based system, while a further 20-30 additional minor components were distinguished by the IEF based fractionation.

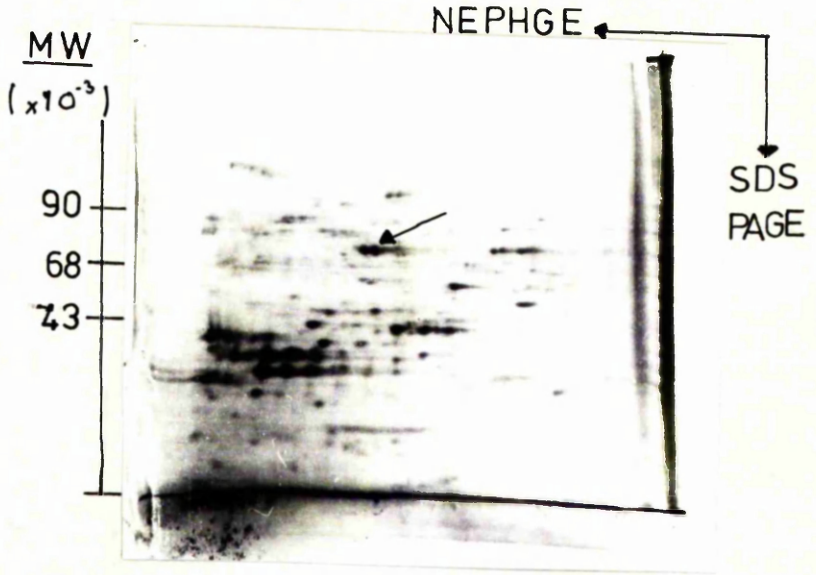
The proteins of the hnRNP particle, therefore, appeared to consist of at least two major, very basic, core polypeptides (which exhibited considerable charge heterogeneity), several less prominent species, which corresponded to those visible on one dimensional SDS-polyacrylamide gels (which also exhibited charge heterogeneity to varying degrees), and a large number of minor species of predominantly acidic pI and mol.wt. above 45,000. Several similar analyses of hnRNP particle proteins have been published since the completion of this work (Suria & Liew, 1979; Lelay & Brunel, 1979; Maundrell et al, 1979; Peters & Comings, 1980). The same general conclusions were reached by all of these investigators.

Fig. 15.

Two-Dimensional Fractionation of Nuclear pH 8·0 Extract.

A pH 8·0 nuclear extract, containing a mixture of non-particulate and hnRNP particle proteins, was subjected to two-dimensional fractionation using the NEPHGE-based two-dimensional gel system (Methods 2.3.2). One millilitre of pH 8·0 extract was ethanol precipitated, and the resulting pellet redissolved in lysis buffer at 10mg. per ml. Forty microlitres (400μg) of protein was applied to the first dimensional gel, and the gel run for 2,000 v.hr.

← 73,000 m.w. protein (See p 85).


NEPHGE
MW
(x10⁻³)
90
68
43
SDS
PAGE

1.5 Comparison of HnRNP Particles Generated by the Extraction and Sonication Techniques.

It has been almost traditional to divide the methods used in the isolation of hnRNP particles into two sections. For the investigation of hnRNP particles from animal tissues such as liver (e.g. Karn et al, 1977), uterus (Knowler, 1976), or blood (Lerner et al, 1980), predominant use has been made of the "Extraction Technique" (See Methods 2.1.2.1), devised by Samarina et al, (1968). Where in vitro cultured cells are the source of the hnRNP particles, the "Sonication Technique" (See Methods 2.1.2.2) of Pederson (1974a) appears to be the method of choice.

In order to assess the merits of these two isolation procedures, comparison of the important sub-nuclear fractions generated in the isolation of hnRNP particles has been made. Two dimensional analysis of the pH 8·0 extract of purified nuclei (Samarina et al, 1968) showed a number of interesting features (Fig.15). The predominance of the hnRNP particle core polypeptides, the presence of other hnRNP particle polypeptides, such as the 73,000 mol.wt. species*, which may be the poly-(A) binding protein, and the familiar triplet of mol.wt. 45,000, indicated that this extract contained high levels of hnRNP particles.

The apparent domination of this profile by hnRNP particle proteins was, however, misleading. Further fractionation on 15% - 30% sucrose density gradients, (as outlined in Fig. 5 & Fig. 7), and comparison of the polypeptide profiles of the two interesting zones (non-particulate proteins, and hnRNP particles), gave a better indication of the complexity of the original extract. The non-particulate material remaining at the top of the gradient exhibited a complex polypeptide profile (Fig.16a). In excess of 200 spots were visualised.

* See arrow in Fig 15.

Fig. 16

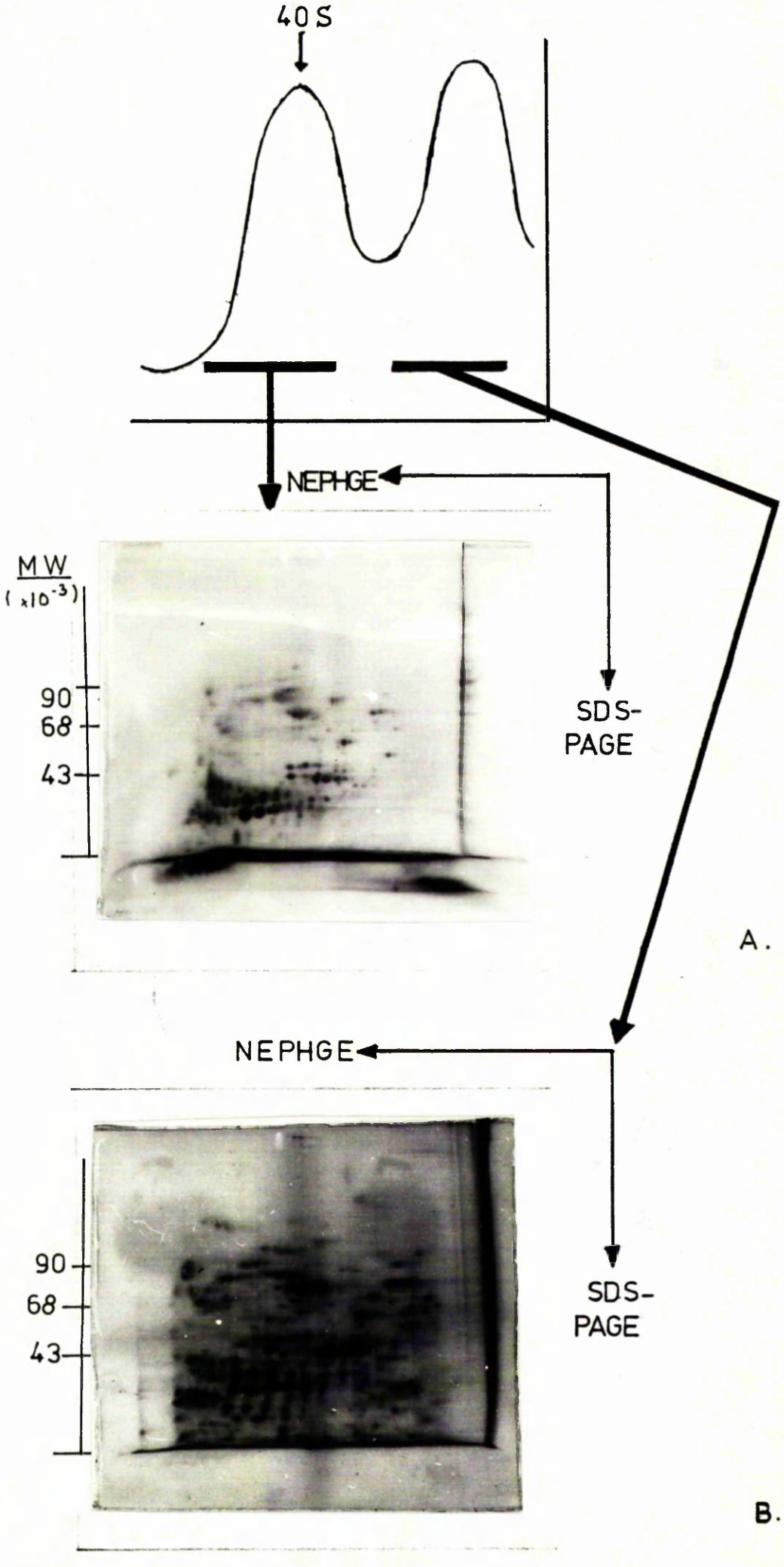
Two Dimensional Analysis of Sucrose Density Gradient Fractions.

I - Extraction Technique.

The 40S hnRNP particle and non-particulate regions were recovered from sucrose density gradients on which a pH 8.0 nuclear extract (Methods 2.1.2.1 p . 42) had been fractionated. Each region of the gradient was precipitated using 2.5 volumes of ethanol at -70°C,and the precipitate redissolved in lysis buffer (9.5M urea, 5% $^{w}/_{v}$ β-mercaptoethanol, 2% $^{w}/_{v}$ nonidet P40, 1.6% $^{w}/_{v}$ pH 5-7 ampholines, 0.4% $^{w}/_{v}$ pH 3.5-10 ampholines),and subsequently analysed by the NEPHGE-based two dimensional fractionation technique (Methods 2.3.2 p . 51).

A - 40S hnRNP particle region - (loading 300 µg: 2,000 v.hr.).

B - Non-particulate region - (loading 500 µg: 2,000 v,hr.).


40 S
NEPHGE
MW
(x10⁻³)
90
68
43
SDS-
PAGE
A.
NEPHGE
90
68
43
SDS-
PAGE
B.

Since this fraction was derived from the apparently simple pH 8·0 extract (Fig.15), one must conclude that these minor species were masked by the relatively high concentrations of hnRNP particle polypeptides present in this extract. The hnRNP particle polypeptides could be detected in the non-particulate fraction but their abundance, with respect to the remaining polypeptides, was greatly reduced.

The presence of hnRNP particle polypeptides in the non-particulate fraction could result from a number of situations. Firstly, the non-particulate hnRNP particle proteins may represent a pool of hnRNP particle proteins, which is drawn upon during the assembly of particles on the nascent RNA chain. Alternatively, they may represent material released in the degradation of the hnRNP particles during their isolation. Such a possibility has been raised by Stevenin et al,(1975), who suggested that the hnRNP particle proteins were not true components of the non-particulate fraction.

One unaccounted for observation from a comparison of the non-particulate polypeptide profile with that obtained from hnRNP particles was the presence of a number of additional components in the core protein A chain. (Compare Fig.16a& Fig.16b). Five derivatives of this core protein could be detected in the hnRNP particle protein profile, however, the three more acidic derivatives (A_3, A_4 and A_5) appeared to be more generously represented in the non-particulate protein profile. The reason for this difference is unknown.

A similar characterisation of the hnRNA particles prepared by the Sonication technique (Pederson, 1974a) appears in Fig.17 . Three regions of the sucrose density gradient are shown. Again, the non-particulate region of the gradient was complex, exhibiting a considerable number of polypeptide species (Fig.17a). The hnRNP particle core polypeptides were present, and appeared to be more

Fig. 17

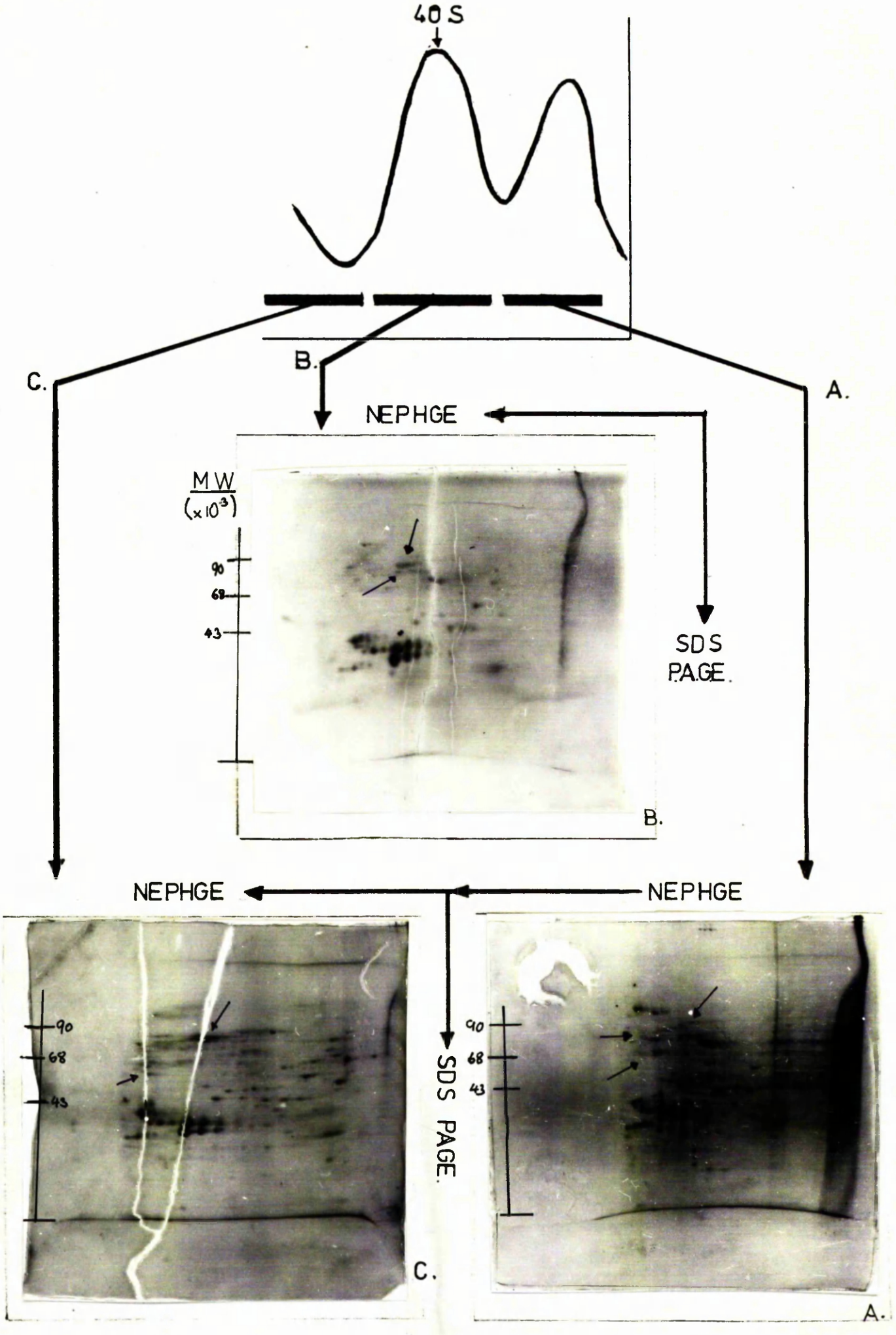
Two Dimensional Analysis of Sucrose Density Gradient Fractions. II - Sonication Technique.

The non-particulate,40S hnRNP particle,and "polyparticle" regions were recovered from sucrose density gradient analysis (See Fig. 5 p . 64) of a post-nucleolar sonicate of rat liver nuclei (Methods 2.1.2.2. p . 43). Each region of the gradient was precipitated using 2.5 volumes of ethanol at -70°C,and the precipitate redissolved in lysis buffer (9.5M urea,5% $^{W}/_{v}$ β - mercaptoethanol, 2% $^{W}/_{v}$ nonidet P40, 1.6% $^{W}/_{v}$ pH 5-7 ampholines; 0.4% $^{W}/_{v}$ pH 3.5-10 ampholines),and subsequently analysed by the NEPHGE-based two dimensional fractionation technique (Methods 2.3.2 p . 51).

A - Non-particulate - (loading 300 µg: 2,000 v.hr.).

B - 40S hnRNP particles - (loading 200 µg: 2,000v.hr.).

C - "Polyparticles" - (loading 200 µg: 2,000 v.hr.).


40 S
B.
C.
A.
NEPHGE
MW
(x10^3)
90
68
43
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PAGE.
B.
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90
68
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SDS PAGE.
C.
90
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43
A.

+

With respect to the statement made on p. 89, line 7, concerning the greater quantities of high molecular weight proteins present in the sonicated extract, this statement was made on the basis of comparison of a number of gels from many preparations. A direct comparison of Fig. 17B and Fig. 16A, appears to contradict this statement. However, it must be stressed that the gels were obtained from different amounts of protein. Perhaps a better example of the "Extraction Method" 40S hnRNP particle protein profile is shown in Fig. 34 p.126, where an equivalent quantity of hnRNP particle proteins has been fractionated to that employed in Fig. 17B (200μg). This observation has also been made by several other authors (Brunel and Lelay, (1979) inter alia).

abundant than in the extracted hnRNP particle profile. This may reflect a more complete release of these proteins from chromatin stores, or may be the result of disruption due to sonication.

Fig. 17b shows that the typical pattern of core polypeptides of hnRNP particle preparations derived from sonicated nuclei was present. However, preparations derived by this method consistently contained greater quantities of high molecular weight protein species.* Which of the two particle preparations most closely represents the true compliment of proteins is unknown. The rapidity of this latter technique coupled with its use in circumstances when endogenous RNAse levels are low (e.g. the majority of all culture lines, neoplastic tissues (Daoust & Lamirande, 1975)) and the brain (Stevenin et al, 1972), allows the preparation of polymer hnRNP particles (polyparticles). However, the majority of animal tissues have somewhat higher endogenous RNAse levels, which preclude the isolation of polyparticles in quantity, regardless of the rapidity of the isolation procedure. The high mol.wt.* species appear to correspond to the nuclear matrix polypeptides described by Peters and Comings (1980).

Fig. 17c illustrates the protein profile of preparations derived from the A_{260nm} absorbing material located at the bottom of a sucrose density gradient upon which sonicated nuclear extracts had been fractionated. They were presumed to represent low levels of polyparticles, known to be liberated by this technique and they revealed a polypeptide composition very similar to that of monoparticles; including the previously described high mol.wt. species.*

Fig. 18 shows the analysis of the RNA component of both sonicated and extracted particles, by formamide gel electrophoresis. Both sonicated and extracted particles contained a similar size distribution, which appeared typical of those previously reported in the literature

*See arrows in Fig 17

Fig. 18.

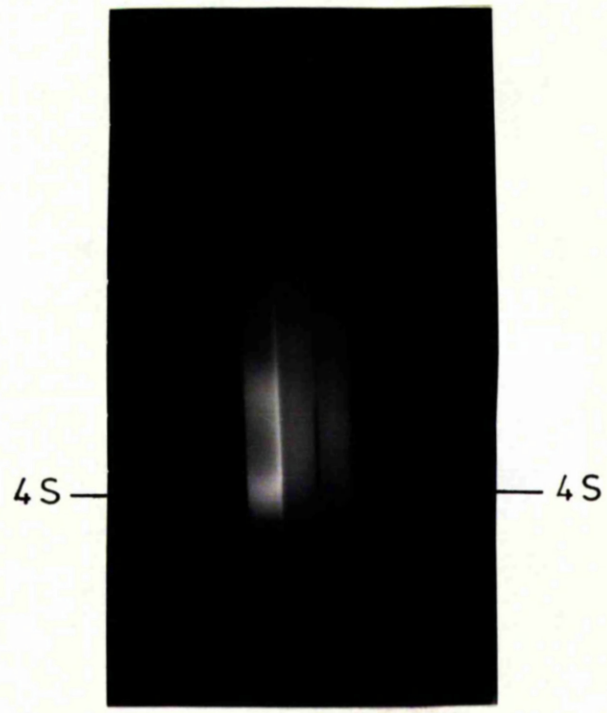
Analysis of the RNA Component of HnRNP Particles by Gel Electrophoresis in 99% Formamide.

The RNA components of both extracted and sonicated hnRNP particles were prepared by phenol extraction of isolated 40S hnRNP particles (Methods:Section 2.4.1), and subjected to polyacrylamide gel electrophoresis in the presence of 99% formamide (Methods: Section 2.4.2). Gels were stained in 0·1% ethidium bromide in 0·1M ammonium acetate and photographed with a Polaroid MP3 land camera.

A - Crude tRNA marker - 40μg.

B - Sonicated hnRNP RNA.

C - Extracted hnRNP RNA.


4S
4S
A B C

(e.g. Samarina et al, 1968; Beyer et al, 1977).

The results presented above indicate, within the limits of the techniques described, that similar hnRNP preparations are recovered by either the extraction or sonication methods. Nevertheless, because more uncertainty exists over the purity of the product of the latter technique, most of the work described throughout the remainder of this thesis employs particles derived by the extraction method.

1.6 Species and Tissue Specificity of HnRNP Particle Proteins.

One approach to the investigation of the function of hnRNP particle polypeptides is an investigation of species and tissue specificity of the protein components. This has been performed using one-dimensional SDS-polyacrylamide gel analysis by a number of authors (Stevenin et al, 1977; Karn et al, 1977; Noll & Lukanidin, 1976). The general conclusion of these investigators has been that the hnRNP particle core polypeptides are apparently universal, although a number of differences have been observed with respect to the minor polypeptides. The application of NEPHGE based two-dimensional fractionation technique to this question seemed appropriate, and was employed in the analysis of rat, rabbit and mouse liver and rat brain hnRNP particle proteins.

HnRNP particles were prepared from purified brain nuclei by the sonication method of Stevenin et al, (1972), and enriched with respect to their 40S monoparticle content by a 15 min. incubation at 22°C prior to sucrose density gradient centrifugation. All liver hnRNP fractions were prepared by the extraction technique (Samarina et al, 1968). Fig.19 compares the polypeptide profile of the 40S hnRNP peak derived from all of these preparations.

Comparison of the rat liver (Fig.13) and rat brain 40S hnRNP particles (Fig.19a) reveals the considerable similarity between the

Fig. 19

Tissue and Species Specificity of HnRNP Particle Proteins.

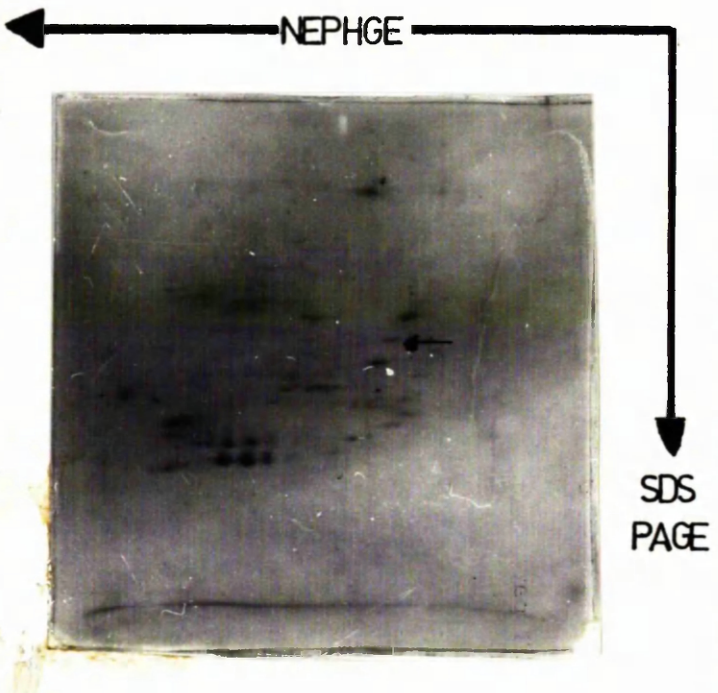
HnRNP particles were prepared from purified mouse and rabbit liver nuclei (prepared by a modification of the method of Chauveau et al (1956), see Methods 2.1.1.1 p . 41) by the low salt extraction technique (See Methods 2.1.2,1 p . 42) and from purified rat brain nuclei (prepared by the method outlined in Methods 2.1.1.2 p . 41) by the sonication technique (See Methods 2.1.2.2 p . 43). The isolated hnRNP particles were precipitated with 2.5 volumes ethanol at -70°C and subsequently redissolved in lysis buffer (9.5M urea, 5% $^{w}/_{v}$ β-mercaptoethanol, 2% $^{w}/_{v}$ nonidet P40, 1.6% $^{w}/_{v}$ pH 5-7 ampholines, 0.4% $^{w}/_{v}$ pH 3.5-10 ampholines) at 10 mg.ml. and submitted to the NEPHGE-based two dimensional fractionation (See Methods 2.3.2 p . 52).

A - Rat brain hnRNP particles - (100 µg protein; 2,000 V.hr.).

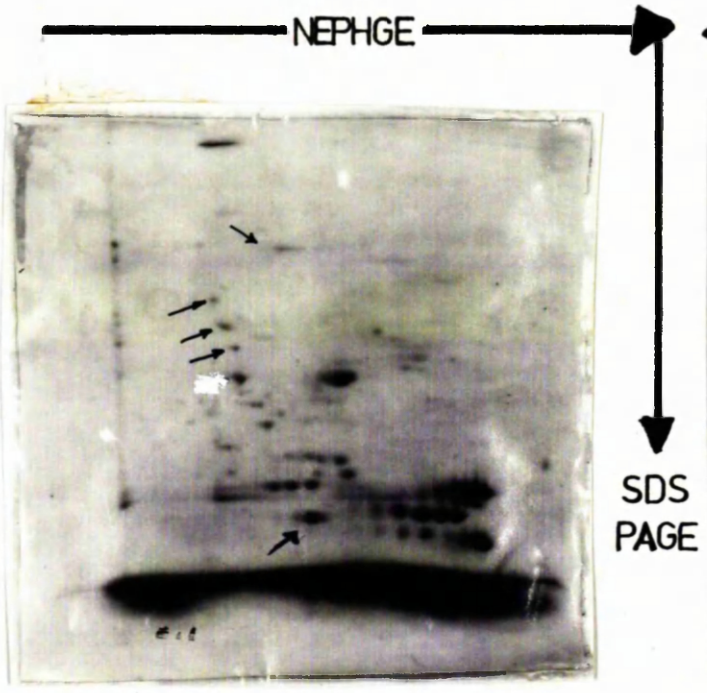
B - Rabbit liver hnRNP particles - (150 µg protein: 2,000 V.hr.).

C - Mouse liver hnRNP particles - (300 µg protein: 2,000 V.hr.).

← Tissue or species specific proteins discussed on p . 93 para. 1,2 and 3.



A



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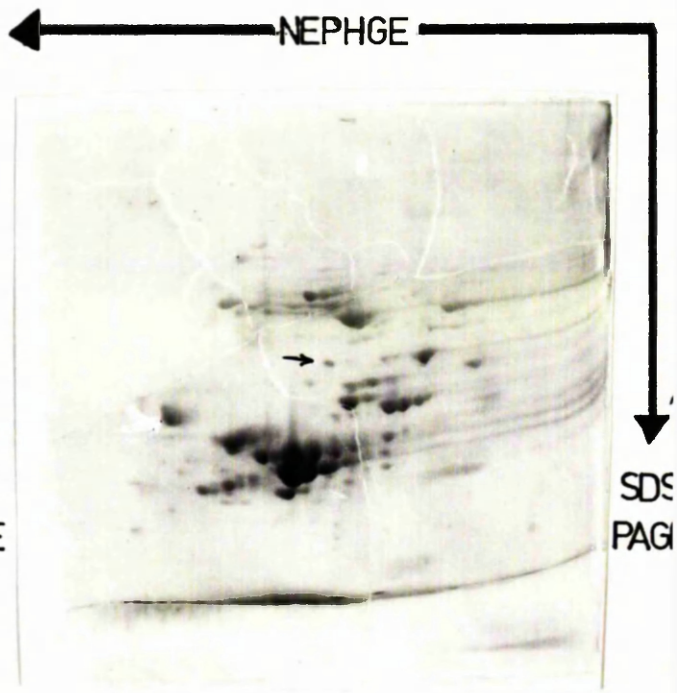


C

two preparations. The predominance of the core polypeptides and the presence of the 45,000 mol.wt. triplet and 73,000 mol.wt. protein are common features. The brain preparation does however contain a number of minor polypeptides not present in liver.

Fig.19 a & b illustrates the protein profile of particles derived from rabbit and mouse liver. Again, the predominant core polypeptides are a common feature, although several interesting minor differences can be seen, particularly with respect to the rabbit liver hnRNP core polypeptides. The majority of the other rat hnRNP particle proteins (Fig.13) are also visible in the mouse and rabbit profiles (Fig.19). However, a number of additional, apparently species specific polypeptides,are resolved (See arrows in Fig.19).

This data suggests that the majority of hnRNP particle proteins are common to all tissues, and are conserved between species. Nevertheless, a number of minor tissue- or species-specific polypeptides were revealed by these analyses, and it is tempting to suggest that these components may play a role in some aspect of the control of specific gene expression.

2. ANALYSIS OF THE HnRNP PARTICLE CORE POLYPEPTIDES.

The core polypeptides constitute between 60 - 70% of the total protein mass of the hnRNP particle. It is now widely accepted, based on a wide spectrum of circumstantial data (See Introduction 4.3.2 and Discussion), that these polypeptides function as RNA packaging proteins, combining with the nascent RNA at an early stage in its synthesis, and protecting the transcript from non-specific hydrolysis during its nuclear lifetime. The importance of these polypeptides in the post-synthetic processes which occur to the nascent transcript is thus a matter of general agreement to investigators, and the study

of the hnRNP particle core polypeptides is becoming increasingly popular (Karn et al, 1977; Fuchs et al, 1980).

2.1 Complexity of the Core Polypeptides.

Two-dimensional gel electrophoresis of the hnRNP particle polypeptides (Fig.13) has revealed a considerable degree of charge heterogeneity amongst the core polypeptides. Close scrutiny of these proteins, which appear as two main bands and , perhaps, five minor bands on one dimensional SDS-polyacrylamide gradient gels (Fig. 7), revealed the presence of approximately 30 polypeptide species. Most of these were components of four chains of spots. A number of phenomena could give rise to this sort of spot grouping. Firstly, post-translational modification of a source polypeptide by a variety of means is known to alter the intrinsic charge of a polypeptide. Secondly, the presence of a gene family (the possible product of an evolutionary gene duplication event) may result in the production of a number of closely similar proteins. Such protein families are known for vitellogenin (Jaggi et al, 1980) and actin (Fyrberg et al, 1980). Finally, the production of slightly different mRNAs from the same gene transcript has been described for some immunoglobulins (Early et al, 1980), raising the possibility that the production of these protein variants could result from post-transcriptional processing of pre-mRNA. The thorough testing of the latter two possibilities is far beyond the scope of this thesis, although some of the results to be described fit more appropriately with these phenomena than with the post-translational modification model. In general, however, the fact that the variation occurs in the charge dimension to a much greater extent than the size dimension, favours the first of the above possibilities and it is this that has been subjected to analysis.

Fig. 20.

In vitro Phosphorylation of HnRNP Particle Core Polypeptides.

HnRNP particles were dialysed against 0·1M sodium acetate, pH 6·5, 10mM $MgCl_2$ and incubated at 30°C for 10 minutes, in the presence of 50μCi. $[\gamma\text{-}^{32}P]$-ATP (5,000 Ci.mMol^{-1}). The phosphorylation products were precipitated with 2·5 volumes of ethanol, at -20°C, and finally dissolved in lysis buffer at 15mg.ml^{-1}. After heating in a boiling water bath for 1 minute, the hnRNP particle proteins were subjected to two-dimensional fractionation as described in the Methods section (2.3.2). The resulting electrophoretogram was stained and dried onto filter paper. The dried gel was photographed and then autoradiographed for 4 days using Kodak X-Omat H film.

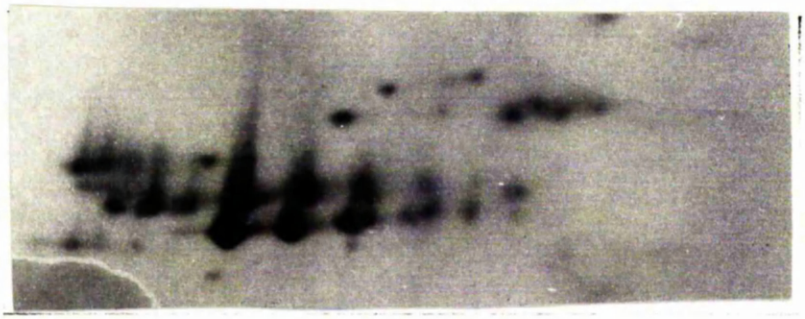
I - Core protein region of stained gel.

II - Core protein region of stained gel (drawing).

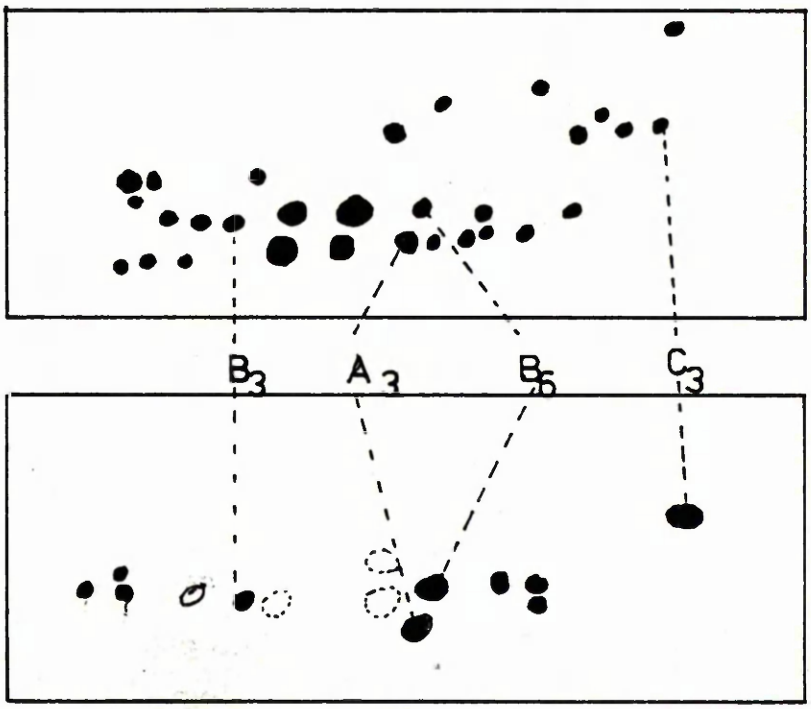
III - Core protein region of autoradiograph (drawing).

IV - Core protein region of autoradiograph.

The proteins are labelled using the nomenclature outlined in Fig.13,(p 79).

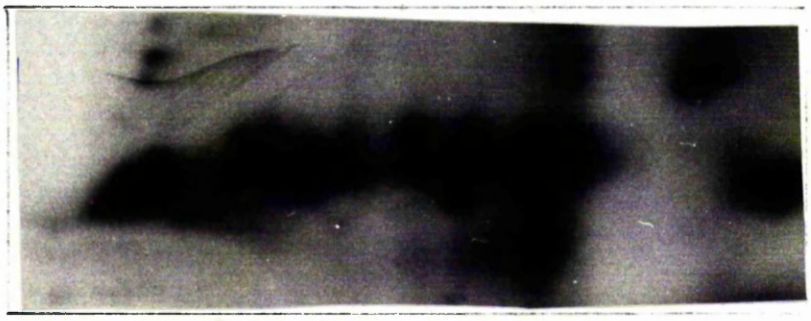


I.



II.

III.



IV.

2.2 Post-Translational Modification of Core Polypeptides.

Several protein modification systems are compatible with the observed gel pattern. These are: phosphorylation, acetylation, glycosylation and ADP-ribosylation.

With regard to glycosylation, ADP-ribosylation and acetylation, In vivo the testing of these possibilities are particularly difficult. Of the three, only glycosylation could be experimentally approached. The use of the Schiff glycoprotein stain on two-dimensional gels proved negative, however, and within the bounds of the sensitivity of the technique, glycosylation appeared not to be involved in the generation of spot heterogeneity.

With respect to phosphorylation, several unsuccessful attempts were made to incorporate ^{32}P into hnRNP particle proteins in vivo. However, even when a total of 10mCi. of ^{32}P was employed (2mCi. per rat), there was insufficient incorporation of ^{32}P into these proteins for the successful autoradiography of a two-dimensional gel (data not shown). Further, the digestion of hnRNP particles by incubation with E.Coli alkaline phosphatase failed to remove the heterogeneity, indicating either that the hnRNP particle core polypeptides were not phosphorylated, or that their phosphate groups were hidden within the hnRNP particle structure, and not accessible for hydrolysis by this enzyme.

In contrast to the negative data concerning the possible in vivo phosphorylation of these polypeptides, however, a number of in vitro phosphorylation studies have revealed considerable phosphorylation of hnRNP particle proteins. The presentation of this data is largely confined to Part 3 of the Results section, however, it is pertinent to consider here some facets of this work, particularly with regard to the substrate core polypeptides,

Figure 20 shows the "core polypeptide" region of the two-dimensional

fractionation of hnRNP particle polypeptides. The hnRNP particles had been incubated in the presence of $[\gamma\text{-}^{32}P]$-ATP under conditions in which a protein kinase activity, which appears to be associated with the hnRNP particles, catalyses the transfer of the radiolabelled phosphate groups onto a variety of hnRNP particle acceptor polypeptides. The composite shown in Figure 20 displays both the stainable pattern and autoradiographs of the ^{32}P-labelled proteins. Four of the main core polypeptides comigrated exactly with radioactive spots; these species were A_3, B_3, B_6 and C_3. The most interesting feature is not which species were phosphorylated, but rather those which were not, notably species A_1, A_2, B_1, B_2, B_4, B_5, C_1 and C_2.

Several explanations for this situation are possible. Firstly, the relative positioning of these polypeptides within the hnRNP particle may pre-dispose some species to in vitro phosphorylation, while protecting others. If this is the case, it would be expected that agents which open up or disrupt the particle might stimulate phosphorylation. Polyamines have been reported to have such an effect on particles and certainly stimulate phosphorylation (Fig. 33 and Section 3). However, they do not change the pattern of phosphorylation. Salt at concentrations greater than 0·2M cause particle dissociation (Beyer et al, 1977) but reduce phosphorylation (Fig. 30 Section 3).

A second possibility is that each of the chains of spots represent different phosphorylated derivatives of a basic polypeptide and that the labelled derivatives in Figure 20 represent those species which can be formed from their precursors, or by phosphate exchange in vitro. In this case, the restriction of phosphorylation to mainly four species (A_3, B_3, B_6 and C_6) may indicate that there are four main groups of polypeptides present. Further support for this possibility has been gathered by tryptic peptide mapping of core protein polypeptides.

Fig. 21.

Tryptic Peptide Mapping of the HnRNP Particle Core Proteins-A.

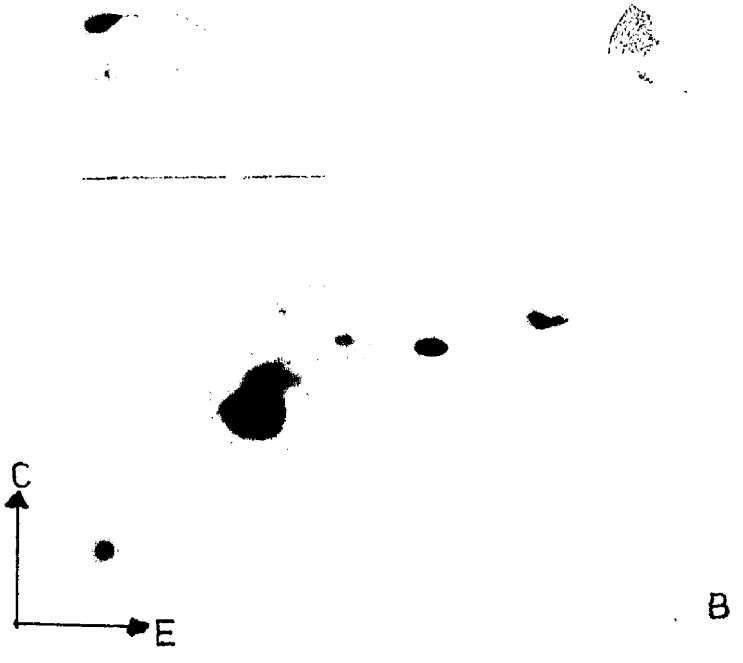
Core proteins were dissected from a two-dimensional gel iodinated and trypsin digested as described in the Methods Section (2.3.3.1). The trypsin digest was then analysed by two-dimensional electrophoresis/chromatography, and the pattern of iodinated tryptic peptides was visualised by autoradiography (at-70°C) using Kodak X-Omat H film, and an X-ray intensifying screen.

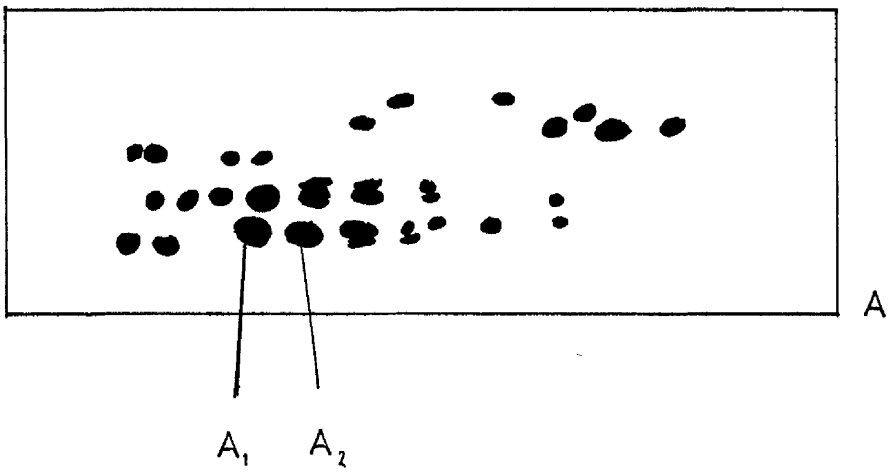
A - Diagram of core protein region of two-dimensional gel.

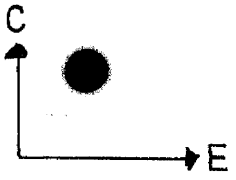
B - Tryptic map of protein A_1.

C - Tryptic map of protein A_2.

D - Generalised diagram of the A group profile.







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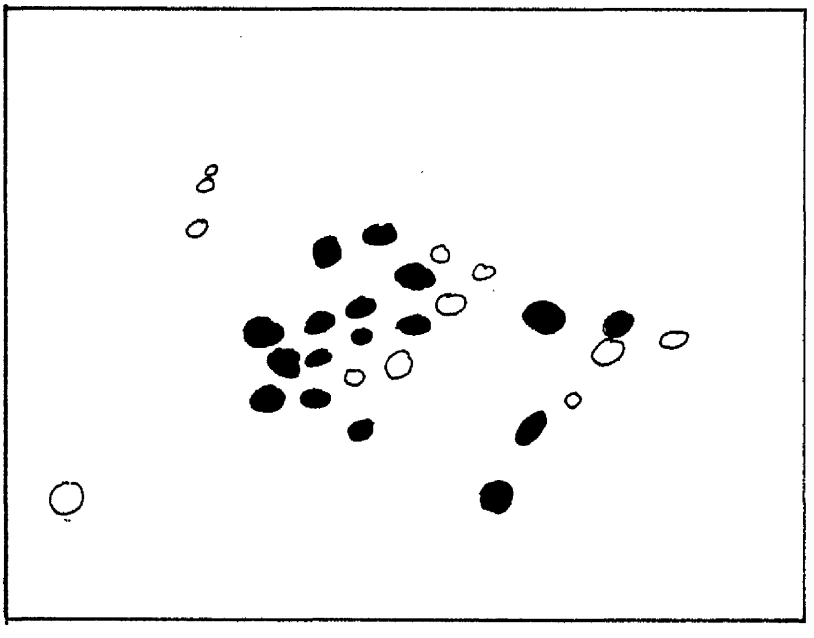


Fig. 22.

Tryptic Peptide Mapping of the HnRNP Particle Core Proteins - B_1 and B_2.

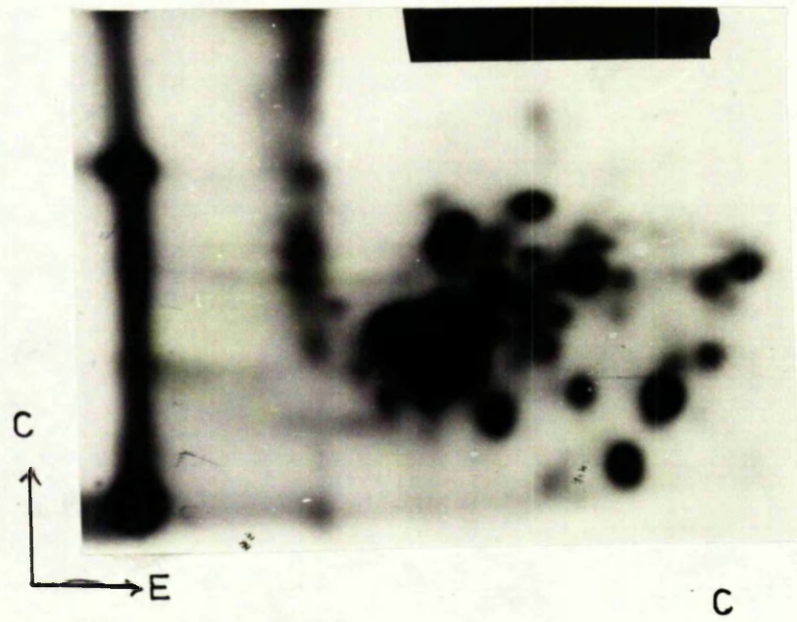
See legend to Fig. 21. for details.

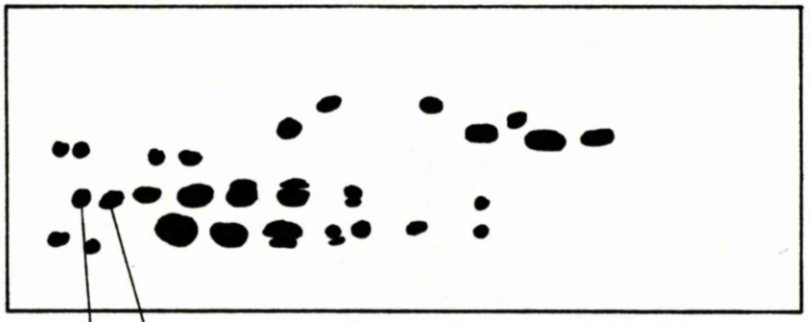
A - Diagram of core protein region of two-dimensional gel.

B - Tryptic map of protein B_2.

C - Tryptic map of protein B_1.

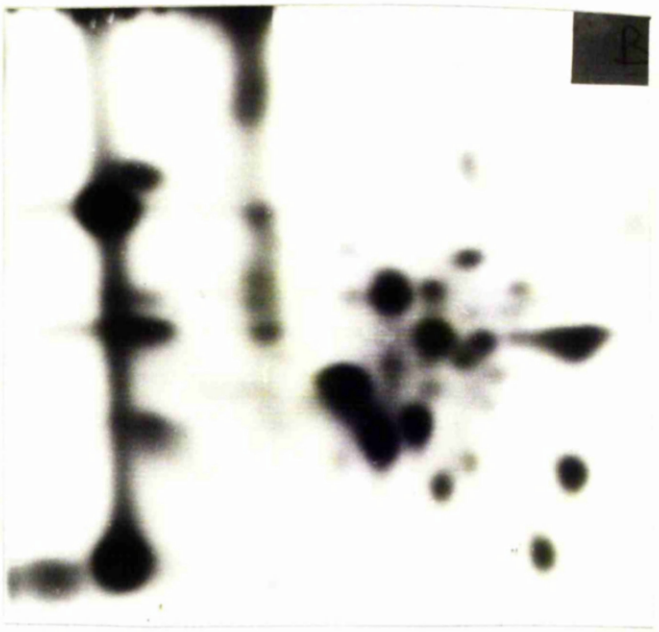
D - Generalised diagram of the group profile.





A.

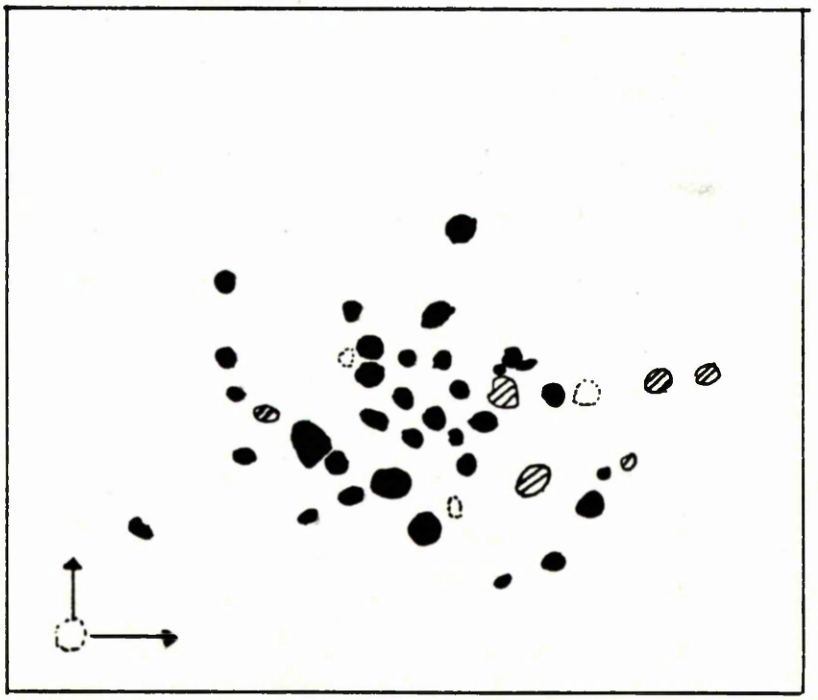
B_1 B_2



C

E

B



D

Fig. 23.

Tryptic Peptide Mapping of the HnRNP Particle Core Proteins - B_4 and B_5.

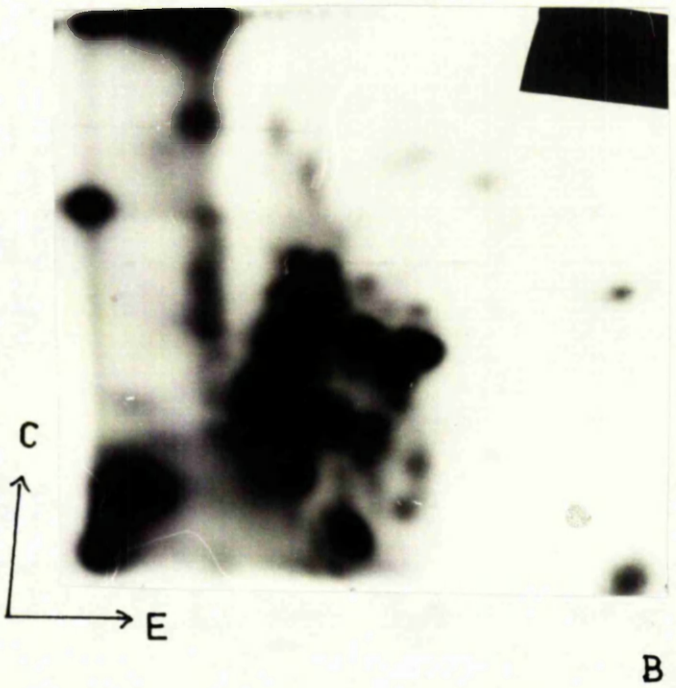
See legend to Fig. 21. for details.

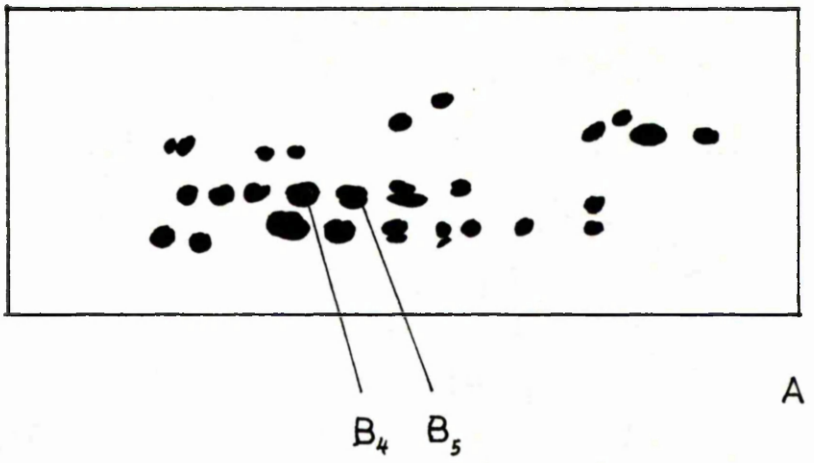
A - Diagram of core protein region of two-dimensional gel.

B - Tryptic map of protein B_4.

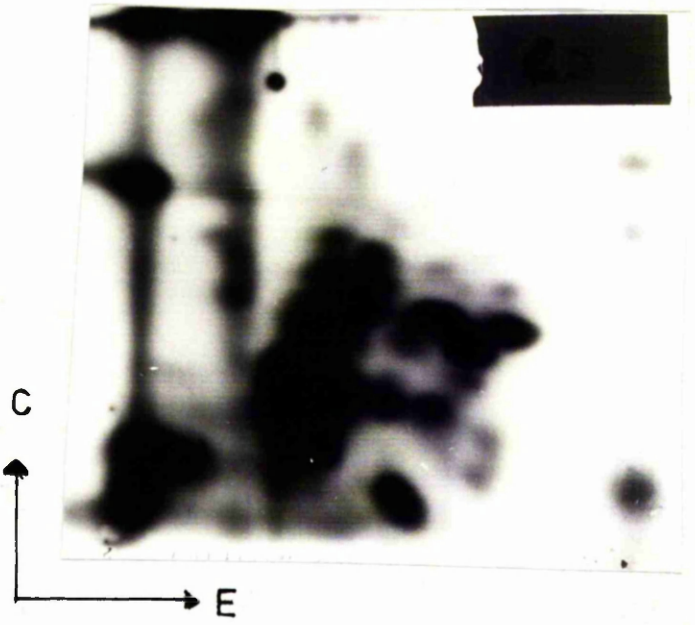
C - Tryptic map of protein B_5.

D - Generalised diagram of the group profile.





A



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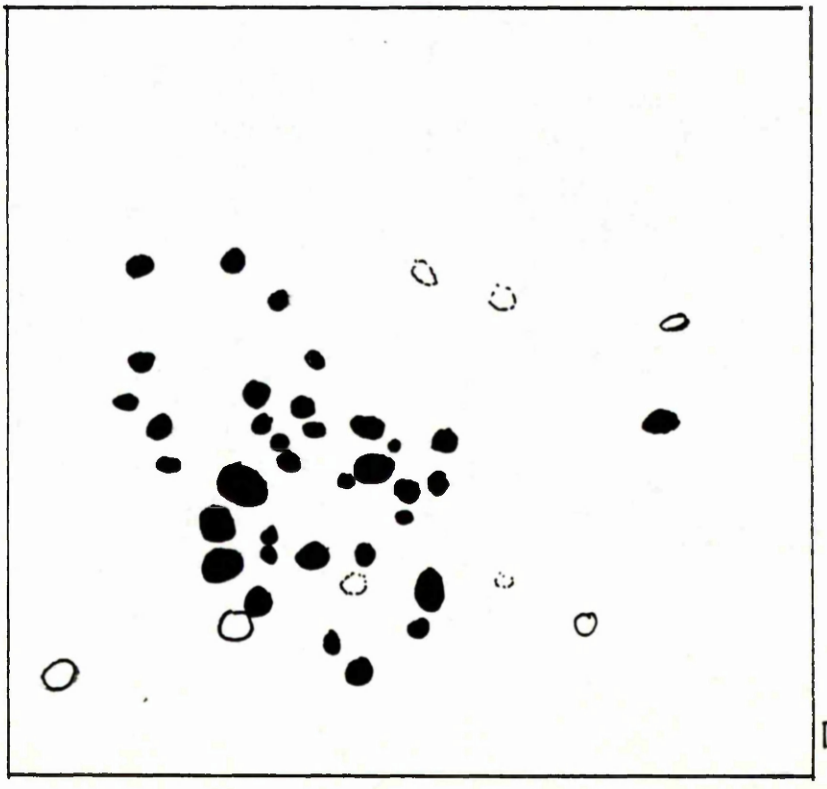


D

Fig. 24.

Tryptic Peptide Mapping of the HnRNP Particle Core Proteins - C_2 and C_3.

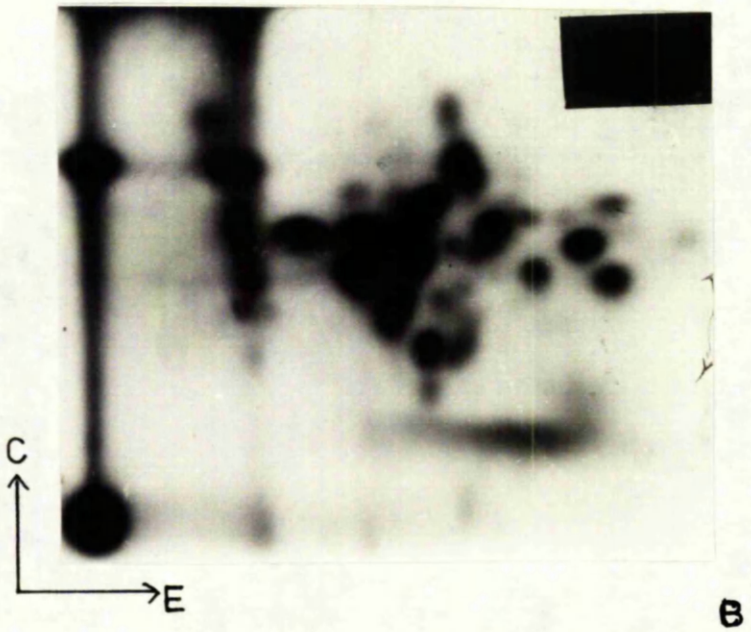
See legend to Fig. 21. for details.

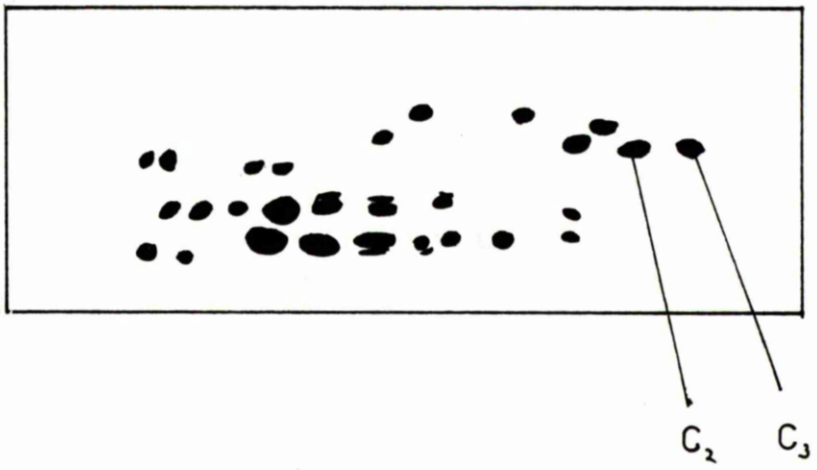
A - Diagram of the core protein region of two-dimensional gel.

B - Tryptic map of protein C_2.

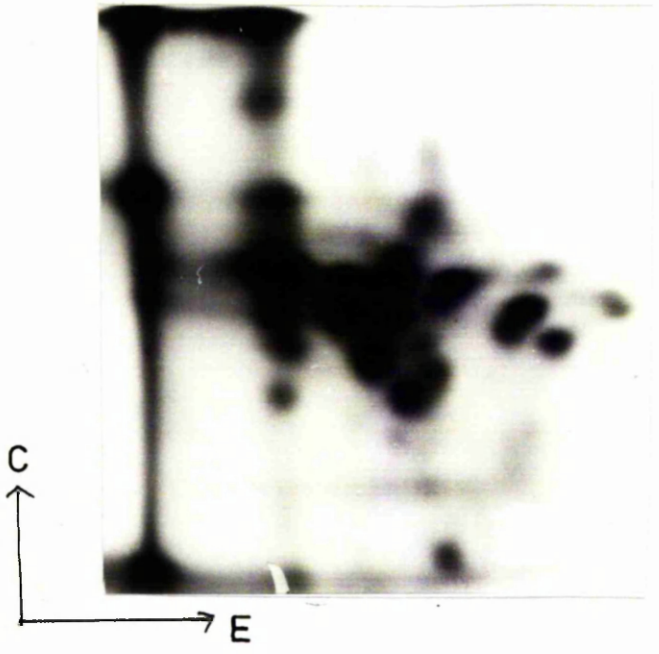
C - Tryptic map of protein C_3.

D - Generalised diagram of the group profile.



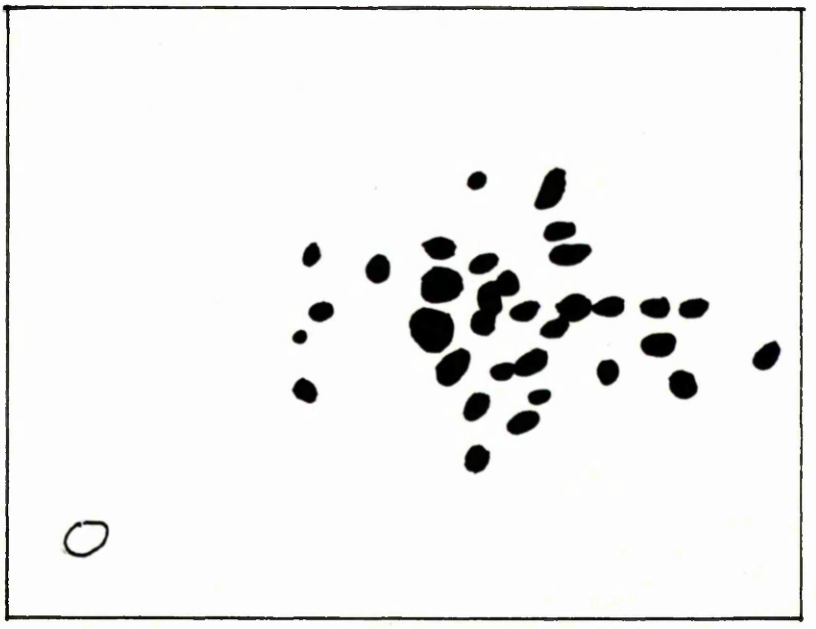


A.



B

C.



D

Fig. 25.

Tryptic Peptide Mapping of the HnRNP Particle Proteins:

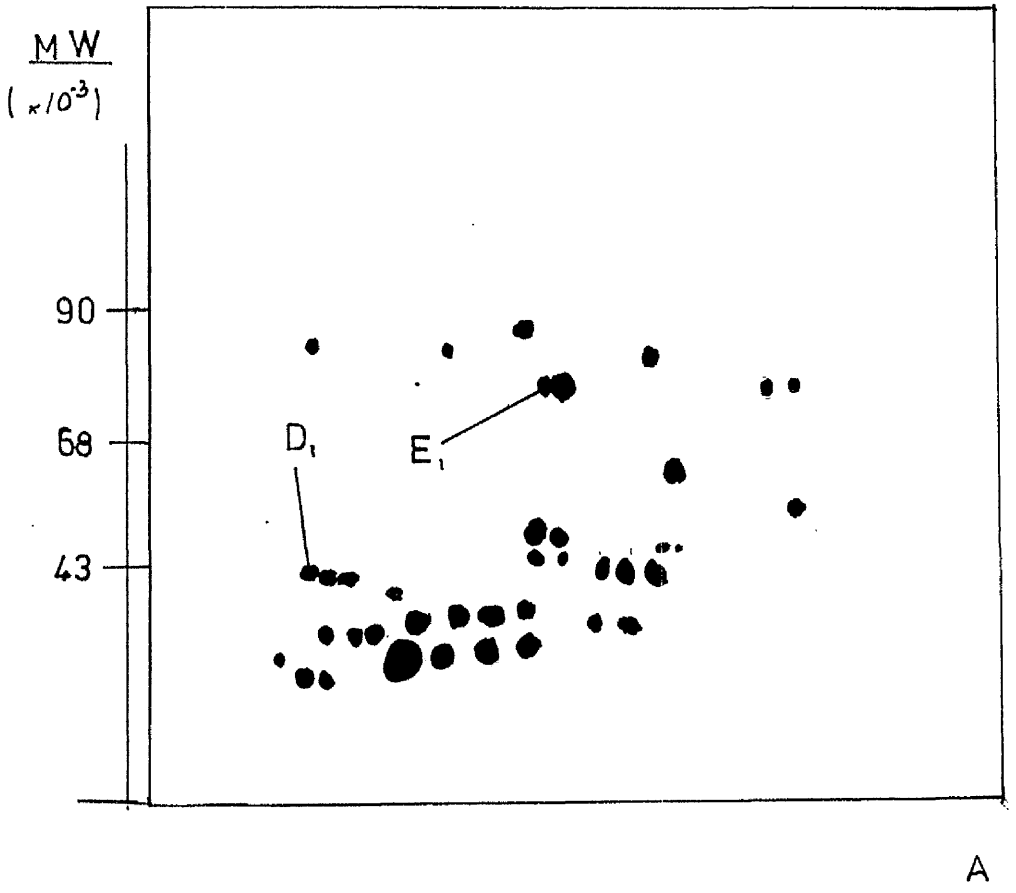
The Non-Core Proteins D_1 and E_1.

See legend to Fig. 21. for details.

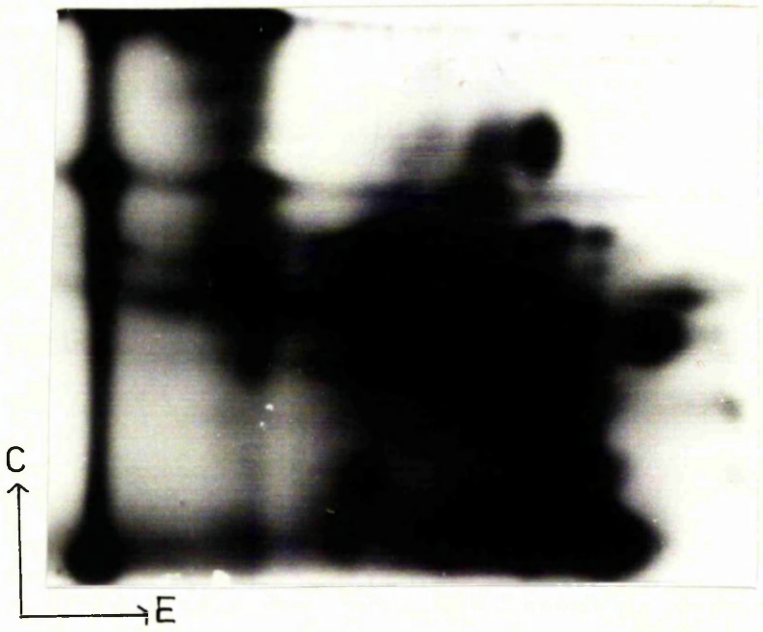
A - Drawing of a two-dimensional gel from which proteins D_1 and E_1 were taken.

B - Tryptic peptide map of D_1.

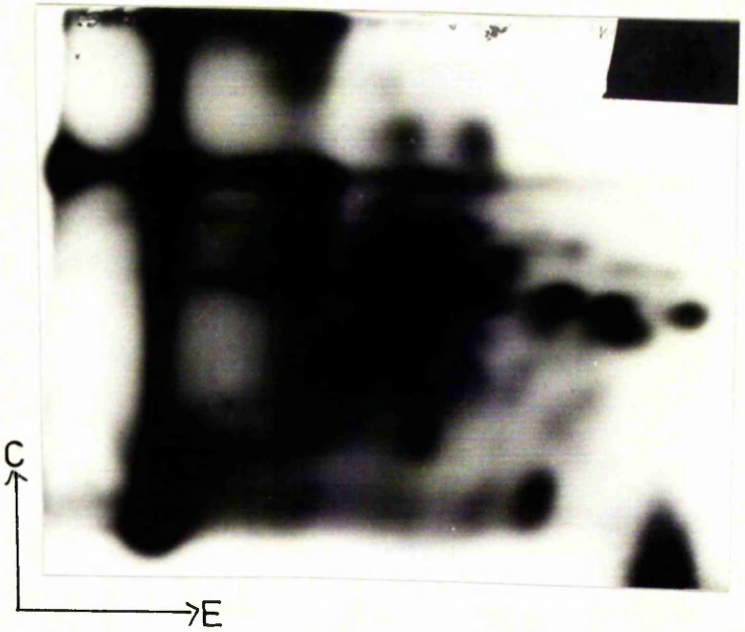
C - Tryptic peptide map of E_1.



A



B.



C

2.3 Tryptic Peptide Mapping of the HnRNP Core Polypeptides.

Tryptic peptide mapping is an analytical method akin to RNA "fingerprinting" or DNA restriction analysis. The specific cleavage of a given polypeptide, at lysine and arginine residues, by trypsin, generates a subset of peptides, of varying charge and hydrophobicity, which can be conveniently fractionated by a two-dimensional electrophoresis/chromatography system. This provides a "fingerprint" of the digested polypeptide which is characteristic of, and specific to, that particular polypeptide primary structure. A further refinement of this method is the chemical incorporation of radiolabelled iodine (^{125}I) into the phenyl-alanine residues of the proteins to be analysed. Thus, in addition to increasing the sensitivity of the technique, a particular subset of peptides (those containing phenyl-alanine) can be highlighted.

A recent extension to this technique has been the analysis of individual polypeptides, isolated by excision from polyacrylamide gels (Elder et al, 1977). In this method the excised gel slice, containing the polypeptide of choice, is sequentially iodinated and trypsin digested while still in situ. Many obvious advantages accrue from this technique, and it has here been used to investigate the core proteins which were individually cut out of two-dimensional gels and processed as described above.

Figures 21 to 25 illustrate tryptic peptide maps of the core polypeptides A_1, A_2, , B_1, B_2, B_5, B_4, C_2 and C_3, and two other hnRNP particle polypeptides. The close similarity (allowing for slightly different running conditions between some samples) of the peptide maps of particular groups of polypeptides (e.g. A_1, A_2 , Fig. 21; B_1 and B_2, Fig 22; B_4 and B_5, Fig. 23; C_2 and C_3, Fig. 24) suggests that these groups are derivatives of the same source polypetide.

There are also certain similarities between groups. The maps

obtained for A_1, and A_2 appear to have some similarities to those obtained for B_1 and B_2 (See Fig.22). There are also a number of similarities between B_1 and B_2, and B_4 and B_5 (Fig.24), and C_2 and C_3 also show one or two similarities with this group. In contrast, the non-core polypeptides E_1 and D_1, show very little sign of homology. Although this data is of a preliminary nature, it suggests that there may be some relationship, not only between the members of chains of spots, but also between the members of the different sets of chains.

In conclusion, several lines of evidence point, albeit inconclusively, to the possibility that the hnRNP particle core polypeptides are composed of four distinct gene products, each of which becomes further derivatised (possibly by phosphorylation) to produce the characteristic two-dimensional gel profile shown in Fig.20. This data should be regarded as interesting, but inconclusive. Further characterisation of the core polypeptides, for example by thermolysin, elastase or cyanogen bromide mapping, may yield a firmer basis for these conclusions. The elucidation of the exact nature of the post-translational modification which results in the variation between group members is also appropriate.

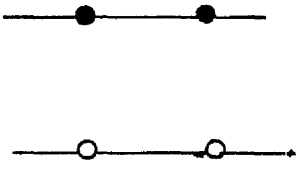
3. ENZYME ACTIVITIES ASSOCIATED WITH RAT LIVER HnRNP PARTICLES.

The possibility that hnRNP particles carry with them enzyme activities associated with hnRNA processing, has been as attractive working hypothesis for many investigators. The scrutiny of the polypeptide components of hnRNP particles for such activities has revealed several candidates for such processing enzymes including; poly-(A)-polymerase (Neissing & Sekeris, 1972), several "capping" enzymes (Bajszar *et al*, 1978) and double-stranded RNA specific RNAses (Rech *et al*, 1979; Molnar *et al*, 1978). In view of the potential involve-

Fig. 26.

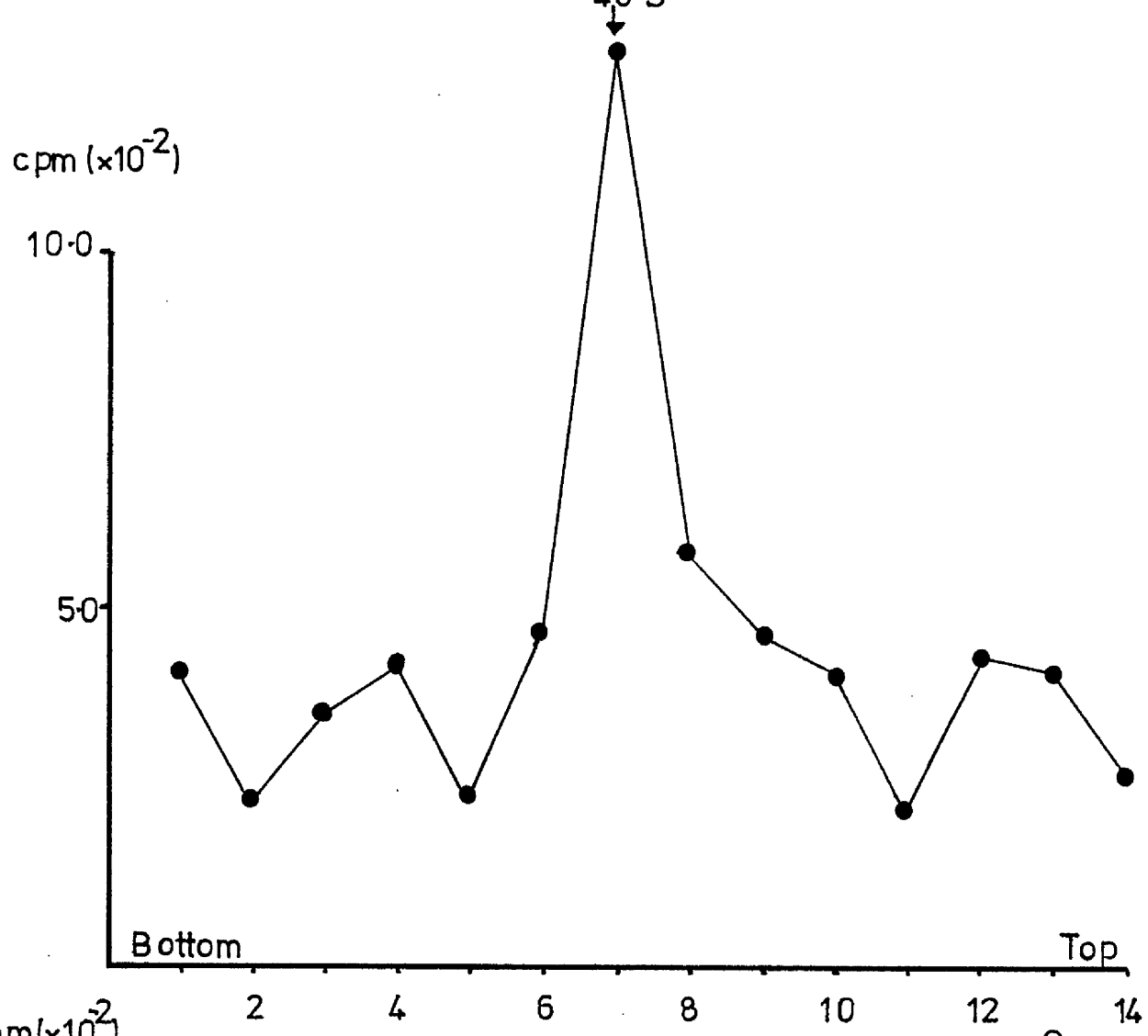
Estimation of Poly-(A)-Polymerase Activity in Each Fraction of a Sucrose Density Gradient Containing HnRNP Particles.

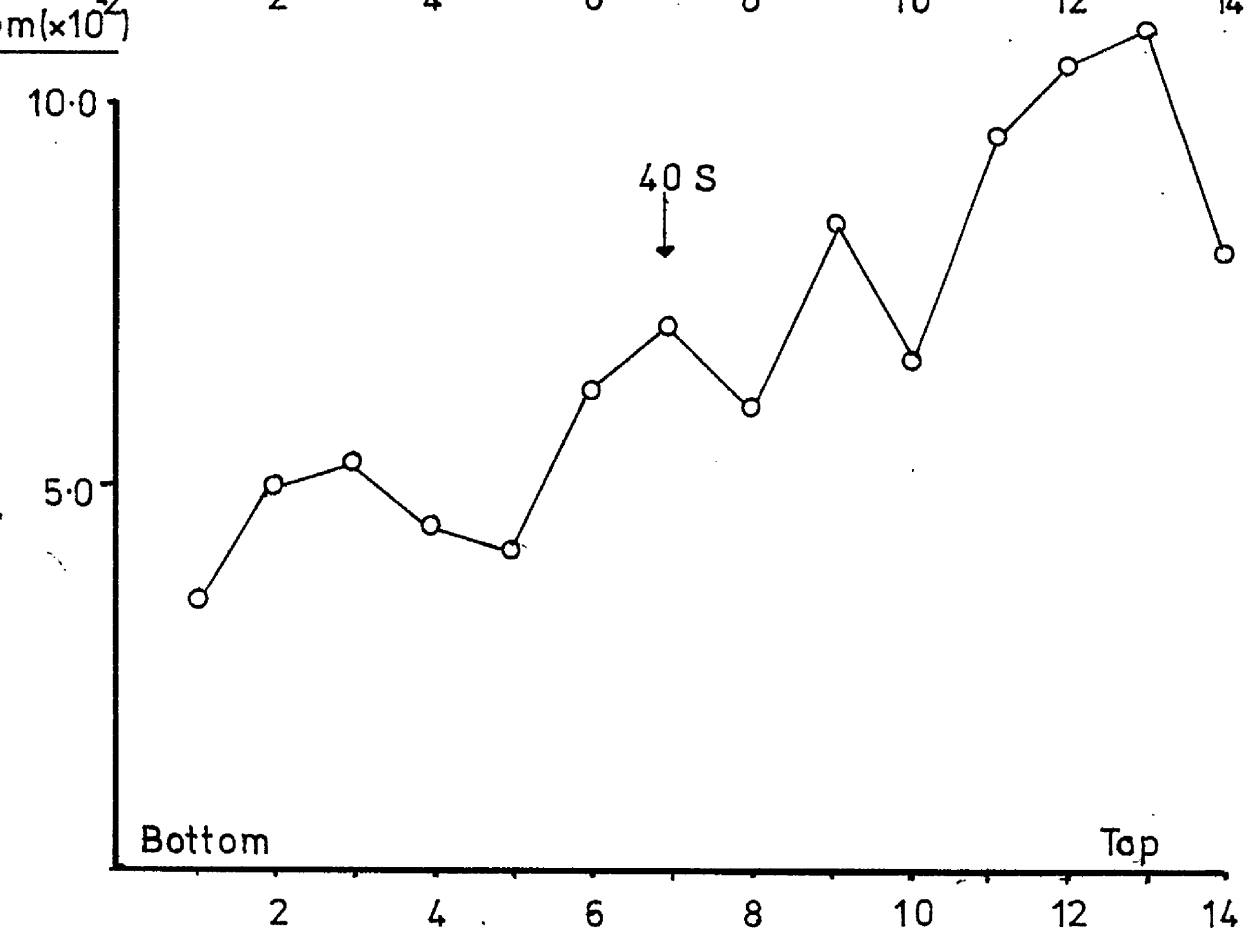
The pH 8·0 nuclear extract was fractionated by sucrose density gradient centrifugation as described in Methods (2.1.2.1). Each fraction of the completed gradient was assayed for poly-(A)-polymerase as described in Methods (2.2.4.2). The position of the 40S hnRNP particle peak is displayed.



Poly-(A)-polymerase activity in the absence of poly-(A) primer.

Poly-(A)-polymerase activity in the presence of poly-(A) primer.


40 S
cpm (×10⁻²)
10·0
5·0
Bottom
Top
2
4
6
8
10
12
14


m(×10⁻²)
10·0
40 S
5·0
Bottom
Top
2
4
6
8
10
12
14

-ment of the hnRNP particle proteins in the control of the rate and extent of hnRNA maturation into mRNA, a particularly interesting observation has been the presence of protein kinase activities in association with HeLa cell, and mouse and rat liver hnRNP particles (Blanchard et al, 1977; Martin et al, 1975; Schweiger & Schmidt, 1974; Karn et al, 1977). This thesis includes an investigation of this protein kinase activity, and also examines some aspects of the poly-(A)-polymerase activity.

3.1 Poly-(A)-Polymerase.

The discovery by Neissing and Sekeris (1972) of the enzyme poly-(A)-polymerase in association with hnRNP particles was the first indication of a known hnRNA processing enzymes in association with hnRNP particles. The further characterisation of this activity was therefore a desirable aspect of the investigation of hnRNP particles in this study.

Initial attempts to repeat the results of Neissing and Sekeris (1972) proved successful (Fig.26a). A distinct peak of poly-(A)-polymerase activity was demonstrated, in the absence of poly-(A) primer. However, the possibility that this apparent comigration of hnRNP particles and polymerase activity was simply an effect of substrate limitation was raised by further analysis of each fraction of the same gradient for poly-(A) polymerase,in the presence of poly-(A) primer. (Fig.26b).

The considerable activity uncovered in the non-particulate region of the gradient by the use of poly-(A) primer, therefore brought into doubt the concept of a specific localisation of the enzyme on the particles. It was decided in the light of this data not to proceed with the further characterisation of this enzyme.

Fig. 27.

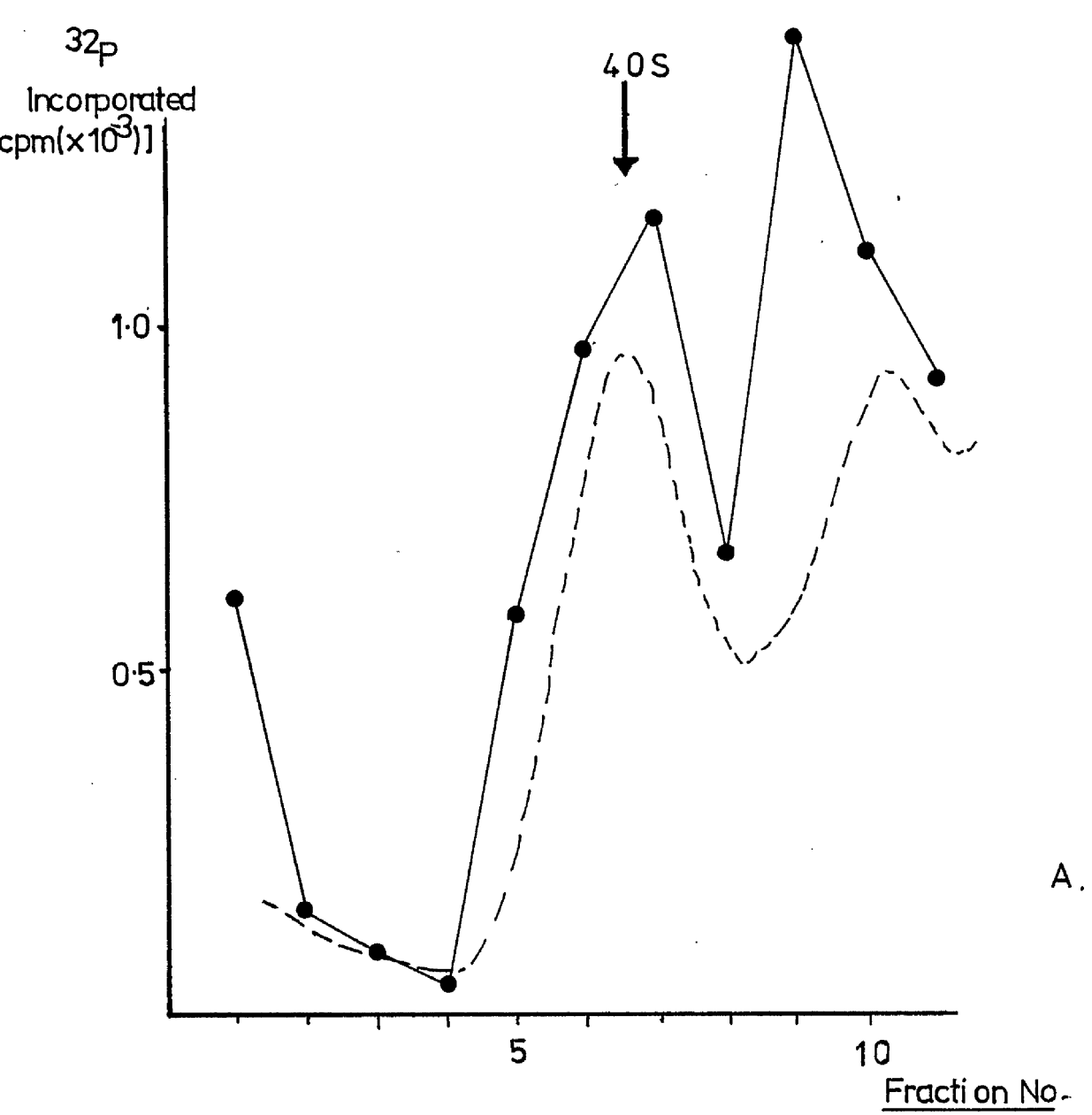
Estimation of Protein Kinase Activity in a Sucrose Density Gradient Containing HnRNP Particles.

One millilitre of a pH 8·0 nuclear extract (Methods 2.1.2.1, p42 was fractionated on a 15% - 30% sucrose density gradient. Each fraction of the gradient was dialysed against 0·1M sodium acetate, pH 6·5, 10mM $MgCl_2$, and assayed for protein kinase activity by incubation at 30°C for 10 minutes in the presence of 0·1mM [γ-^{32}P]-ATP (1μCi. per assay). Incorporation of ^{32}P was estimated by the filter disc assay described in Methods 2.2.4.1.(p47 Fig. 28b shows the kinase activity in each fraction of a similar sucrose density gradient, upon which the 40S hnRNP particles from a previous gradient were recentrifuged.

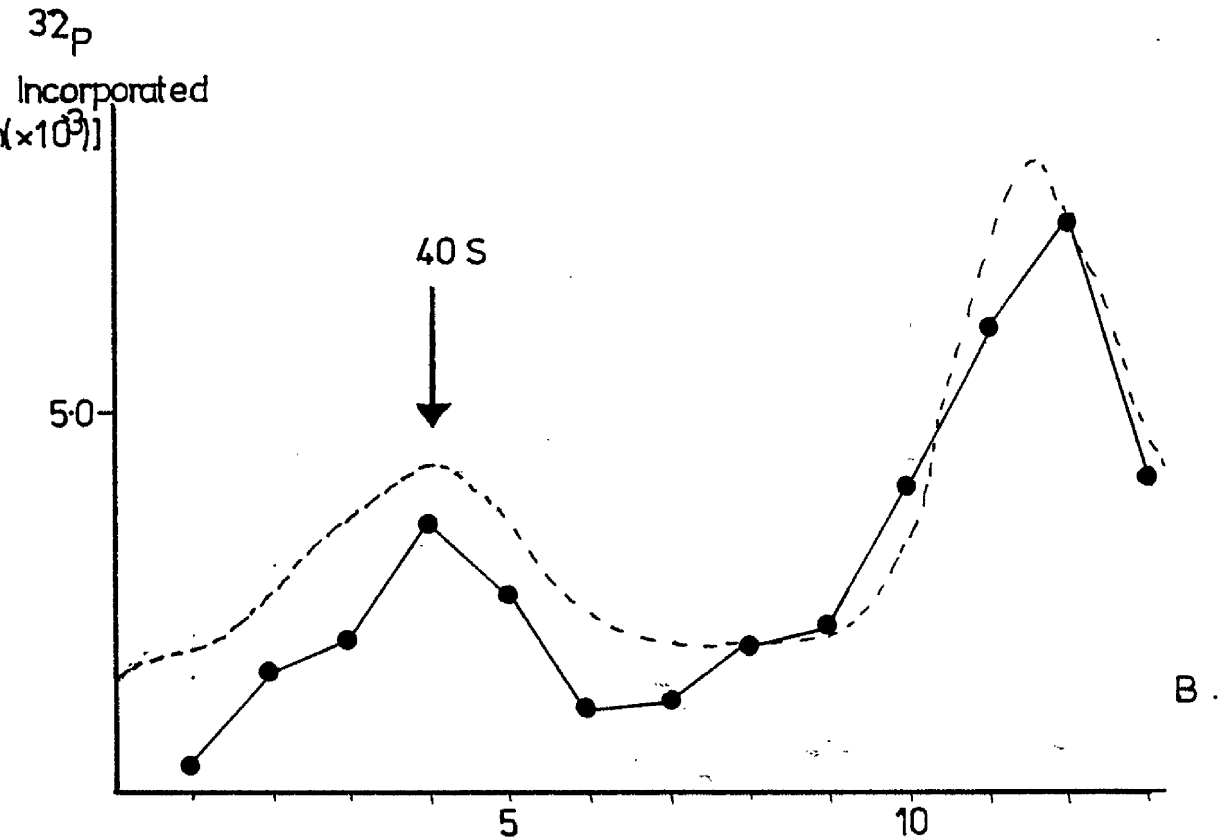
OD_{260} trace.

Incorporation of ^{32}P (cpm).

Each point was the average of two determinations made using one-tenth aliquots of each fraction.


32P
Incorporated
cpm(x10-3)]
40 S
1·0
0·5
5
10
Fraction No.

A.


32P
Incorporated
(x10-3)]
40 S
5·0
5
10

B.

3.2 HnRNP Particle-Associated Protein Kinase.

The "autophosphorylation" of hnRNP particles was first noted by Schweiger and Schmidt (1974), by the simple expedient of incubating rat liver hnRNP particles at 37°C in the presence of $[\gamma\text{-}^{32}P]$-ATP. The autophosphorylation reaction was not further characterised by these investigators. However, an apparently similar kinase activity in HeLa cell hnRNP particles was extensively analysed by Blanchard et al, (1975 and 1977), and evidence for this enzyme's close association with these particles was presented. The experiments described here are a similar characterisation of the rat liver hnRNP particle protein kinase activity.

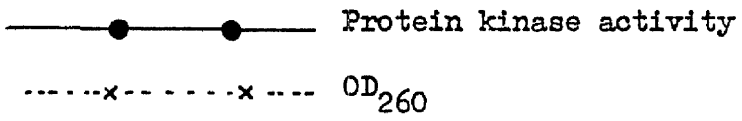
3.2.1 Evidence for the Association of Protein Kinase Activity with 40S HnRNP Particles.

Several lines of evidence point towards the association of a protein kinase activity with rat liver hnRNP particles. Firstly, analysis of each fraction of an analytical sucrose density gradient containing hnRNP particles for the presence of kinase activity revealed a peak of incorporation from $[\gamma\text{-}^{32}P]$-ATP coincident with the 40S hnRNP particle peak (Fig. 27). A second peak of radioactivity was associated with the top of the gradient, indicating that kinase activity not bound to hnRNP was also extracted from the nuclei. At present it is difficult to rule out the possibility that the particle-associated kinase was not adventitiously bound during extraction, or the reverse possibility that the unbound kinase was leached from the particles. It has been demonstrated, however, that a very different and more complex spectrum of proteins remain on the top of the gradient (Fig. 16a). Also, if particles were extracted in the presence of radioactive total cellular proteins, radioactivity did not adventitiously associate with the particles.

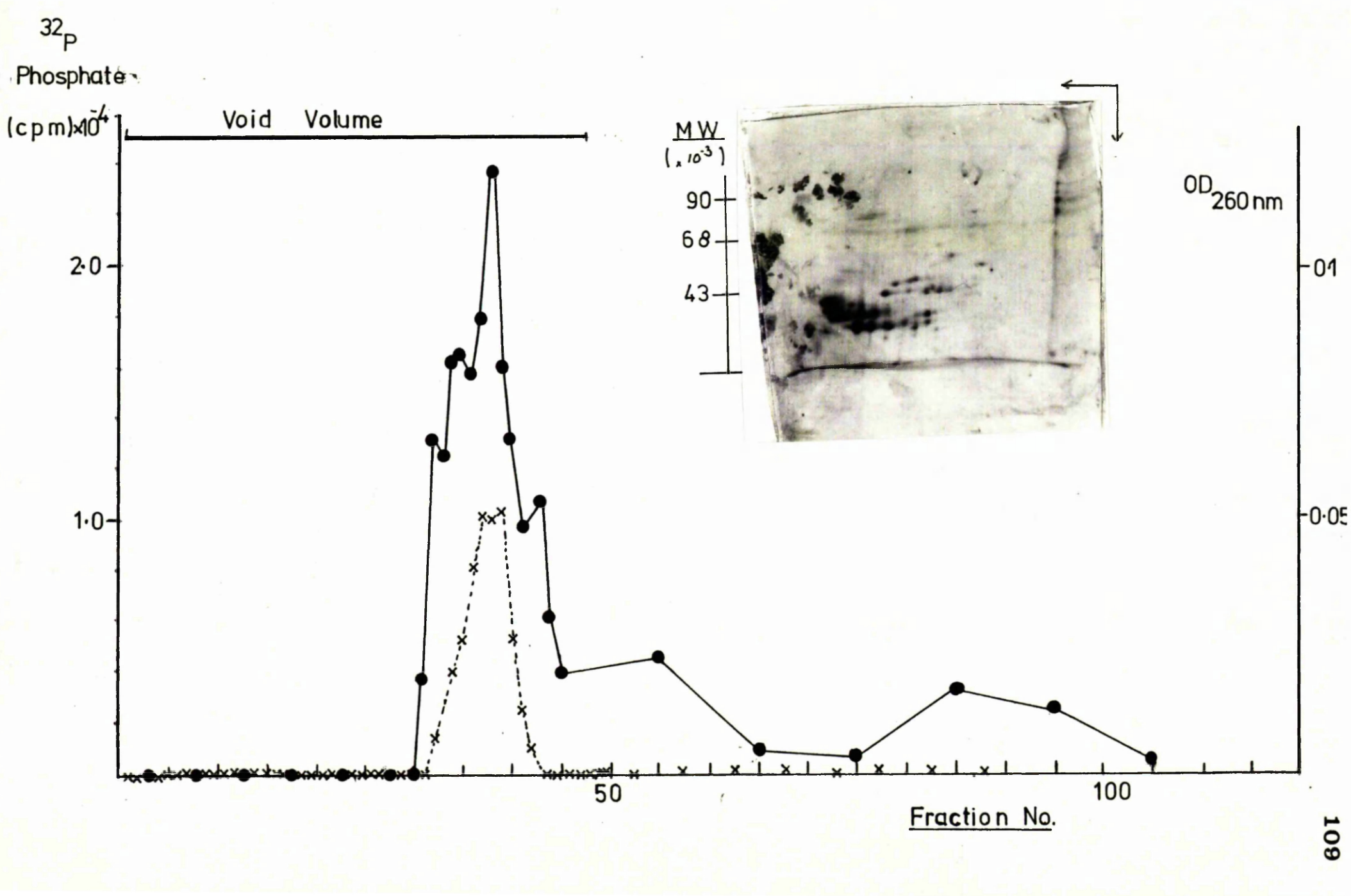
Fig. 28.

Gel Exclusion Chromatography of Purified HnRNP Particles

Purified hnRNP particles were subjected to gel exclusion chromatography as described in Methods section 2.2.2. One milligram of protein was applied to an 8cm x 3cm column of Biogel A-0·5M (BioRad Ltd.) and 0·5 ml fractions were collected. The flow rate was 10 cm per hour. Each fraction was assayed for OD_{260nm}, protein concentration, and , after microdialysis (Methods section 2.5.1, p.58) against 0·1M sodium acetate, pH 6·5, 10mM $MgCl_2$, for protein kinase activity. Two-dimensional polyacrylamide gel electrophoresis (Methods 2.3.2) of the excluded material from a similar experiment is shown in Fig. 28 A.



Each point on the graph represents a single determination of protein kinase activity, using a one-tenth aliquot [i.e. 50µl] of each fraction.


32P
Phosphate
(cpm)×10⁻⁴
Void Volume
2·0
1·0
MW
(×10⁻³)
90
68
43
OD260nm
01
0·05
50
100
Fraction No.

Recentrifugation of the 40S hnRNP particle peak, after dialysis against STM 8·0 buffer, and subsequent analysis of the second gradient fractions for kinase activity revealed that approximately 30% of the protein kinase activity remained with the particle, the rest was located at the top of the gradient (Fig.27b). While this was good evidence that at least some of the enzyme activity was a component of the 40S hnRNP particle, the loss of the remaining 70% was worrying. One possible explanation for this result is the known instability of hnRNP particles in solution. Patel and Holoubeck (1977) found that centrifugation on sucrose density gradients of hnRNP particles prepared by gel filtration on Biogel A-50M, resulted in the loss of the minor, high molecular weight polypeptides. Combined recentrifugation enhanced this loss, until the predominant core polypeptides were virtually the only polypeptide components observed. If the protein kinase activity is one of these minor components, its subsequent loss, as detected by the incorporation of ^{32}P from[γ-^{32}P]-ATP, would not be surprising.

To further analyse the association of the protein kinase activity with the hnRNP particles, isolated 40S particles recovered from sucrose density gradients as described in the Methods section, were subjected to gel exclusion chromatography on BioRad Biogel A-0·5M . Two-dimensional protein analysis confirmed the material recovered from the column as hnRNP particles (Fig.28a). Fig.28 shows an analysis of the fractions obtained from such a column with respect to kinase activity. It revealed that, with this system, a larger percentage of the kinase activity remained associated with the particles through the second fractionation step. Nevertheless, some activity was retained by the resin and was, therefore, probably in the form of free protein when eluted.

Treatment	% Radioactivity Bound
Cold TCA (0°C)	100%
Hot TCA (100°C)	70.2%
RNAse	97.6%
PRONASE	1.9%
ALKALINE PHOSPHATASE	43.5%

Table 4

Effect of Various Enzyme Treatments on the Products of the in vitro Phosphorylation of HnRNP Particles.

HnRNP particles, phosphorylated in vitro by incubation at 30°C in 0.1M sodium acetate pH 6.5, 10mM $MgCl_2$, in the presence of 1 μCi [γ-^{32}P] ATP (0.1mM final concentration), were subjected to a variety of treatments. Hot and cold 5% TCA treatments were preformed as outlined for the protein kinase activity assay (Methods 2.2.4.1. p . 47). Duplicate aliquots of in vitro phosphorylated hnRNP particles were further incubated in the presence of pancreatic RNAse (10 μg per assay; ambient temperature for 20 minutes). A further duplicate set of aliquots were microdialysed against alkaline phosphatase buffer, (0.1M glycine pH 10.5; 0.1mM $ZnCl_2$) and subjected to alkaline phosphatase digestion (5 μg per assay; 30°C for 1 hr.). The enzyme treated fractions were spotted onto filter discs and protein bound ^{32}P was estimated by treatment with cold 5% TCA, followed by ethanol and ether washes, and preparation for scintillation counting as described in Methods 2.2.4.1. p . 47).

Other techniques widely employed in the proof of the association of a particular enzyme activity with a sub-cellular organelle have proved to be impracticable. Thus, metrizamide has been reported to generate artefacts with hnRNP particles (Gattoni et al, 1977), and CsCl buoyant density gradient centrifugation of uncrosslinked hnRNP particles simply leads to their dissociation. In addition, poly-(U)-Sepharose columns (Blanchard et al, 1977; Cardelli & Pitot, 1980) are inappropriate since 40S hnRNP particles appear to lack significant poly-(A) tails (Samarina et al, 1973; Molnar & Samarina, 1975). However, one other possible approach which deserves investigation is the use of reversible crosslinkers. Such a system would depend on the crosslinker not irreversibly inactivating the kinase but if this could be demonstrated it would allow the extensive purification of particles before reversal of linkage and analysis of enzyme activity. Time constraints has precluded the inclusion of such an analysis as a part of this study.

3.2.2 Characterisation of the *in vitro* Phosphorylation of HnRNP Particles.

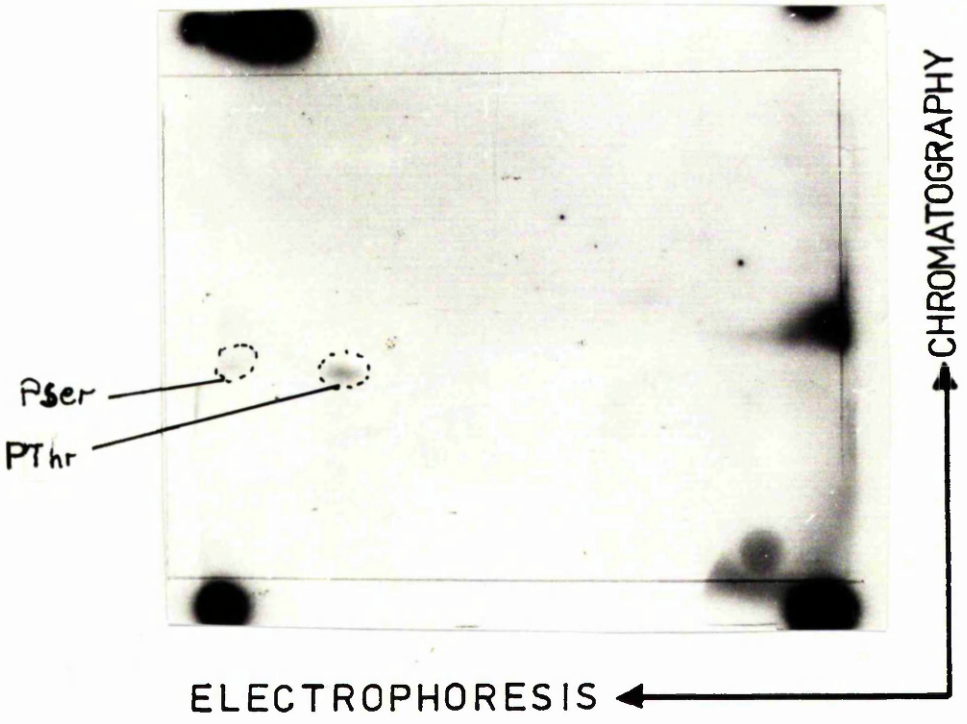
3.2.2.1 Nature of the Products of *in vitro* Phosphorylation.

Evidence for the transfer of ^{32}P from $[\gamma\text{-}^{32}P]$-ATP into covalent linkage with the protein component of hnRNP particles has come from a number of approaches. The simplest evidence for this transfer is shown in Table 4 . HnRNP particles, after incubation at 30° C in the presence of $[\gamma\text{-}^{32}P]$-ATP, were dialysed against an appropriate buffer, before being treated with either RNAse, Pronase or alkaline phosphatase. Both Pronase and alkaline phosphatase release significant quantities of radioactivity (99% and 70% respectively), however, RNAse treatment fails to reduce the acid insoluble radioactivity. These data coupled

Fig. 29.

Identification of in vitro Phosphorylation Acceptor Amino Acids.

HnRNP particles (100µg.) were phosphorylated *in vitro* as described in the Methods section (2.2.4.1), and precipitated by the addition of 2·5 volumes of ethanol. The precipitates were resuspended in 1ml. of 6M HCl, by warming in a 60°C water bath. The proteins were then hydrolised at 110°C in an evacuated "cold finger" for 2 hours, the residue rotary evaporated under vacuum and submitted to electrophoresis/chromatography on cellulose-backed TLC plates as described in the Methods section (2.3.2.2). Plates were stained with 1% $^{w}/_{v}$ ninhydrin dissolved in acetone to show the phosphoserine and phosphothreonine markers and autoradiographed using Kodak X-Omat H X-ray film.


Pser
PThr
CHROMATOGRAPHY
ELECTROPHORESIS

with the stability of the majority of the incorporated ^{32}P to hot (100°C) 5% $^{W}/_{v}$ trichloroacetic acid treatment strongly suggests that the phosphate acceptors are proteinaceous. (The losses (30%) which occur during this treatment may be due to the presence of acid labile phosphate derivatives in this preparation, or lack of stringency in the cold acid washes).

Perhaps the strongest evidence in support of this observation comes from the two-dimensional amino acid analysis of *in vitro* ^{32}P-labelled hnRNP particle polypeptides. Precipitated ^{32}P-labelled hnRNP particles were dissolved in 6M HCl. by warming in a boiling water bath. When the pellet was fully dissolved the solution was transferred to a "cold finger", the tube evacuated, sealed, and acid hydrolysis of the peptides performed for 2 hrs. at 110°C. Concentration of the hydrolysate on a rotary drier attached to a vacuum line, was followed by resuspension in 10mM HCl. and subsequent analysis by two-dimensional electrophoresis/chromatography on TLC plates. Simultaneous fractionation of the unlabelled markers phosphothreonine and phosphoserine, provided an internal standardisation The autoradiograph obtained from the exposure of such a TLC plate is shown in Fig. 29. The two phospho-amino acids, phosphothreonine and phosphoserine, despite the use of relatively mild hydrolysis conditions, which do not result in the degradation of this compound, suggests that the kinase activity of hnRNP particles is unlike that of, for example, the Rous Sarcoma virus *src* gene product (Hunter & Bartholomew, 1980), or polyoma middle T antigen (Eckhart *et al*, 1980), which produce significant quantities of this derivatised amino acid. The hnRNP particle kinase and its cognate acceptor proteins, thus appear to be similar to the majority of previously characterised kinase/protein substrate pairs, including the HeLa cell hnRNP particle kinase (Blanchard *et al*, 1977).

Fig. 30.

Effects of pH and Salt Concentration on the in vitro Phosphorylation of HnRNP Particles.

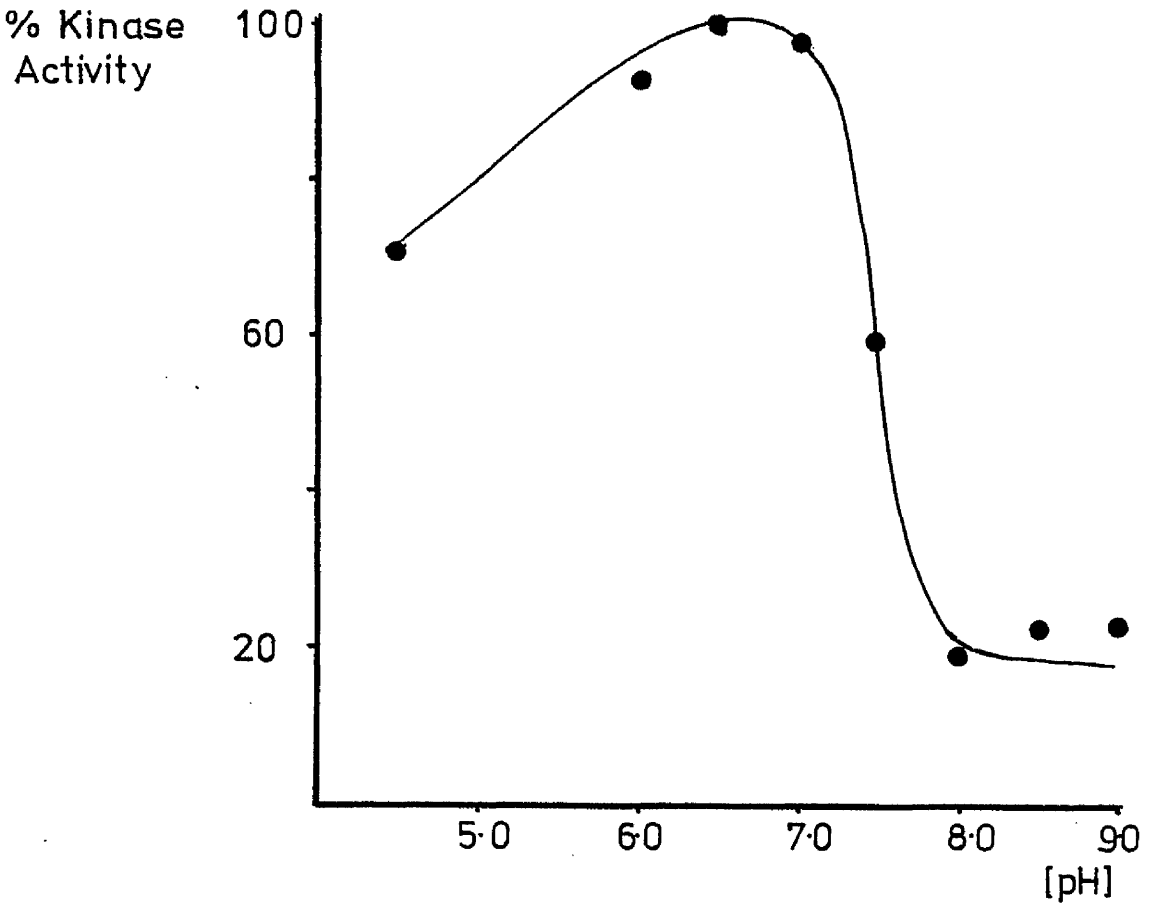
A - Effects of pH.

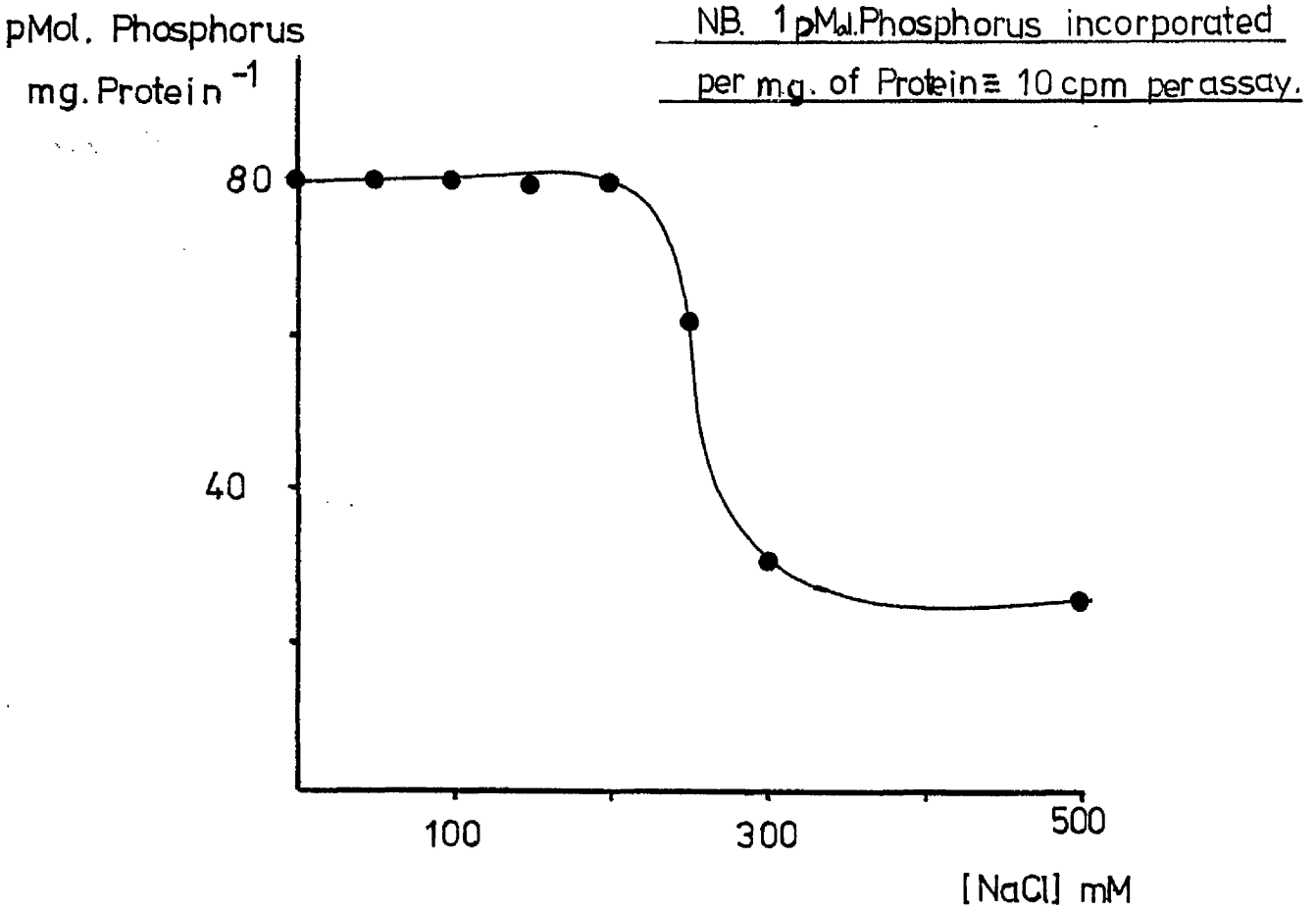
HnRNP particles were dialysed against 10mM $MgCl_2$, and adjusted to a protein concentration of $0{\cdot}5\mu g.\mu l^{-1}$ by further dialysis against polyethylene glycol 6000. Fifty microlitre ($25\mu g$) aliquots were added to $5\mu l$ of 1·0M sodium acetate, 0·1M Hepes which had been adjusted to the appropriate pH with either acetic acid or sodium hydroxide. Finally, $1\mu Ci$. $[\gamma\text{-}^{32}P]$-ATP was added to a concentration of 0·1mM, and the assay incubated at 30°C for 10 minutes. Estimation of incorporation was as described in the Methods section (2.2.4.1).

B - Effects of Salt Concentration.

HnRNP particles were dialysed against 0·1M sodium acatate, pH 6·5, 10mM $MgCl_2$, and adjusted to a protein concentration of $0{\cdot}5\mu g.\mu l$. by further dialysis against polyethylene glycol 6000. Fifty microlitre aliquots were taken and adjusted to the appropriate salt concentration by addition of 1M or 5M NaCl. The total volume of each aliquot was adjusted to $55\mu l$ and $1\mu Ci$. $[\gamma\text{-}^{32}P]$-ATP was added for a final concentration of 0·1mM. Incubation of each sample was at 30°C for 10 minutes, and incorporation of radiolabel was estimated as described in the Methods section (2.2.4.1).

The data shown in Fig. 30B are averages of two measurement Those in Fig. 30A were single determinations; however, each "half" of the curve was quantitatively and qualitatively reprodu using each component of the buffer "cocktail" (sodium acetate and Hepes) individually.


% Kinase Activity
100
60
20
5·0
6·0
7·0
8·0
9·0
[pH]


pMol. Phosphorus mg. Protein -1
NB. 1 pMol. Phosphorus incorporated per mg. of Protein ≡ 10 cpm per assay.
80
40
100
300
500
[NaCl] mM

3.2.2.2 Characterisation of the *in vitro* Phosphorylation of HnRNP Particles with respect to pH, Time and Salt Concentration.

An important aspect of the characterisation of the *in vitro* phosphorylation reaction was the optimisation of the system for pH. Using a composite Hepes/sodium acetate buffering system (at concentrations of 10mM and 100mM respectively), the pH profile (Fig. 30a) of the reaction exhibited a broad optimum value of around pH 6·0 - 7·0, with a rapid fall in the incorporation of radioactivity between pH 7·0 and pH 8·0. This type of curve is not atypical of the pH profiles of many other protein kinase activities, however, it was surprising to find a decrease in apparent kinase activity at pH values higher than pH 7·0. The HeLa cell hnRNP particle protein kinase has been reported to have a pH optimum at pH 8·5 (Blanchard *et al*, 1977). There are several possible explanations for this finding. Firstly, there may also be a phosphoprotein phosphatase in association with the isolated hnRNP particle. Rech *et al*, (1977) have reported such an activity in the HeLa cell system. The pH optimum for this enzyme, (pH 7·4), corresponds with the region of least kinase "activity" shown in Fig. 30a. The pH profile of the kinase activity shown in Fig. 30a may thus be a composite of incorporation (protein kinase activity) and release (phosphoprotein phosphatase activity) of protein-bound ^{32}P. Alternatively, the pH optimum may be a genuine value, and the enzyme activity, here described, may be different from that observed in the HeLa cell system.

The time course of phosphate incorporation (Fig. 31a) also exhibited some features which were different from those reported for the HeLa cell hnRNP particle kinase. While the incorporation of ^{32}P from $[\gamma\text{-}^{32}P]$-ATP was rapid and linear for the first 10-15 minutes of the reaction, a rapid decrease of protein-bound phosphate could be observed after 15 minutes. After one hour, the protein-bound phosphate

Fig. 31.

Time Course of Incorporation of ^{32}P into HnRNP Particle Proteins in vitro.

A -

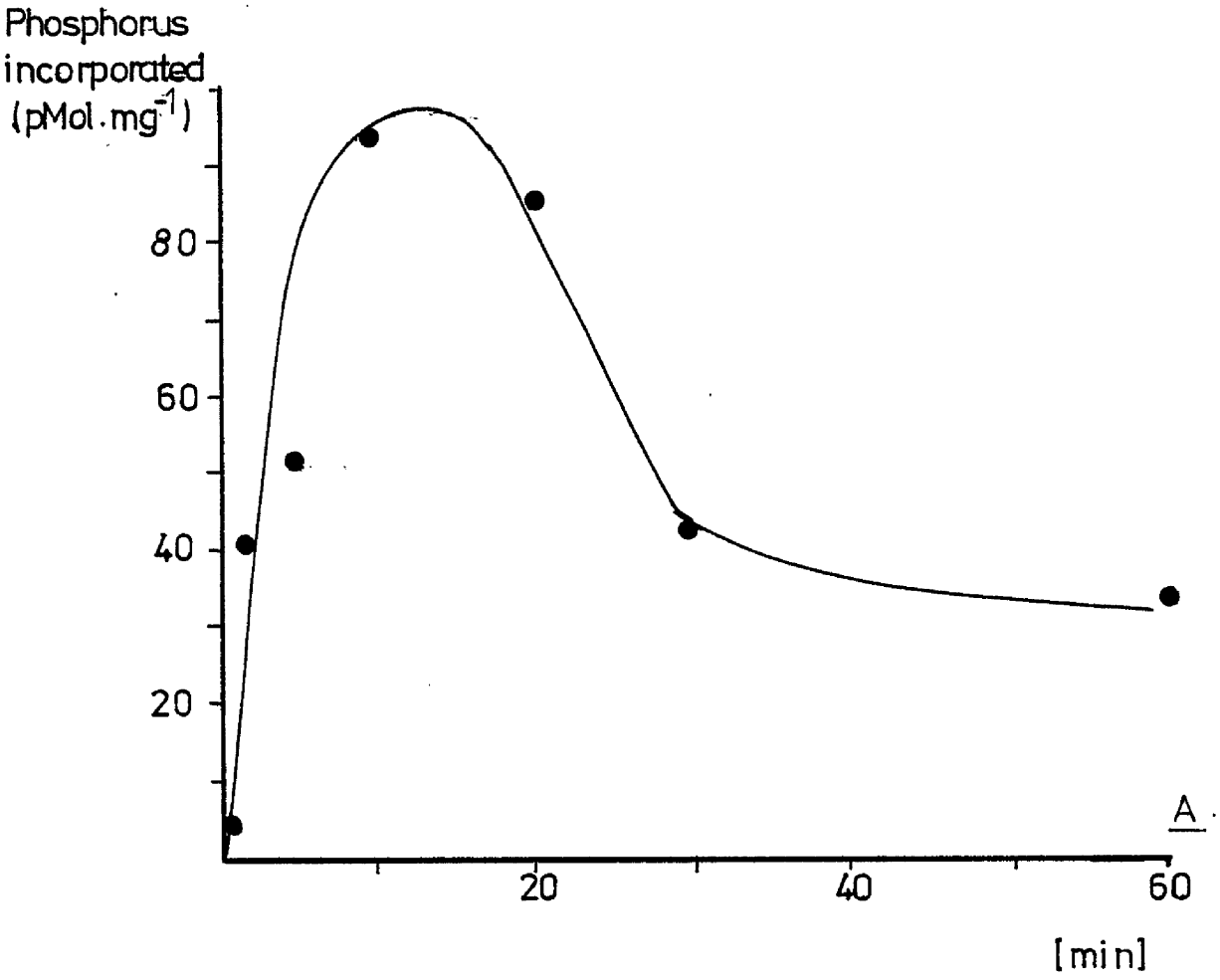
Two hundred micrograms (0·4 $mg.ml^{-1}$) of hnRNP particles (measured with respect to protein) were incubated at 30°C in 100mM sodium acetate, pH 6·5, 10mM $MgCl_2$ in the presence of 25 μCi. $[\gamma\text{-}^{32}P]$-ATP (final concentration 0·1mM). Aliquots of 50μl were removed at various time points and the contents assayed for the presence of protein-bound ^{32}P (Methods 2.2.4.1).

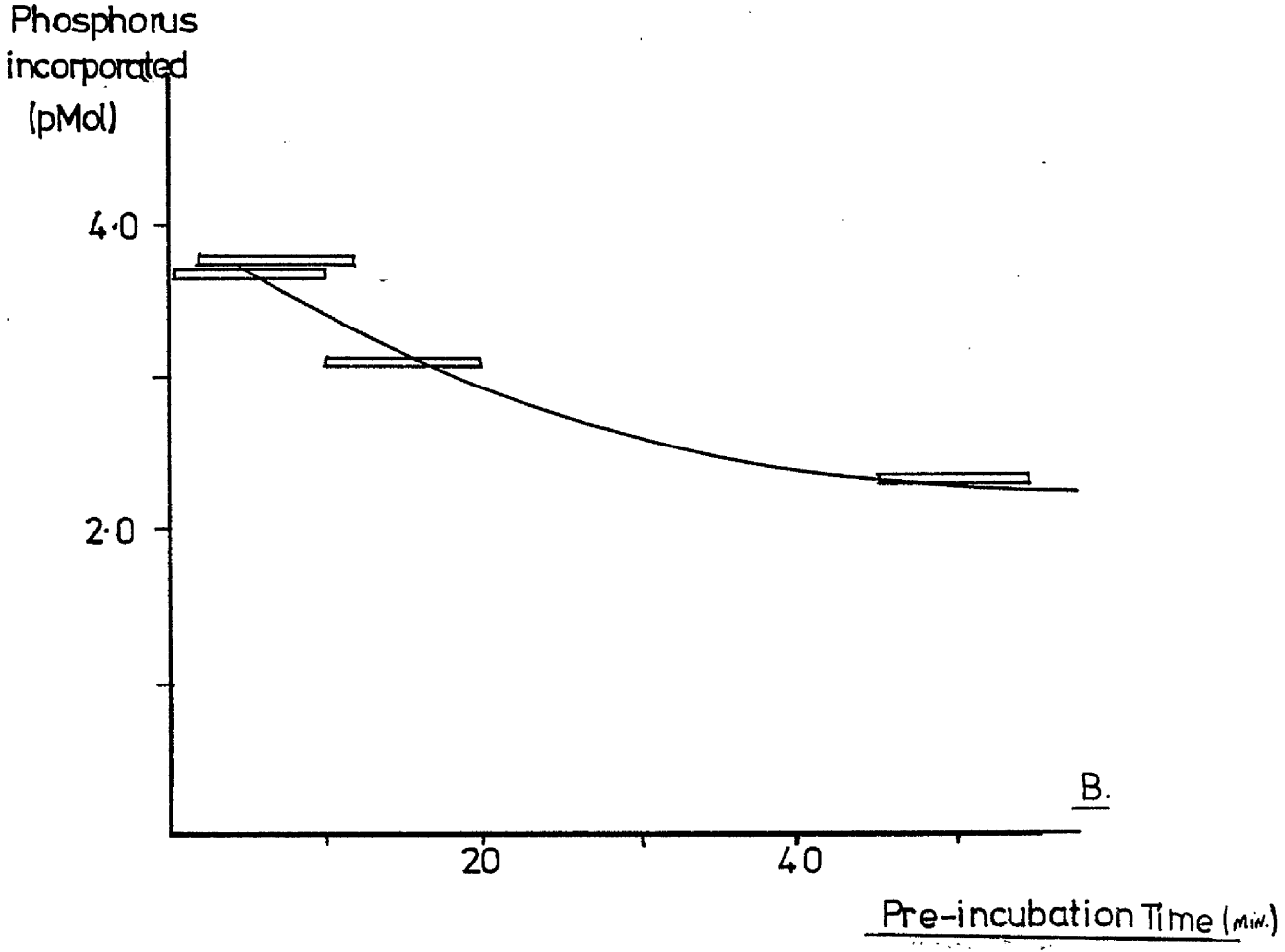
B -

Fifty microgram aliquots of hnRNP particles (measured with respect to protein) were pre-incubated for various lengths of time at 30°C, before the addition of 1μCi $[\gamma\text{-}^{32}P]$-ATP to a final concentration of 0·1mM. Incorporation of ^{32}P into hot 5% $^{w}/_{v}$ trichloroacetic acid insoluble material during a further 10 minute incubation at 30°C was then measured as described in Methods (2.2.4.1).

The time points shown on both graphs represent the average of two determinations.

N.B. 1pMol. Phosphorus . mg. Protein^{-1} ≡ 10cpm per assay.


Phosphorus
incorporated
(pMol.mg^{-1})
80
60
40
20
A
20
40
60
[min]


Phosphorus
incorporated
(pMol)
4·0
2·0
B.
20
40
Pre-incubation Time (min.)

incorporated was down to only 40 % of the value at 15 minutes. Neither PMSF (a protein inhibitor) nor sodium fluoride (a phosphatase inhibitor) prohibited the decrease, although the interpretation of this data must be meted with the knowledge that neither inhibitor is effective for all proteases or phosphatases. The measurement of protein concentrations at each time point, however, suggested that there was little or no protein degradation throughout the first 45 minutes of the reaction (data not shown).

Fig. 31b shows the effect of pre-incubation at 30°C upon the incorporation of ^{32}P -ortho-phosphate during a further 10 minute incubation. This experiment was designed to detect any instability of the kinase activity with respect to time. A considerable loss of kinase activity is observed, resulting in a 38% loss of activity after 45 minutes of incubation. This loss of kinase activity, presumably due to enzyme instability over long periods of incubation, may well explain the time course obtained. Whatever the reason for the time course profile, the use of a 10 minute incubation seemed appropriate for all further investigations.

Figure 30b shows the effect of salt concentration on hnRNP protein kinase activity and confirms the data of Blanchard et al (1977) for the HeLa cell hnRNP particle kinase. Salt concentrations in excess of 250mM markedly diminished phosphate incorporation, and this value corresponds to that reported by Beyer et al (1978) to initiate the dissociation of hnRNP proteins from RNA. It thus seems likely that the integrity of the particle is an important requirement for optimal in vitro phosphorylation.

In summary, these experiments suggested the use of a pH of 6·5, in combination with a salt concentration of less than 250mM. In practice this was accomplished by the use of 100mM sodium acetate

Fig. 32.

Effects of Divalent Cations on the in vitro Phosphorylation of HnRNP Particles.

A - Effects of Mg^{++} and Mn^{++}.

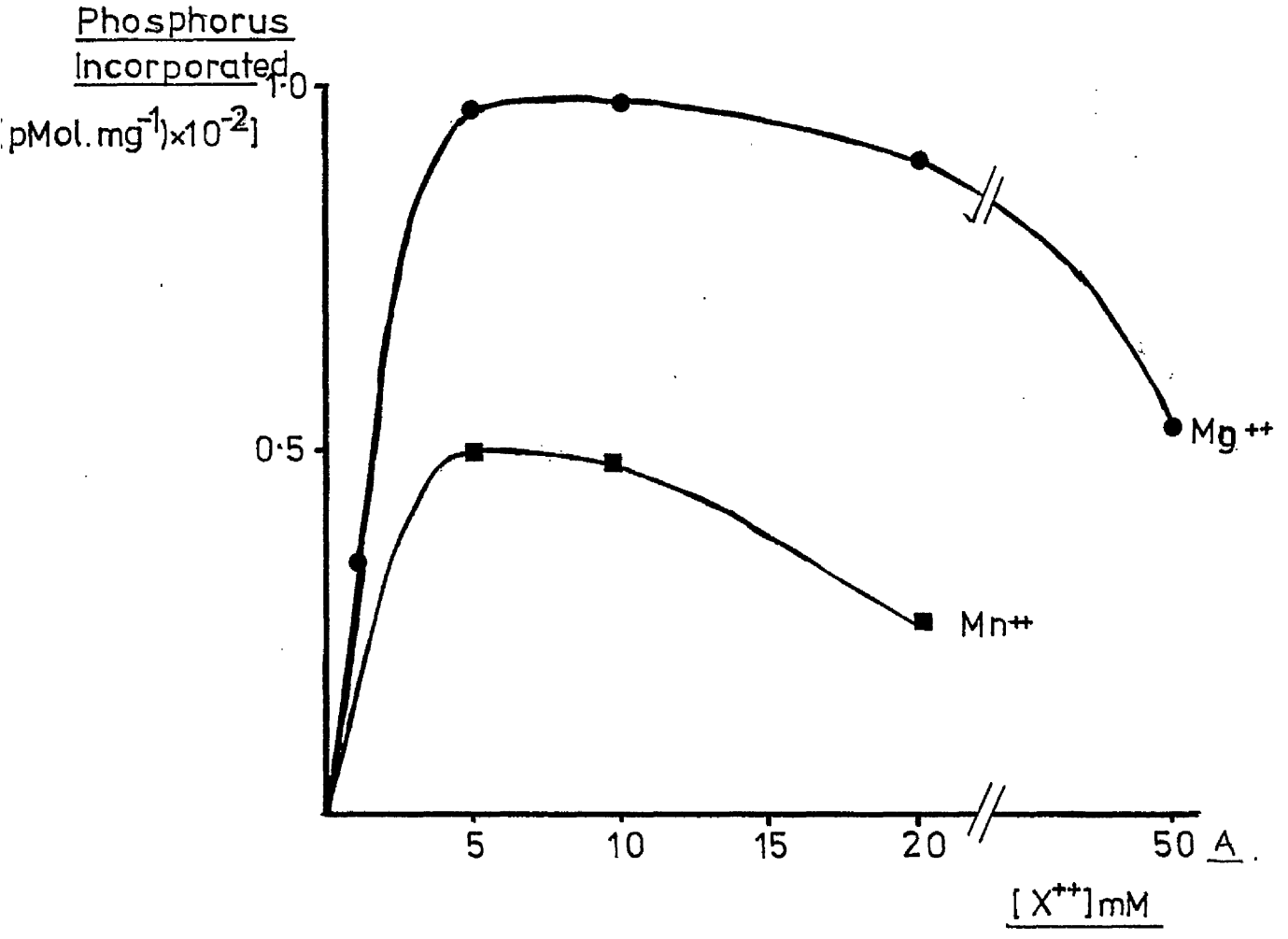
HnRNP particles were dialysed against 0·1M sodium acetate, pH 6·5 and 50µl aliquots containing 25µg. of protein were adjusted to the appropriate $[Mg^{++}]$ or $[Mn^{++}]$ concentration by the addition of 0·1M solutions of their respective chlorides. Volumes were adjusted to 55µl and the assays incubated at 30°C for 10 minutes in the presence of 0·1mM $[\gamma\text{-}^{32}P]$-ATP (1µCi. per assay). Incorporation of ^{32}P was estimated as described in Methods 2.2.4.1.

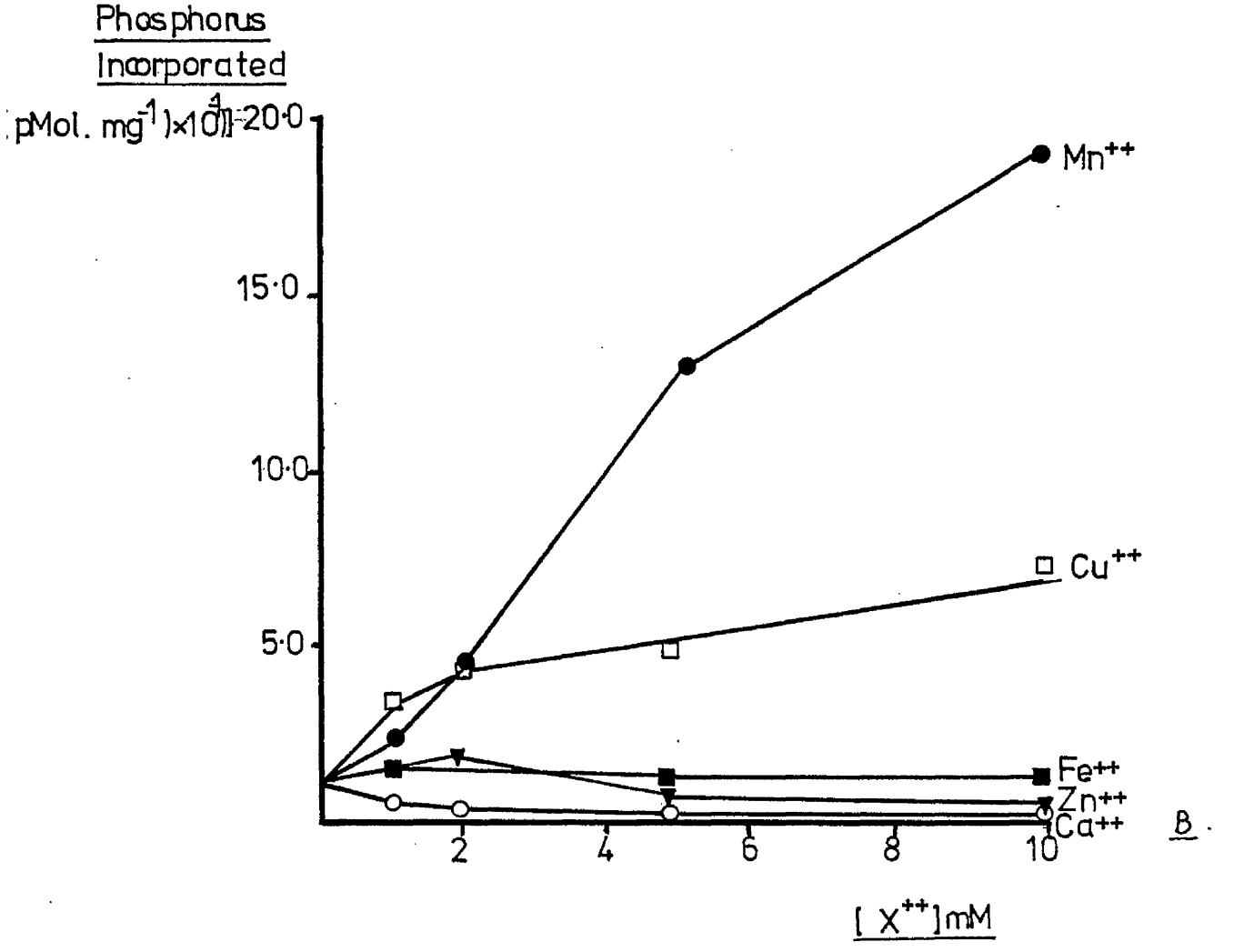
B - Effects of Supplementary Divalent Cations in the Presence of Optimal Mg^{++} Ion Concentrations.

HnRNP particles (as above), suspended in 0·1M sodium acetate pH 6·5, 10mM $MgCl_2$, were adjusted to the appropriate supplementary divalent cation concentration by the addition of the respective chloride. Volumes were adjusted to 55µl, and the assays incubated at 30°C for 10 minutes in the presence of 0·1mM $[\gamma\text{-}^{32}P]$-ATP (1µCi. per assay). Incorporation of ^{32}P was estimated as described in Methods 2.2.4.1.

Each point on the graphs represents a single determination. Each set of data proved to be entirely reproducible, however.

NB. 1 pMol. Phosphorus : mg. Protein^{-1} ≡ 10 cpm per assay


Phosphorus
Incorporated
(pMol. mg⁻¹)×10⁻²]
1·0
0·5
Mg ++
Mn++
5
10
15
20
50
A.
[X++]mM


Phosphorus
Incorporated
(pMol. mg⁻¹)×10⁻²]
20·0
15·0
10·0
5·0
Mn++
Cu++
Fe++
Zn++
Ca++
2
4
6
8
10
B.
[X++]mM

buffer pH 6·5, and incubations were carried out at 30°C. for 10 minutes.

3.2.2.3 Divalent Cation Requirements of the *in vitro* Phosphorylation of HnRNP Particles.

Fig. 32 and Table 5a show the effects of the presence of a number of divalent cations on the incorporation of phosphate into the proteins of isolated hnRNP particles. From the data in Table 5a it is clear that Mg^{++} ions are the most efficient as cofactors for kinase activity. The dependence of protein kinases upon Mg^{++} ions as cofactors seems to be a general rule; although exceptions are known (Kang *et al*, 1974). Manganese ions could partially replace magnesium, as could Cu^{++} and Fe^{++} to a much smaller extent. The optimal concentrations of Mg^{++} and Mn^{++} ions was 5-10mM (Fig. 32a). Above this range they become inhibitory. It is seen that Mn^{++} ions, and to a lesser extent Cu^{++}, exhibited a profound additive effect on the enzyme activity, such that 5mM Mn^{++} in the presence of 10mM Mg^{++} caused a 20 fold increase in activity over that in the presence of Mg^{++} alone. Ca^{++} inhibited Mg^{++}-stimulated activity as did Zn^{++} at higher concentrations. Fe^{++} and Co^{++} had little effect. The reasons for this Mn^{++} ion effect is unknown, although effects of supplementary ions on the specificity and extent of phosphorylation of proteins has been recorded elsewhere (Singh & Wong, 1980) particularly with respect to membrane bond kinases (Lam & Kaspar, 1980). The phenomenon was not, however, observed by Blanchard *et al* (1977) for the HeLa cell hnRNP protein kinase.

3.2.2.4 The Effects of Cyclic Nucleotides and Polyamines on the *in vitro* Phosphorylation of HnRNP Particles.

Protein kinases usually serve as an amplification mechanism for

Table 5.

Effects of Divalent Cations, Cyclic Nucleotides and Polyamines on the in vitro Phosphorylation of HnRNP Particles.

A - Effects of Divalent Cations.

HnRNP particles, dissolved in 0·1M sodium acetate, pH 6·5, were divided into 50µl (25µg. of protein) aliquots and adjusted to 10mM with respect to the various divalent cations listed by addition of 5µl of their respective chlorides (0·1M). Each aliquot was incubated at 30°C for 10 minutes in the presence of 1µCi. $[\gamma\text{-}^{32}P]$-ATP (0·1mM) and the incorporation of ^{32}P quantified as described in Methods section 2.2.4.1.

B - Effects of Cyclic Nucleotides and Polyamines.

Fifty microlitres (25µg. of protein) aliquots of hnRNP particles dissolved in 0·1M sodium acetate, pH 6·5, 10mM $MgCl_2$, were adjusted to the appropriate concentration of cyclic nucleotide, polyamine, kinase inhibitor or EDTA, and incubated at 30°C for 10 minutes in the presence of 0·1mM $[\gamma\text{-}^{32}P]$-ATP (1µCi, per assay). Incorporation of ^{32}P into protein was estimated as described in the Methods section 2.2.4.1. Figures in brackets indicate the number of separate estimations of incorporation.

N.B. 1pMol. Phosphorus. mg.Protein^{-1} ≡ 10 cpm. per assay

Addition.	Kinase Activity ($pMol.P.mg^{-1}.Prot.$)	Percentage Activity
Mg^{++}	91.3	100.0%
Mn^{++}	50.45	55.67%
Co^{++}	8.96	9.82%
Cu^{++}	1.72	1.89%
Fe^{++}	3.90	4.28%
Zn^{++}	2.40	2.63%
Ca^{++}	0.36	0.40%

Addition	Concentration	Kinase Activity ($pMol.P.mg^{-1}.Prot.$)	Percentage Activity
None	-	86.92 ±8.98 (4)	100%
cyclic AMP	1μM	309.72 ±16.50(3)	356.3%
dibutyryl cAMP	10μM	335.29 ±110.13(4)	386.3%
* cAMP-dependent kinase inhibitor	10μg per assay	34.61 (1)	39.8%
Putrescine	5mM	457.63 ±94.90(4)	526.5%
Spermidine	5mM	234.25 ±59.71(4)	269.5%
EDTA	10mM	1.96 (1)	2.3%
Putrescine dibutyryl cAMP	5mM 10uM	190.36 (1)	219.0%

*Protein inhibitor of cyclic-AMP-dependent kinase

Source	Addition	Kinase Acitivity (pMol.P.mg.$^{-1}$Prot.)	Percentage Activity
Column Eluate	-	-	-
Control	-	97.28 ± 14.2 (3)	100.0%
Mn^{++}	5mM	698.60	713.0%
cAMP	1µM	222.41	227.15%
Putrescine	5mM	586.46	598.55%
Detergent-Washed Nuclei			
Control	-	85.25	100.0%
cAMP	1µM	185.0	217.0%

Table 5c

Effects of Cyclic AMP, Putrescine and Mn^{++} on the *in vitro* Phosphorylation of HnRNP Particles Isolated by Gel Exclusion Chromatography, or from Detergent-Washed Nuclei.

HnRNP particles, purified by gel exclusion chromatography, (Methods 2.2.2), or isolated from detergent-washed nuclei, were examined with respect to their *in vitro* phosphorylation, in the presence of Mn^{++}, putrescine or cyclic AMP. Estimation of the extent of *in vitro* phosphorylation was by means of the filter disc assay described in the Methods section (2.2.4.2).

All the data shown in Tables 5A, 5B and 5C are averages of two or more determinations. Where more than two determinations were made the exact number is shown in brackets.

the response to a particular stimulus. The regulation of protein kinase activity is therefore an important aspect of metabolic control and is brought about by a variety of signals. These include calcium ions (Schulman & Greengard, 1978), polyamines (Kuehn et al, 1979), & the cyclic nucleotides, cyclic AMP and cyclic GMP (see Ruben & Rosen, 1975). It was therefore of interest to investigate the potential stimulation of the in vitro phosphorylation of hnRNP particles by the most common of these signals, cyclic AMP and cyclic GMP. In addition, the recent observation of polyamine mediation of the phosphorylation of a nucleolar protein in P. polycephalum (Keuhn et al, 1979), suggested their investigation as possible stimulators of the hnRNP particle kinase.

Fig.33b shows the effects of cyclic AMP and cyclic GMP on the in vitro phosphorylation of hnRNP particles. Although cyclic GMP shows no stimulatory effect, cyclic AMP at 1×10^{-6} M markedly stimulates the incorporation of phosphate into protein. Table 5b shows the effects of both cyclic nucleotides and polyamines, and also the protein inhibitor of cyclic AMP-dependent kinase (the kind gift of Dr. H. G. Nimmo of this department) on rat liver hnRNP protein kinase activity. The reduction in kinase activity obtained using the inhibitor confirms the likely presence of cyclic AMP-dependent kinase, however, the failure of this inhibitor to abolish entirely this activity could be interpreted as evidence for a second, cyclic AMP-dependent kinase activity.

Polyamines, particularly putrescine, were also able to stimulate the kinase and appeared to be more effective than cyclic AMP.(Fig.33a. and Table 5b). While the presence of a cyclic AMP stimulated kinase activity could, because of numerous precedents, be interpreted as being due to the presence of a classical cyclic AMP-dependent protein kinase, the mode of action of the polyamine mediated stimulation could be explained in a number of ways. The possibility that polyamines act by a similar mechanism to cyclic AMP, is suggested by

Fig.33.

Effect of Increasing Polyamine and Cyclic Nucleotide Concentrations on the in vitro Phosphorylation of HnRNP Particles.

HnRNP particles were dialysed against 0·1M sodium acetate, pH 6·5, 10mM $MgCl_2$, and 50µl aliquots containing 25µg of protein were adjusted to the requisite polyamine or cyclic nucleotide concentrations by the addition of the appropriate volume of polyamine (100mM) or cyclic nucleotide (100µM) stock solution. Volumes were adjusted to 55µl, and the assays incubated at 30°C for 10 minutes in the presence of 0·1mM $[\gamma\text{-}^{32}P]$-ATP (1µCi. per assay). Incorporation of ^{32}P was estimated as described in Methods 2.2.4.1.

A - Polyamines

—●———●— Putrescine

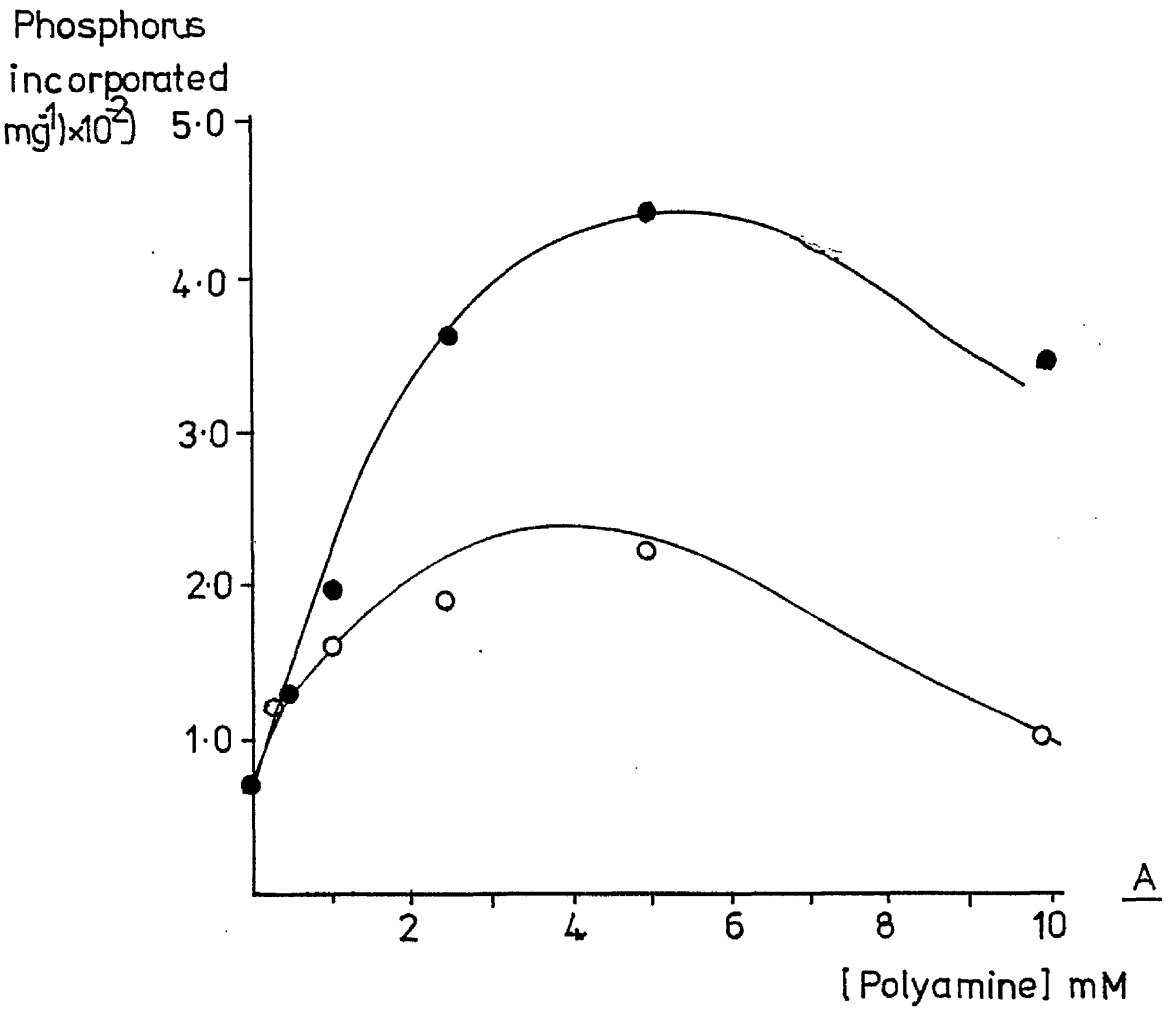
—○———○— Spermidine

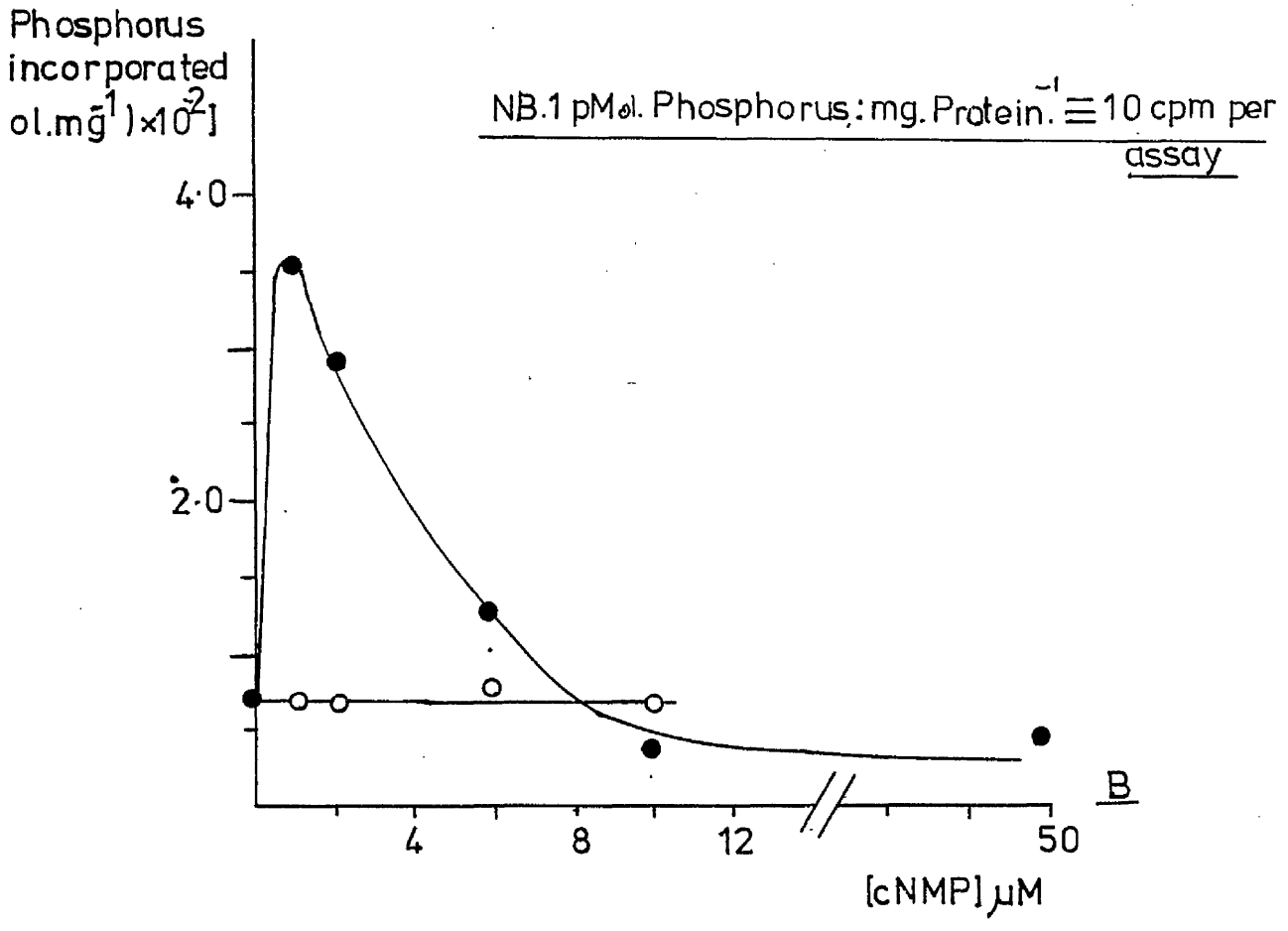
B - Cyclic Nucleotides

—●———●— Cyclic AMP

—○———○— Cyclic GMP

Each point on the graphs represents a single determination. Each set of values has been repeated several times, however, and these data appear in tabulated form in table 5 5 [p . 121]


Phosphorus
incorporated
mg⁻¹)×10⁻²)
5·0
4·0
3·0
2·0
1·0
2
4
6
8
10
A
[Polyamine] mM


Phosphorus
incorporated
ol.mg⁻¹)×10⁻²]
NB.1 pMol. Phosphorus: mg. Protein.⁻¹ ≡ 10 cpm per assay
4·0
2·0
4
8
12
50
B
[cNMP] µM

the non-additive affect of their simultaneous addition (Table 5 b). The basic proteins, histones and protamines have been shown to dissociate the regulatory and catalytic subunits of cyclic AMP-dependent kinase, and it is conceivable that polyamines could do the same. Secondly, polyamines have previously been employed at a concentration of 100mM to completely dissociate hnRNP particles (Schweiger & Kostka, 1976). Their presence in the lower concentraions used here (0-10mM) may result in the "loosening" of the hnRNP particle structure and could be an important factor in the stimulation of kinase activity. An hnRNP protein kinase, the activity of which is stimulated by cyclic AMP and polyamines, has not previously been reported. It was important therefore, to confirm that the effects were genuine. The most clear cut way to achieve this would be to purify the enzyme, but this has not yet proved possible. However, Table 5c shows that, if 40S particles, recovered from sucrose density gradients, are further purified by gel exclusion chromatography on BioRad Biogel A-0·5, the particulate kinase activity is still markedly stimulated by both cyclic AMP and polyamines.

Table 5c shows data on kinase activity of hnRNP particles extracted from nuclei, which had been subjected to a more rigorous purification in which the nuclear membrane and adhering cytoplasmic tags had been removed by Triton X100, as described by Blobel and Potter (1966). It is seen that such particles still exhibit a protein kinase activity which is stimulated by cyclic AMP and polyamines.

3.2.3 Characterisation of the Proteins Phosphorylated During *in vitro* Phosphorylation of HnRNP Particles.

Some aspects of the *in vitro* phosphorylation of the core polypeptides have been discussed elsewhere (Results: Section 2). However,

Fig. 34.

Two Dimensional Fractionation of in vitro Phosphorylated HnRNP Particle Proteins.

I. NEPHGE-Based Analysis.

HnRNP particles,isolated from a sucrose density gradient similar to that shown in Fig. 5 (p . 64) were dialysed against 2 changes of 500 volumes of 0.1M sodium acetate pH 6.5,containing 10mM $MgCl_2$. After concentration of the dialysate to 0.5 mg.ml. by further dialysis against polyethylene glycol 6000 (See Methods 2.2.4.1 p . 48),500 µg of protein were incubated at 30°C for 10 minutes in the presence of 50µCi (γ-^{32}P ATP (5,000 Ci.m,Mol.$^{-1}$). The proteins were then precipitated with 2.5 volumes of ethanol and subsequently resuspended in lysis buffer (9.5M urea, 5% ^{W}/v β-mercaptoethanol, 2% ^{W}/v nonidet P40, 1.6%^{W}/v pH 5-7 ampholines, 0.4% ^{W}/v pH 3.5-10 ampholines) at a concentration of 10 mg.ml.). Two dimensional fractionation was performed on 200 µg of phosphorylated protein using the NEPHGE-based technique (See Methods 2.3.2 p . 51). The final gel was stained,destained and dried and photographed as described in Methods 2.3.2.4 p . 54. Radiolabelled phosphoprotein products were detected by autoradiography (8 days) using Kodak X-Omat H film.

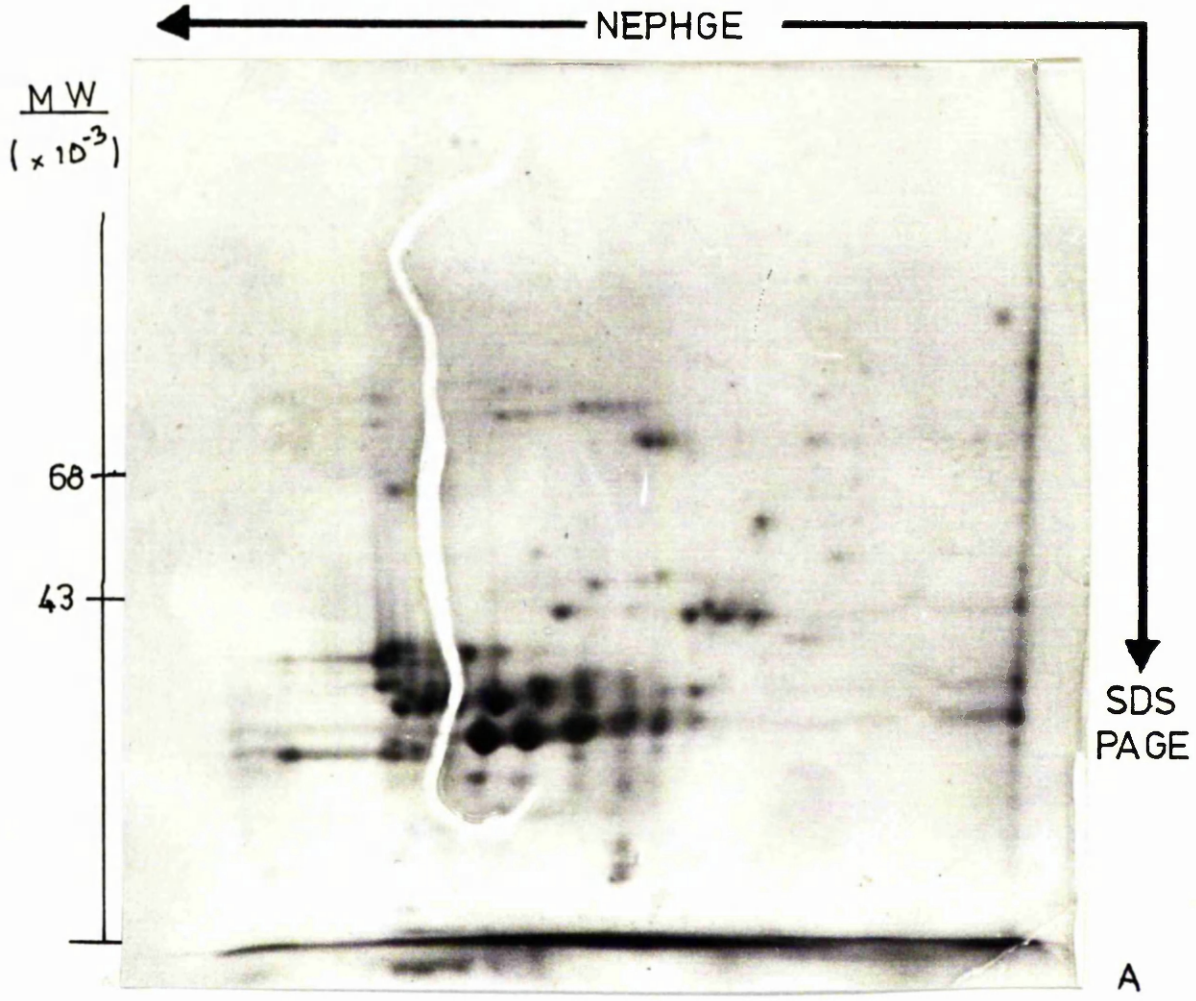
A - stained gel.

B - autoradiograph.

Core protein phosphorylation discussed on p . 128 para. 4.

Acidic protein phosphorylation discussed on p . 128 para. 5.

Minor low mol. wt. species discussed on p . 130 para.1.


NEPHGE
MW
(×10⁻³)
68
43
SDS
PAGE
A

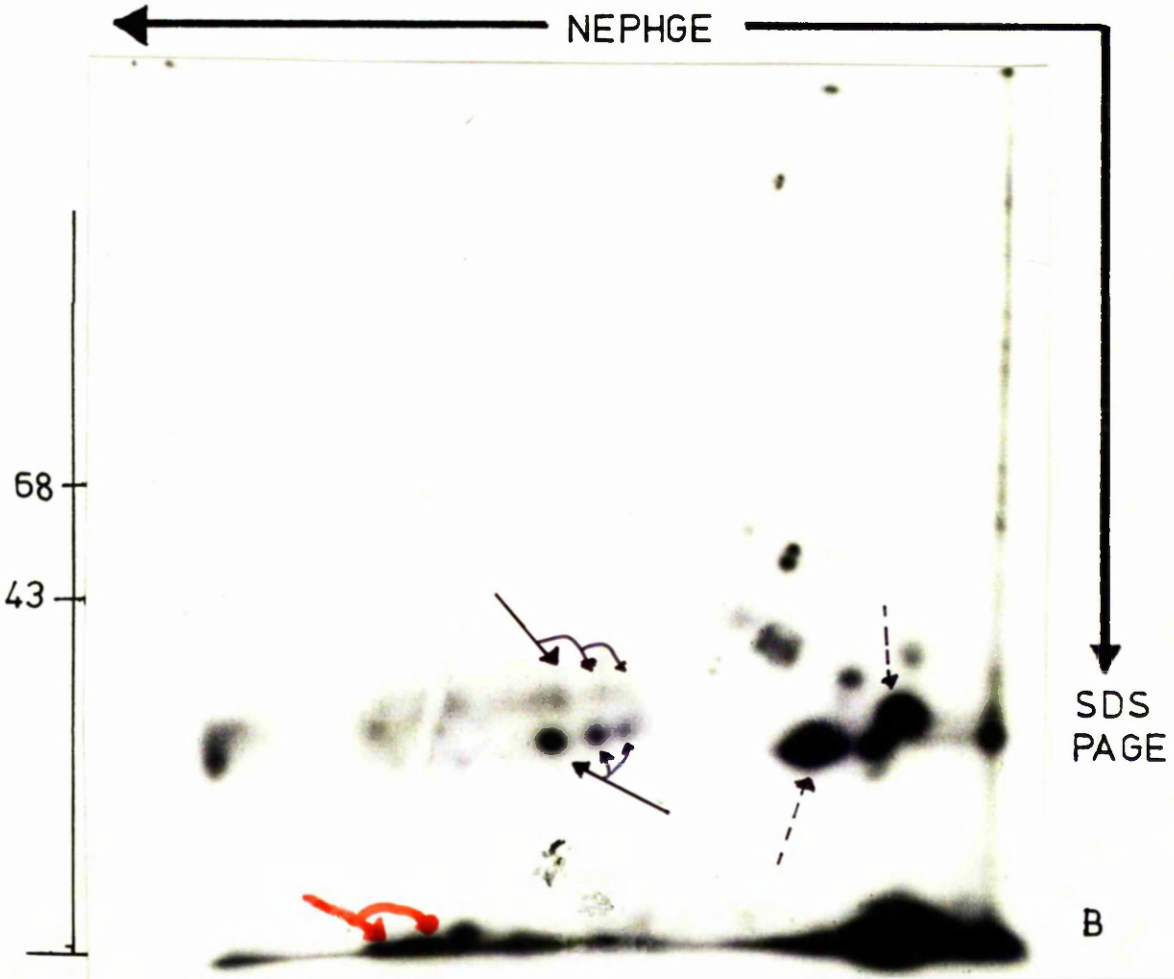

NEPHGE
68
43
SDS
PAGE
B

Fig. 35

Two-Dimensional Fractionation of in vitro Phosphorylated HnRNP Particle Proteins.

II - IEF-Based Analysis.

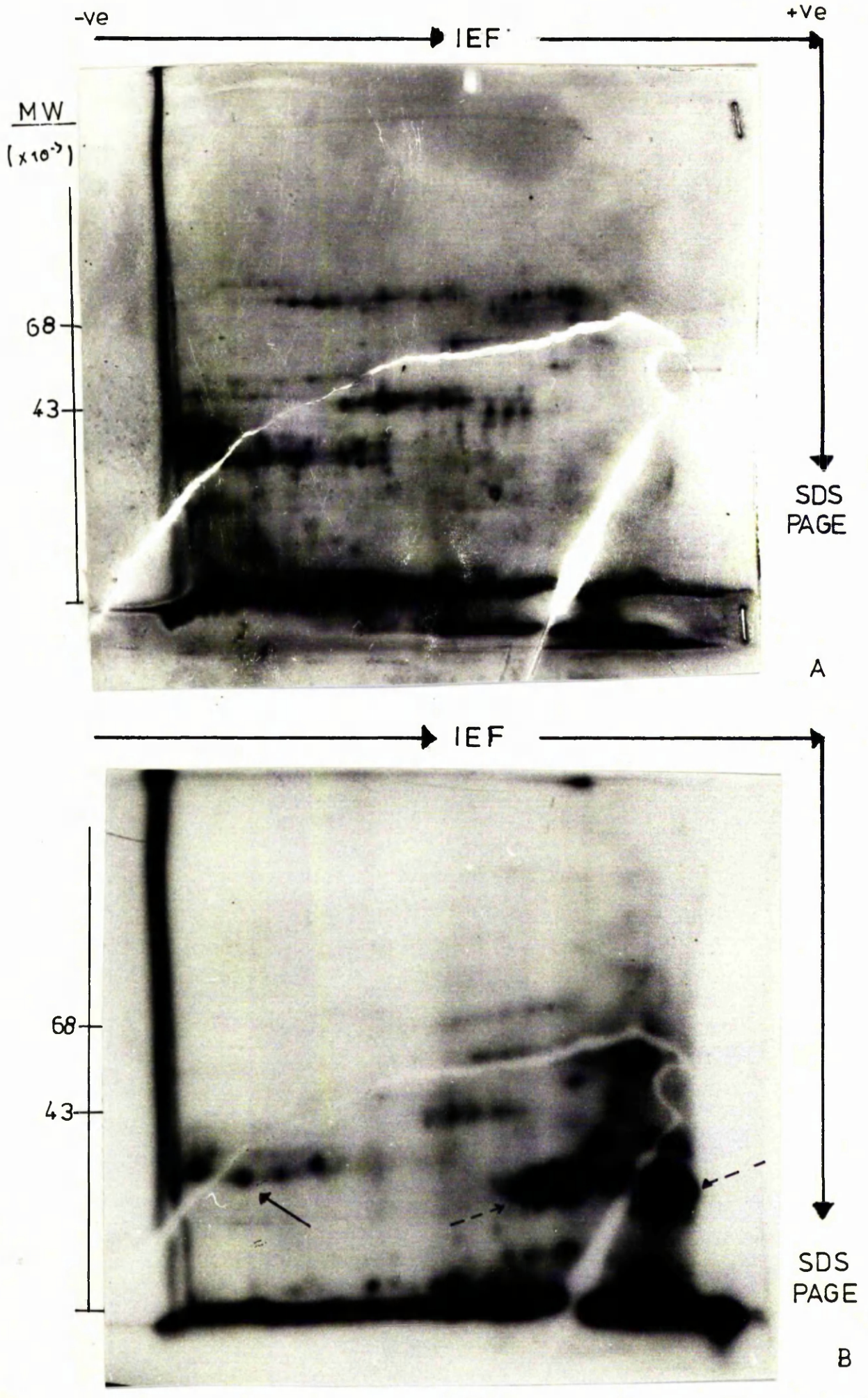
The sample employed in the analysis described in Fig. 34 was additionally fractionated by means of the IEF-based two-dimensional fractionation technique. Three hundred micrograms of in vitro phosphorylated proteins were loaded onto the first dimension gel, and the completed gel processed as described in Fig. 34.

A - Stained gel.

B - Autoradiograph.

Core protein phosphorylation, discussed on p .128, para 4.

Acidic protein phosphorylation, discussed on p .128, para 5


-ve
IEF
+ve
MW
(x10⁻³)
68
43
SDS
PAGE
A
IEF
68
43
SDS
PAGE
B

the full extent of the phosphorylation reaction has been characterised by two-dimensional gel electrophoresis, and will be discussed here with respect to the effects of cyclic AMP, polyamines and Mn^{++} ions.

Fig.36a shows the pattern of phosphorylated proteins which could be detected if a pH 8·0 nuclear extract was incubated with $[\gamma\text{-}^{32}P]$-ATP, without fractionation on sucrose density gradients. Fig.36b shows the pattern of phosphorylated proteins detected after the incubation had been performed on the non-particulate proteins left on top of a sucrose density gradient after removal by centrifugation of the hnRNP particles. Since the profiles of stainable proteins detected on both gels were very similar to those previously displayed for these subnuclear fractions (Fig.15 and Fig.16a), only the autoradiographs are shown.

Both autoradiographs are complex with respect to the number of phosphoproteins present, and are extremely difficult to interpret. However, the lack of significant phosphorylation of the hnRNP particle core polypeptides is interesting.

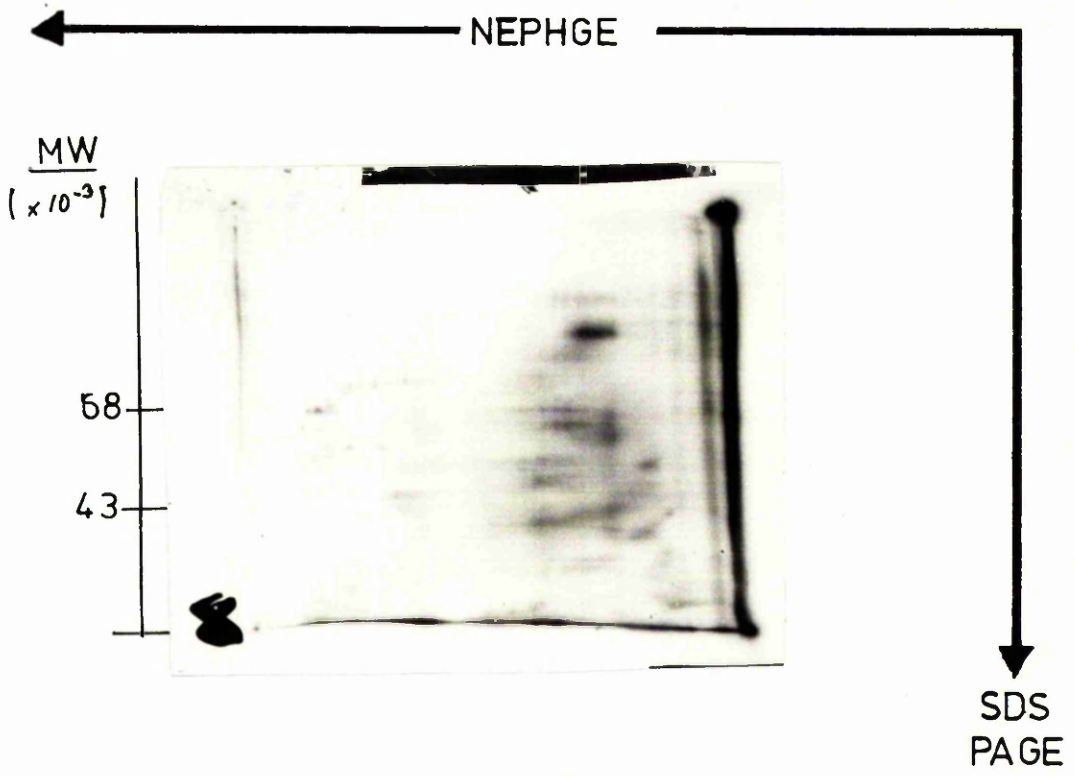
Figs.34 and 35 show respectively the NEPHGE and IEF-based two-dimensional fractionations of hnRNP particle proteins after incubation in the presence of $[\gamma\text{-}^{32}P]$-ATP. In contrast to the autoradiographs of the pH 8·0 nuclear extract and non-particulate fractions, there is considerable phosphorylation of the core polypeptides (See also Results: Section 2).

The majority of phosphoproteins produced by the <u>in vitro</u> reaction are acidic and are best fractionated in the IEF-based fractionation system. It is interesting that the most highly phosphorylated protein component is a rather acidic pI 4·5 species of mol.wt. 32,000. The close similarity in molecular weight between this species and the A-group core polypeptides highlights the profound advantage of the two-

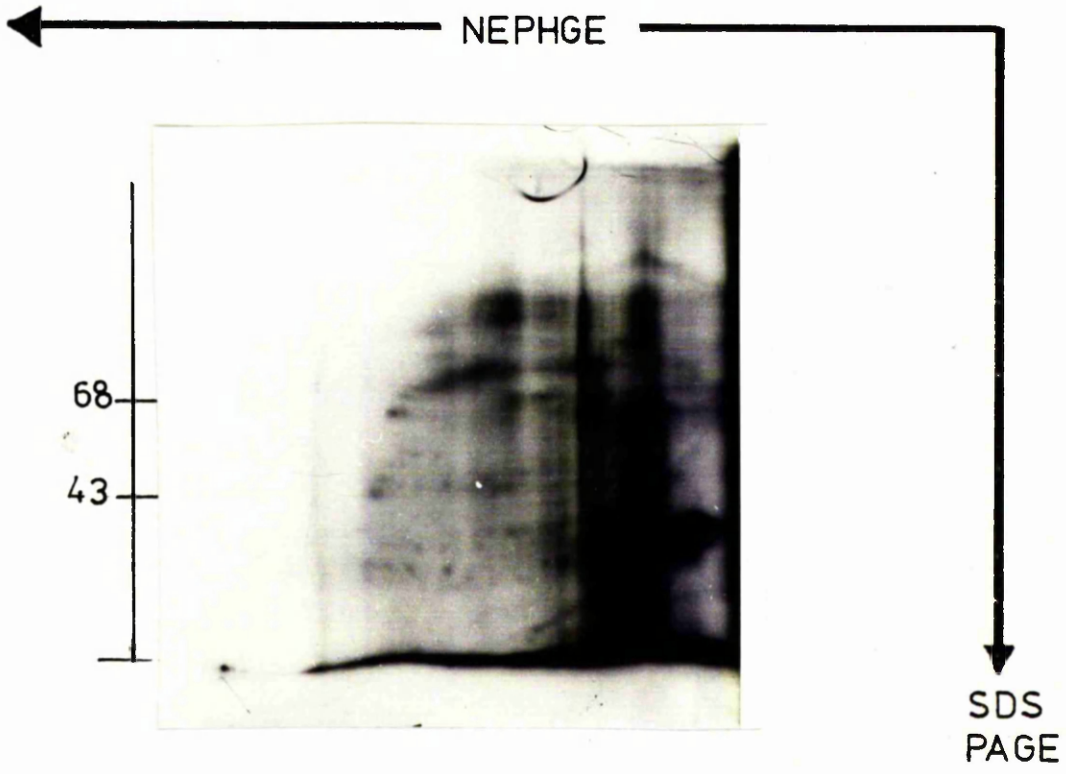
Fig. 36

Two Dimensional Fractionation of Phosphoproteins Generated During in vitro Phosphorylation of a Nuclear pH 8.0 Extract and the Non-Particulate Region of a Sucrose Density Gradient Containing 40S HnRNP Particles.

A nuclear pH 8.0 extract (A) (See Methods 2.1.2.1 p. 42) and the pooled non-particulate region (<20S) of a sucrose density gradient similar to that shown in Fig. 5 (p . 64)(B) were both dialysed against 2 changes of 500 volumes of 0.1M sodium acetate, pH 6.5, containing 10mM $MgCl_2$. The dialysed material was then incubated for 10 minutes at 30°C in the presence of 20 µCi (γ-^{32}P) ATP 2,000 Ci m.Mol.$^{-1}$ The protein concentrations were 1.2mg.ml. for the pH 8.0 extract and 0.1mg.ml^{-1} for the non-particulate material. After precipitation of the proteins with 2.5 volumes of ethanol and subsequent resuspension of the precipitated material in lysis buffer (9.5M urea,5% $^{w}/_{v}$ β-mercaptoethanol,2% $^{w}/_{v}$ nonidet P40,1.6% $^{w}/_{v}$ pH 5-7 ampholines,0.4% $^{w}/_{v}$ 3.5-10 ampholines) at a concentration of 10mg.ml, 200 µg (measured with respect to protein) of each sample was subjected to NEPHGE-based two dimensional fractionation (Methods 2.3.2 p . 51). The completed gels were stained,destained,dried and autoradiographed as described in Methods 2.3.2.4 (p. . 54). Since the stained patterns for each gel were very similar to those shown in Figs. 16B (p . 86) and Fig. 15 (p. . 84) only the autoradiographs are shown.



A.



B.

dimensional fractionation system employed over one dimensional systems. It appears very likely that the highly phosphorylated species observed on one-dimensional gels by Karn et al (1977) and Blanchard et al (1977), and considered by them to represent core protein phosphorylation, in fact represented phosphorylation of the minor non-stainable species which would migrate with core protein on a one dimensional gel. Furthermore, the previously proclaimed absence of hnRNP protein species of molecular weight less than 25,000 (Karn et al, 1977; Beyer et al, 1977, inter alia; See Fig. 7), is shown to be incorrect on the basis of the autoradiographs shown in Figs.34 and 35 . Such low molecular weight proteins are not detected by staining but the presence of 12,000 to 15,000 molecular weight species is revealed by in vitro phosphorylation. These proteins clearly make little contribution to the mass of the hnRNP particle, but the role of two highly phosphorylated, low molecular weight, basic proteins in hnRNP particles may repay further investigation.

The effects of cyclic AMP and polyamines appear to be purely quantitative. Fig. 37 shows the autoradiographs obtained after fractionation of the in vitro phosphorylation products of hnRNP particles that were incubated in the presence of 1μM cyclic AMP or 5mM putrescine. No qualitative differences were observed, again suggesting the similar mode of action of these two compounds in the stimulation of in vitro phosphorylation. Manganese ions also show little or no qualitative effect on the in vitro phosphorylation, and the massive stimulation of phosphorylation by manganese appears to be reflected in the majority of phosphoprotein products. There are slight indications that in the presence of Mn^{++} ions the two minor species of mol.wt. 13,000 to 15,000 have increased levels of phosphates, relative to the other phosphoprotein species. However, this has not resulted in a significant charge alteration. More experimentation would be required for a definite assertion that this minor alteration was real.(Fig.38)

Fig. 37.

Effects of Cyclic AMP and Putrescine on the Products of the in vitro Phosphorylation of HnRNP Particles.

Isolated hnRNP particles were dialysed against 0·1M sodium acetate, pH 6·5, 10mM $MgCl_2$ and concentrated to 0·7mg. protein per ml. by dialysis against dry polyethylene glycol 6000. The particles were then phosphorylated in vitro in the presence of 1µm cyclic AMP or 5mM putrescine, by incubation at 30 C for 10 minutes in the presence of 0·1mM [γ-^{32}P]-ATP (10µCi). After precipitation, 150µg. of protein was examined by two-dimensional protein fractionation, using a NEPHGE first dimension (Methods 2.3.2). Autoradiographs (12 days exposure) of the phosphoprotein profile obtained are displayed:

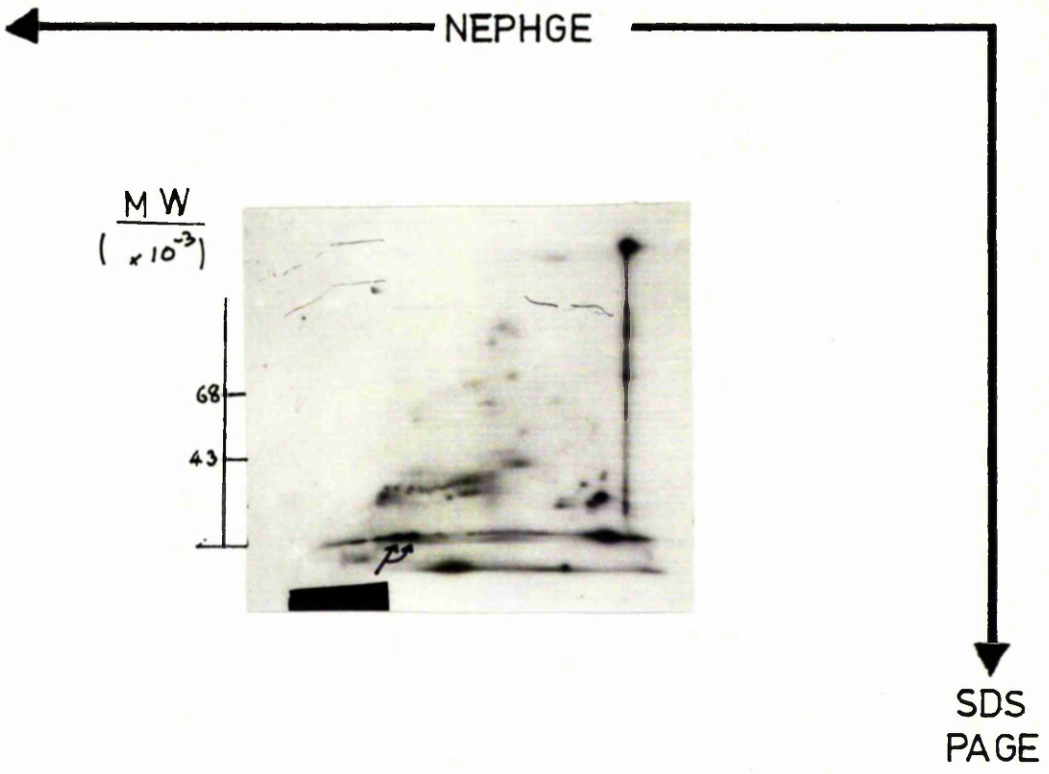
A) - in the presence of 1·0µm. cyclic AMP.

or

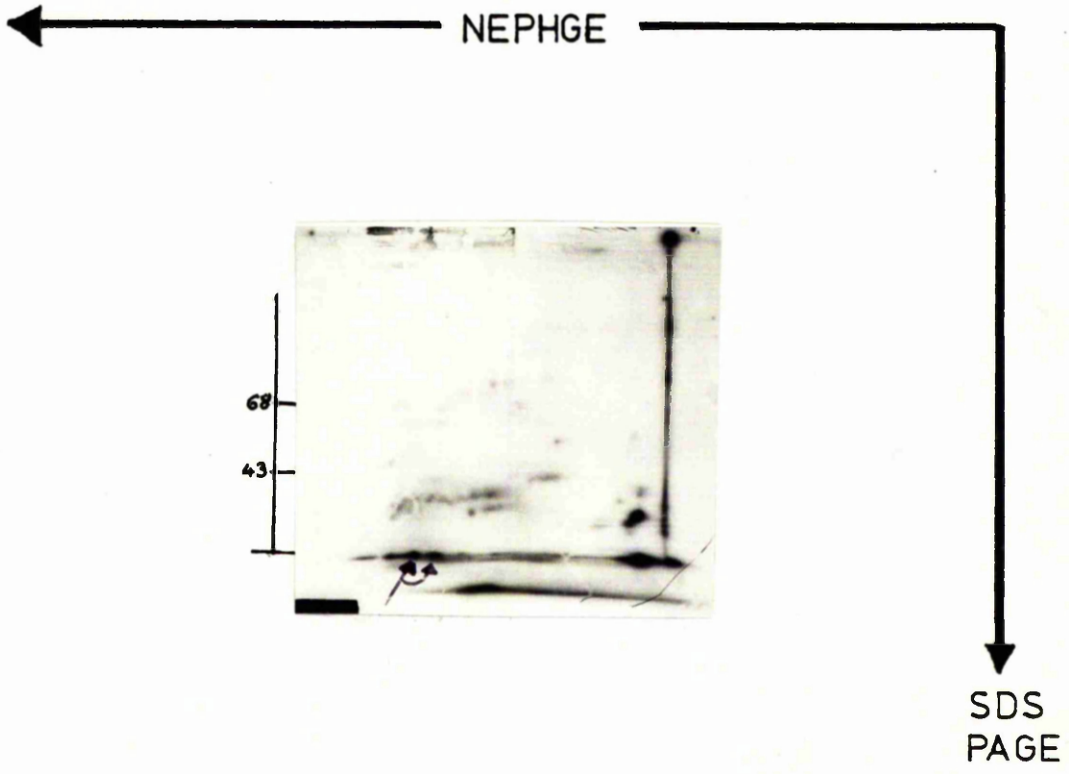
B) - in the presence of 5mM putrescine.

The "control" gel for this experiment is shown in Fig 38 B'

→ indicates low mol.wt. proteins discussed on p.130 paragraph 2.



A.



B.

Fig. 38.

Effects of Manganese Ions on the Products of the in vitro Phosphorylation of HnRNP Particles.

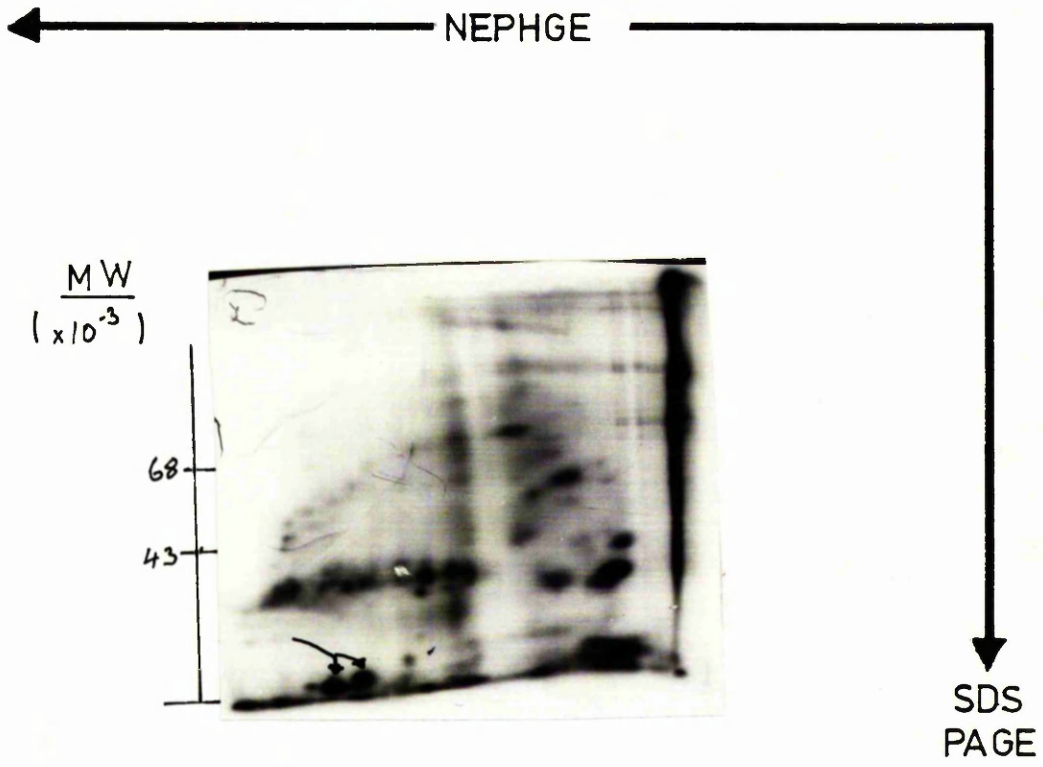
HnRNP particles isolated from a sucrose density gradient and dialysed against 2 changes of 500 volumes of 0.1M sodium acetate pH 6.5,containing 10mM $MgCl_2$,were phosphorylated as described in Fig. 37,in the presence (A) or absence (B) of 10mM $MnCl_2$. The proteins were precipitated by the addition of 2.5 volumes of ethanol and subsequently re-suspended at 10 mg.ml. in lysis buffer. After fractionation of 150 μg of these samples by NEPHGE-based two-dimensional fractionation,the gels were stained, destained,dried and autoradiographed as described in Methods 2.3.2.4 (p. 54). The autoradiographs obtained are displayed.

A). Phosphorylation in the presence of 10mM $MnCl_2$.

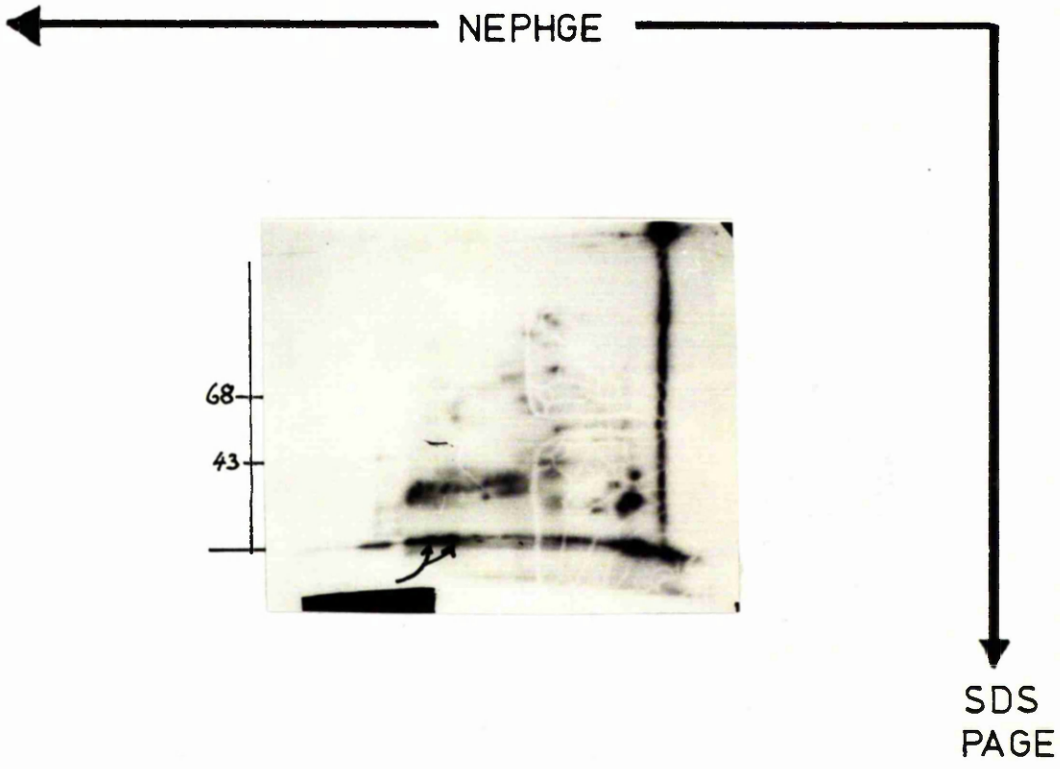
B). Phosphorylation in the absence of any added stimulator of phosphorylation.

Fig. 38B may be considered as the control experiment for Figs. 37A and B and Fig. 38A.

indicates low mol.wt. proteins discussed on p. 130 paragraph 1. and p. 130 paragraph 2.



A.



B.

A number of possible approaches suggest themselves for the continuation of the work presented in this thesis. Firstly, the employment of a second site specific protease (e.g. elastase or thermolysin), or CNBr peptide mapping, would help to resolve the question of the relationship between the four groups of hnRNP core polypeptides. In addition, an attempt to label the hnRNP particle peptides, by administering high levels of ^{32}P would clarify the possibility that phosphorylation is the cause of the charge heterogeneity. Finally, an attempt to purify the protein kinase(s) present in association with hnRNP particles, may lead to an elucidation of its possible role in hnRNP function.

DISCUSSION

1. DO HnRNP PARTICLES EXIST IN VIVO?

There are considerable ultrastructural data supporting the idea that the nascent transcripts of eukaryotic structural genes become complexed with proteins at an early stage in their existence (Miller & Hamkalo, 1972; Malcolm & Sommerville, 1974, 1977). The nature of this ribonucleoprotein complex has been the subject of intensive research in recent years, since it is only by the investigation of the ribonucleoprotein form of hnRNA that a full understanding of mRNA formation can emerge.

Although electron-microscopic analysis of isolated hnRNP particles and their most likely in vivo correspondent, the perichromatin fibril, shows that, with respect to size distribution (10-30nm) and general morphology, these structures are almost identical (Stevenin & Jacob, 1979), the investigation of isolated hnRNP particles as representative isolated sub-nuclear organelles has not been straightforward. Uncertainty about the relevance of the isolated hnRNP particle to the in vivo situation has been fired by the apparent inconsistency of some of their properties. The number of protein species present in the hnRNP particle, for example, has varied between one (Samarina et al, 1968) and 45 (Stevenin et al, 1970).

It has become clear now that many of these discrepencies were due to the wide variation in the techniques employed by different workers (e.g. low pH urea gel electrophoresis versus high pH SDS gel electrophoresis), and also, in some measure, to genuine tissue and species specific differences in these proteins. However, the precise relationship between the isolated complex and the structures observed under the electron-microscope remains unknown, and it seems pertinent here to critically appraise the possible nature of this relationship.

There are two main areas of uncertainty. Firstly, there is the possibility that contamination during isolation of the hnRNP particles, by any of a variety of other structures, could lead to the false attribution of certain properties to the particles. Secondly, the generation of artefactual structures during the isolation of these complexes could render the isolated hnRNP particle unrepresentative of the in vivo structure. Both possibilities would diminish the value of the investigation of the isolated hnRNP particle.

1.1 Contamination of HnRNP Particles.

There are several possible sources of contamination or modification of hnRNP particles. These are:-

1). Contamination of isolated particles by other subcellular organelles,

2). Contamination of the protein components of particles by by adsorption of foreign protein,

3). Loss of specific proteins or partial degradation of proteins during isolation.

The first possibility remains the most easily tested, and the most easily avoided problem. Likely sources of contamination include ribosomes, pre-ribosomal particles, chromatin fragments, membrane fragments, mRNP particles and protein aggregates. Several approaches have been employed in the exclusion of these sources as contaminants. The absence of significant levels of DNA and lipid (Moulé & Chauveau, 1968) in the particles precludes the possibility of chromatin fragment and membrane fragment contamination, while problems with ribosome and pre-ribosome contamination have been shown unlikely by CsCl buoyant gradient centrifugation, which is able to distinguish effectively between ribosomes and hnRNP particles by virtue of their characteristic protein:RNA ratios (See Fig. 6).

Particular difficulty has been encountered in excluding the possibility of mRNP particle contamination. The absence of a difference in the buoyant density between hnRNP particles and mRNP particles (both around 1·4 g.cm $^{-3}$) means that the other techniques must be employed. Of these, the most significant is the use of analytical gel electrophoresis. A number of mRNP proteins have been characterised by molecular weight and appear to be universally present. Their absence from hnRNP particle preparations has therefore been used to indicate a lack of mRNP contamination (Liautard et al, 1974; Schweiger & Kostka, 1980).

Stevenin et al (1974) have presented data in support of the possibility that the 40S hnRNP particle may be contaminated by protein aggregates. The extensive ribonuclease treatment of (^{3}H)-protein labelled hnRNP particles of various size classes (70S, 50S and 40S), and subsequent recentrifugation on sucrose density gradients, appears to reveal the presence of protein aggregates in the 50S and 40S particle populations, where none exist in the 70S region. Several criticisms of this work can be made, however, and the results must remain contentious. Firstly, the relatively short centrifugation employed for the preparation of polyparticle (>70S) hnRNP particles predisposes the monoparticle (40S) population isolated from similar preparative gradients to contamination by non-particulate material. Secondly, other data within the same paper, shows that recentrifugation of ribonuclease-treated 70-85S double-labelled particles results in the isolation of a 40S protein "aggregate", suggesting the alternative possibility that the 40S "aggregate" is really an artefact of the RNAse digestion procedure.

The possibility of non-specific and specific adhesion of proteins to hnRNP particles during their isolation is a particularly difficult

one to exclude. Since there is no functional assay available to distinguish or define an hnRNP particle protein as distinct from, for example, a chromatin protein, a useful approach to answer this question is difficult to conceive. The situation is all the more complex because some of the proteins may be true components of more than one sub-nuclear structure. The solution to this problem would only emerge after a detailed consideration of individual proteins, their functions and locations within the cell. In lieu of such evidence, the findings presented in this thesis have provided support for the absence of non-specific binding of proteins to hnRNP particles during their isolation (Fig.12).

The possibility that proteins are lost during the isolation of particles is also very difficult to exclude. Indeed, the following section presents evidence on how this may come about. Also, some of the results presented in this thesis provide evidence for instability in particle composition indicating, for instance, that particle-associated protein kinase may be partially removed during ultracentrifugation and gel exclusion chromatography. Similar data on the loss of minor proteins during purification of hnRNP have been presented by others (Stevenin et al, 1975).

1.2 Rearrangement of HnRNP Particles.

Recently, Stevenin et al (1979) have made a strong case for the possible rearrangement of hnRNP particles due to RNAse hydrolysis of the RNA component. Early work by these investigators employed ribonuclease treatment of 30S - 50S rat brain hnRNP particles, and appeared to distinguish two classes of particles (Stevenin et al, 1977b). However, reappraisal of these data in the light of a more thorough examination of each class, with respect to sedimentation coefficient

and protein complement, lead to the suggestion that one class, (the M_{α}-Ribonucleoprotein) did not pre-exist, and was, in fact, an artefactual rearrangement product, (Stevenin et al, 1979). Indeed, this rearrangement phenomenon may explain some of the variability exhibited by the protein components of hnRNP particles discussed previously. The M_{α}-Ribonucleoprotein had a simple protein profile, consisting predominantly of the hnRNP particle core polypeptides. The M_{β}-Ribonucleoprotein, however, contained both the core proteins and a number of additional, higher molecular weight, species. The possibility that the protein profile exhibited by the 40S hnRNP particles, isolated by different workers, represented the combination of various quantities of M_{α} and M_{β} particles, was raised. The simpler polypeptide profile, therefore, appears to be an index of the levels of ribonuclease present during the preparation of the particles.

In defence of the data presented in this thesis, however, it is important to point out one or two salient features of the protein complement of the hnRNP particles analysed herein. Firstly, the hnRNP particle protein profile of the liver particles reveals approximately 22 bands clearly visible on one dimensional gels (Fig. 7.) and in excess of 90 visible by two-dimensional analysis (Fig. 13.). These patterns are totally reproduc ible in every case. Secondly, the data presented in Fig. 12. concerning the non-specific binding of labelled protein to hnRNP particles during their isolation, confirms the possible generation of artefactual structures after ribonuclease treatment (Fig.12c). However, it is noteworthy that no such structures are generated during the normal isolation procedures routinely employed. Indeed, the isolation of significant levels of the rearrangement products described by Stevenin et al, required the use of ribonuclease levels many times higher than those that can be

reasonably assumed to be present *in vivo*.

On balance, therefore, it seems that the analysis of the isolated hnRNP particle is a reasonable model for the investigation of nuclear RNA metabolism, and may lead to a more comprehensive understanding of the functioning of the nucleus.

2. MODELS FOR THE STRUCTURE AND FUNCTION OF HnRNP PARTICLES.

A corollary to the acceptance of the isolated hnRNP particle as being representative of an *in vivo* structure, has been the presentation of a variety of structural models. The earliest model was put forward by Samarina *et al* (1968), and was based on their observation of a single polypeptide component of 40,000 molecular weight in 40S hnRNP particles. Approximately 40 copies of this single species of protein (informatin) were arranged as a core complex around which the hnRNA was wound, (See Introduction 4.4. ; Fig. 2α). In support of this model was the apparently high sensitivity of the RNA component to RNAses, and the isolation of RNA-free protein beads which could be isolated after high salt dissociation of these particles (Lukanidin *et al*, 1973). However, these data must be regarded with caution. The chemical iodination of hnRNP particles employed by Lukanidin *et al*, may result in artefactual crosslinking between proteins (Martin *et al*, 1977; T. Martin, personal communication). The possibility of artefactually created salt-stable structures can not, therefore, be ruled out.

A more systematic study of hnRNP particle structure has been carried out by the groups of Monique Jacob and Peter Heinrich. Stevenin and Jacob (1974), observed that the RNA component of hnRNP particles was in fact, not completely available to RNAse digestion. This data was interpreted as favouring the internal location for at least some

of the RNA molecule. This data has been extended by other workers (Augenlicht, 1979; Wahrmann & Augenlicht, 1979), and evidence for a degree of sequence specificity in the protected RNA obtained. It is likely, therefore, that the RNA interacts with the hnRNP core polypeptides in a non-random manner, and the two components are subsequently enfolded into the beaded structures which are characteristic of isolated hnRNP particles.

The use of proteinases as probes for the packaging of the proteins on hnRNP particles has been employed by Northemann and Heinrich (1979). A battery of three proteases mainly degraded three polypeptides, indicating an exterior location for these species. The ribonucleoprotein complex is assumed, therefore, to be ordered with respect to protein distribution as it is with respect to the location of RNA. The individual components which contribute to the structure and function of hnRNP particles will now be discussed.

2.1 The HnRNP Particle Core Proteins.

The core polypeptides are almost certainly the major structural components of the hnRNP particle. Their predominance with respect to the remaining proteins (e.g. Fig.13c), their universal occurrence (See Introduction:Section 4.2.2. and Fig.19), their basicity (Fig.13), the presence of high levels of glycine and the derivatised amino acid $N^G N^G$-dimethyl arginine (Lestourgeon et al, 1977) and their ability to bind RNA (Bowen et al, 1980) have all been cited as evidence for a structural role. Data presented in this thesis appears to favour the presence of several groups of polypeptides amongst the core protein population (See Results:Section 2). If this possibility is proved correct, the implications for the structural models proposable for the hnRNP particle are considerable. One may speculate, for example,

whether every species of polypeptide is present in every particle or whether a more limited subset might be represented. If the latter were the case, for instance, the presence in a particle of core protein A_1 rather than A_2 - A_5, would affect the conformation of the particle, and the fate of the hnRNA. Alternatively, it is conceivable that different subsets of proteins might associate with different types of nuclear RNA.

The *in vitro* phosphorylation (Section 2) of the core polypeptides suggests that phosphorylation may be the cause of some of the heterogeneity within each group. The further implications of such a modification are unclear. Obviously, this modification could modulate the interaction of this protein and the RNA to which it binds. The gross observation that the phosphoprotein components of hnRNP particles are more strongly bound to the RNA component than the non-phosphorylated species (Gallinaro-Metringe *et al*, 1975; Fuchs & Jacob, 1979), confirms this possibility. However, it has yet to be demonstrated that these core proteins are phosphorylated *in vivo*. Two-dimensional gel electrophoresis of *in vivo* ^{32}P-labelled (36hr.) hnRNP particle proteins suggests that this is not the case (Brunel & Lelay, 1979). In contrast, Karn *et al*, (1977) have identified phosphothreonine and phosphoserine in some of the core polypeptides after a labelling time of 45 minutes. The literature on this subject is thus inconclusive.*

While the position of the core polypeptides within the hnRNP particle is unknown, their close proximity, one to another, is revealed by their ability to become crosslinked in the presence of weak oxidising agents such as chloramine T (Lukanidin *et al*, 1972; Martin *et al*, 1977). The contribution of the core polypeptides to the structure of the hnRNP particle is probably considerable. Circular

* See Addendum p. 148–149

dichroism measurements (Northemann et al, 1978) show that the RNA component is constrained (i.e. packaged). The instability of "40S hnRNP particles" generated in vitro, by the simple mixing of hnRNA and hnRNP particle proteins, (Zawislak et al, 1974), suggests that the binding of core proteins to the RNA component is highly ordered. In this context it is interesting to note that in Drosophila Melanogaster embryo, the non-nucleolar transcripts appear to assume the typical ribonucleoprotein form in a non-random manner (Beyer et al, 1980). Specific foci for ribonucleoprotein particle formation may, therefore, be present, and, although the mediation of specificity of interaction could be performed by a minor protein component, it is conceivable that the ordered nature of the hnRNP particle could be a consequence of the specific interaction of the core polypeptides (or their insect equivalents) with these foci.

2.2 Minor Protein Components of the HnRNP Particle.

With the possible exception of the 73,000 molecular weight poly-(A)-binding protein, no single minor component of the proteins associated with hnRNP particles has been ascribed a function. However, they probably fulfil a number of functions. Some may be purely structural and may be involved in the attachement of the hnRNP particles to other sub-nuclear organelles (for example, chromatin or the nuclear matrix). Alternatively, they may perform or control the post-transcriptional processing events by which hnRNA matures into mRNA and leaves the nucleus. The identification of enzyme activities within the protein components of hnRNP particles has already been presented (Introduction: Section 4 and Results: Section 3) and will be discussed with respect to the present work in the next section.

3. ASSOCIATION OF ENZYMES WITH HnRNP PARTICLES.

A number of enzyme activities have been isolated in association with hnRNP particles; these are listed in Table 2 of the Introduction. The majority of these activities have been noted in preparations from abundant sources of hnRNP particles (e.g. suspension-culture HeLa cells or rat liver). With regard to the enzymes potentially involved in the metabolism of hnRNA, it is not difficult to draw the conclusion that their association with hnRNP particles is of functional significance.

In at least two cases enzymes have been shown to be distributed between both 40S hnRNP particles, and the non-particulate fraction. These enzymes, the capping enzymes (Bajszar et al, 1978) and poly-(A) polymerase (this thesis:Fig. 26), are obvious candidates for hnRNA processing enzymes. Their distribution between hnRNP particles (their site of action) and the non-particulate region (their probable site of storage) could thus be regarded as a genuine functional distribution. An alternative possibility, and one that should be seriously considered with all of the enzymes isolated from 40S hnRNP particles, is that of cross-contamination. The possible isolation of material from the non-particulate region in fractions of the gradient presumed to contain only hnRNP particles, is a potential source of such contamination and stringent control of this factor should be an important facet of the investigation of the distribution of these enzymes. Indeed, results presented in this thesis (Fig.26b) initially indicated the presence of a poly-(A) polymerase confined to the particulate portion of liver nuclear extracts. Further experiments revealed, however, that activity was present throughout a sucrose density gradient on which the extract was fractionated. Clearly, reservations must be expressed about the specific association of poly-(A) polymerase with rat liver hnRNP.

With the possible exception of the poly-(A) polymerase and capping enzymes, the enzymes associated with hnRNP particles are particularly difficult to assess with respect to their role in RNA processing. The discovery of various general, and double-stranded RNA specific, RNAses requires further characterisation, particularly with respect to site specificity, before any significance can be attached to their presence. It may be that other factors also require consideration; the contribution of the structural proteins to the site specificity of these enzymes, for example, remains to be assessed.

A similar difficulty is faced in the case of the protein kinase and phosphotase activities, which may regulate some presently uncharacterised functions of the hnRNP particle. However, the potential importance of these enzymes in metabolic regulation may, by their investigation, shed some light on the processes of hnRNA maturation.

3.1 Nuclear Protein Kinases.

Evidence that metabolism can be regulated in eukaryotic cells by protein phosphorylation has been accumulating over recent years (for review, see Ruben & Rosen, 1975). Thus glycogen synthesis, glycogenolysis and lipolysis are, to some extent, controlled by protein kinase mediated phosphorylation of one, (or more), enzyme(s) in each pathway (Walsh et al, 1968; Sodeling et al, 1970). Protein kinase activities have also been found in nuclear extracts (Takeda et al, 1971; Kimayama et al, 1971; Desjardins et al, 1972; Kish & Kleinsmith, 1974). These nuclear enzymes exhibit a variety of substrate and co-factor requirements, and one study (Kish & Kleinsmith, 1974) could distinguish 12 distinct kinase activities by phosphocellulose chromatography alone.

There is good evidence that many non-histone chromosomal proteins are phosphorylated, and that fluctuations in this phosphorylation

can be correlated with, for example, cell cycle stage (Platz et al, 1973), liver regeneration (Chiu et al, 1975) and hepatocarcinogenesis (Chiu et al, 1973). Similar fluctuation in nuclear kinase activity have also been noted (Thompson et al, 1975; Costa et al, 1977; Phillips et al, 1979). If it is accepted that it is the fluctuation in the kinase activities which is responsible for the altered level of phosphoproteins, it follows that these kinase activities have important regulatory roles in gene expression.

So far, no function has been ascribed to hnRNP-associated protein kinase. The HeLa cell hnRNP particle kinase has been partially purified (Periasamy et al, 1979) and bears a strong resemblance to protein kinase NKI, previously isolated from rat liver nuclei (Thornburg et al, 1977). It should be noted, however, that, with the possible exception of their substrate specificity, the HeLa cell hnRNP particle kinase and that described in this thesis, from rat liver hnRNP particles, share few properties in common. The cyclic AMP dependence of this rat liver enzyme (Figs. 33b and Table 5.) has not been noted in any other hnRNP particle-associated kinase. However, in the present work, the stimulation of activity by both cyclic AMP and polyamines is reproducible and preserved throughout detergent treatment of nuclei, purification of particles through successive sucrose gradients, or through a sucrose gradient followed by gel exclusion chromatography. The rat liver enzyme also differs from the HeLa cell enzyme in its pH optimum, its ionic requirements and its stability (Blanchard et al, 1977; Fig. 30).

Although it differs strikingly from the HeLa enzyme and from nuclear kinase I of rat liver (Thornburg et al, 1977), it does show some properties in common with an enzyme, isolated from mouse spleen cell nuclei (Ohtsuki et al, 1980) by a method which could be

expected to extract proteins previously associated with hnRNP particles (i.e. sonication of the isolated nuclei in 20mM Tris. HCl. pH 7·5 containing 2mM DTT, 0·5M KCl and 1·5mM MgAcetate). This enzyme, designated Enzyme I was cyclic AMP-dependent, required Mg^{++} ions or Mn^{++} ions for activity, and was capable of phosphorylating non-histone protein as well as histones.

A second previously characterised enzyme to which the rat liver hnRNP particle kinase shows some similarity, is the cyclic AMP-dependent "Histone-specific" nucleoplasmic protein kinase (Neumann et al, 1978) isolated by low salt extraction of purified rat liver nuclei. This enzyme is cyclic AMP-dependent, requires Mg^{++} ions (although Mn^{++} ions will substitute to some degree) and has a pH optimum of 6·5 with histone H_1 as a substrate. The cyclic GMP and Fe^{++} ion stimulation demonstrated with this enzyme was not, however, shown by the hnRNP particle kinase. Until the hnRNP particle kinase is purified, its exact nature will remain uncertain and this should be the main thrust of continuing work in this field.

3.2 The Significance of the in vitro Phosphorylation of HnRNP Particle Proteins.

Several observations regarding the in vivo phosphorylation of hnRNP particle proteins makes the phenomenon of their in vitro phosphorylation particularly interesting. Firstly, the observation that phosphorylated proteins were more tightly bound to RNA in the presence of high concentrations of NaCl (Gallinaro-Metringe et al, 1975), suggested that phosphorylation may dictate the degree of RNA-protein interaction within the particles. Secondly, the phosphorylation of a 35,000 molecular weight nuclear protein in Chinese hamster ovary (CHO) cells, markedly decreases after heat shock at 43°C. Since heat shock in CHO cells results in the cessation of

mRNA transport into the cytoplasm, the gradual recovery, in parallel, of both the mRNA transport and the phosphorylation of this species after release from heat shock suggests that these two phenomena are related (Caizergues-Ferrer et al, 1980). Thus, phosphorylation may play an important part in mRNA transport to the cytoplasm. In this context it is of interest to note that the exit of mRNA sequences from the nucleus may be ATP-dependent, (Ishikawa et al, 1969; Raskas, 1971).

The present state of knowledge regarding the kinase activities presumed to be in association with hnRNP particles precludes any specific statement as to their mode of action. However, it is surprising that so many of the protein components are phosphorylated. It is conceptually difficult to visualise how a particle-bound enzyme manages to phosphorylate so many other particle-bound acceptor molecules, given the currently accepted model of hnRNP particle structure. There are, however, several possible mechanisms by which this can occur; Firstly, the kinase may be rather loosely bound to the particle and simply pick its way around the complex interacting with its acceptor molecules as it proceeds through the nucleus. The partial loss of kinase activity from the particle after recentrifugation (Fig. 27) or gel exclusion chromatography (Fig. 29) may be a reflection of this phenomenon. However, the specific activity of the kinase (pMol. P. mg^{-1} protein) remains similar throughout the treatment, and it seems likely that the loss of kinase activity is simply one aspect of the general instability of the hnRNP particle in vitro.

An alternative model for the mechanism of action of the particle bound kinase is based on a more flexible arrangement of the particle components. Here, the phosphorylation would be an interaction between particles. The kinase enzyme residing on a given particle may, therefore, phosphorylate acceptor molecules on other particles. This is perhaps the model most pertinent to a possible role in hnRNA maturation. Spirin's

model for cytoplasmic mRNP particles (omnia mea mecum porto) in which he suggests that the mRNA molecule at different stages of its lifetime carries on itself the proteins which are required for its own biogenesis, processing and transport, and for its functioning as a template, may thus hold for hnRNP particles.

By whatever mechanism the phosphorylation occurs there is a precedent in the pyruvate dehydrogenase complex, in which pyruvate dehydrogenase is itself phosphorylated and its activity modulated by a particle-bound kinase (for review see Denton et al, 1975).

The function of the kinase activity, its distribution amongst the hnRNP particle populations and its mechanism of action remains unknown. However, with the current emphasis of research on the delineating of the mechanisms of hnRNA maturation, it may be expected that answers to these questions will be vigorously pursued.

Addendum.

Critical appraisal of the methods available for the phosphorylation of hnRNP particles in vivo

This project is solely concerned with the *in vitro* phosphorylation of hnRNP particles from rat liver nuclei. Considerable advantage would have been gained in being able to compare the *in vitro* results with those obtainable *in vivo*. Indeed, several such *in vivo* studies have been performed. Brunel and Lelay (1979) have been able to label hnRNP particles from HeLa cells using 5mCi per litre of culture and 36 hours labelling time. Cell culture techniques, however, have the profound advantage of coupling the use of relatively low levels of radiolabel with the facility of extending the labelling period, thereby labelling proteins whose phosphorylation rate is slow. Animal studies, on the other hand, are complicated by several factors. Firstly, administration of the label to the required tissue (in my case, the liver) is via the blood stream. Radiolabel, therefore, becomes spread throughout the animal, effectively diluting the dose of radiolabel reaching the 'target' tissue. Secondly, handling and management of such experimental animals becomes difficult and even dangerous. Animal experiments are thus less attractive propositions, although clearly not impossible.

One such experiment, performed in order to study the *in vivo* phosphorylation of hnRNP particles, exemplifies the limitations of the *in vivo* approach, as applied to animals. Karn *et al* (1977) employed 10mCi per animal (no. of animals unspecified) and labelled for 45 minutes. Examination of the phosphoprotein products of this experiment by SDS gel electrophoresis suggests that only a low level of ^{32}P is incorporated into hnRNP particles under the conditions employed. In the study described in this thesis an attempt was made to reproduce these results. Due to the (understandable) reluctance of the members of staff responsible for radiation safety to permit such high levels (10mCi per animal) of radioactivity to be used in the available laboratories,

and in the absence of appropriate specialised equipment, the experiment performed was limited to 2mCi per animal (5 animals). The subsequent failure of this experiment indicated that however desirable such experiments were, they could not hope to be done without specialised apparatus, which was at that time, unavailable.

A number of alternative strategies were considered. The use of chopped liver cube cultures was abandoned after what appeared to be signs of necrosis in all but the outer layers of cells. Liver perfusion techniques were also considered. Difficulties in obtaining appropriate apparatus and technical instruction prevented what appeared to be a good idea being carried further, however.

Aside from the technical difficulties of employing whole animals in hnRNP particle protein labelling experiments, there appears to be a discrepency between the results obtained in cell culture and with whole animals (See p. 141). These differences may be due to species differences (Human v Rat) or differences in the labelling time (36 hr. v 45min.).

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