ENDOGENEOUS KINASE ACTIVITY OF HETEROGENEOUS RIBONUCLEOPROTEIN PARTICLES

by

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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^1-methylene bisacrylamide
cpm	counts per minute
cyclic AMP	Adenosine 3'-5' monophosphate
cyclic GMP	Guanosine 3'-5' monophosphate
CM Cellulose	Carboxymethyl Cellulose
DEAE Cellulose	Diethylaminoethyl Cellulose
DNase	Deoxyribonuclease
EDTA	Ethylene diamine tetra acetate
Hepes	N-2-hydroxethylpiperazine N-2-ethane-sulphonic acid
HnRNA	Heterogeneous nuclear ribonucleic acid
HnRNP	Heterogeneous nuclear ribonucleoprotein
IEF	Isoelectric focusing
MES	2 [N-Morpholino] ethanesulphonic acid
MOPS	3 [N-Morpholino] propanesulphonic acid
NEPHGE	Non-equilibrated pH gradient electrophoresis
OD	Optical density
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	NNN'N' tetramethyl ethylenediamine

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SUMMARY

The aim of this work was to investigate a protein kinase activity found associated with hnRNP particles from rat liver nuclei. The location of this enzyme activity coupled with the known role of protein kinases in controlling other cellular events has led to the suggestion that an hnRNP associated kinase might influence the maturation of hnRNA to mRNA.

HnRNP particles were isolated from purified rat liver nuclei by the method of Samarina et al., (1968) and further purified by centrifugation on 15-30% w/v sucrose density gradients.

When analysed on a 2-dimensional fractionation system, which employed non-equilibrated pH gradients in the first dimension and SDS polyacrylamide gels in the second, hnRNP particles exhibited a heterogeneous protein profile dominated by the core proteins which appeared as chains of spots showing charge heterogeneity. Isolated hnRNP particles exhibited an endogenous protein kinase activity which was capable of phosphorylating added casein or histone but also phosphorylated hnRNP particle proteins. When the proteins which had been subjected to in vitro phosphorylation were separated on 2-dimensional gels it was seen that the more acidic species in the chains of core proteins were phosphorylated.

Evidence/

Evidence for the association of the protein kinase with 40S hnRNP particles came from the fact that a peak of kinase activity coincided with the 40S peak of hnRNP from sucrose density gradients and was still associated with the particles when hnRNP were isolated from one gradient and rerun on a second sucrose gradient. Kinase activity was still found associated with the particles even after gel filtration on a Bio Gel column although losses of kinase activity suggest that the enzyme is only loosely bound. A band of kinase activity corresponding to the stained hnRNP band was detected on non-denaturing polyacrylamide gels.

The fractionation of hnRNP particle proteins to yield reasonable quantities of purified proteins is not easy as the proteins have strong affinities for each other. HnRNP particles could be completely dissociated with high salt concentrations. However dissociation was partially reversible and removal of salt led to reaggregation of a substantial percentage of the proteins.

In order to try to fractionate hnRNP particle proteins, the particles were treated with 1M NaCl before being loaded on to a Sephadex G100 column in the presence of 1M NaCl. When the fractions from the column were analysed for protein kinase, 2 peaks of activity were seen - 'A' and 'B'. Both peaks contained a different set of polypeptides as seen on SDS polyacrylamide gels, and although 'A' and 'B' had similar pH, Mg^{2+}, time and temperature profiles their response to Mn^{2+} and their substrate specificity appeared to differ.

INTRODUCTION

INTRODUCTION

The aim of the work described in this thesis was to investigate, in greater detail, a protein kinase activity found associated with nuclear hnRNP particles. The location of this enzyme activity, coupled with the known role of protein kinases in controlling other cellular events, has led to the suggestion that a hnRNP-associated kinase might influence the maturation of hnRNA to mRNA. The introduction to this thesis therefore attempts to revise what is known of mRNA maturation as well as the structure and possible role of ribonucleoprotein in the maturation pathway. It also briefly reviews known examples of protein kinase involvement in the control of cellular processes.

1. Nuclear RNA

RNA in eukaryotic cells is present in a number of different classes which can be distinguished on the basis of physical and chemical properties. The majority of the RNA is functionally expressed in the cytoplasm, i.e. the rRNA and tRNA which play important roles in the translational apparatus, and the mRNA template which is used to synthesise the protein chain. However, 5% of the RNA exists in the nucleus and includes precursors of the cytoplasmic species.

1.1 HnRNA

The idea that the primary transcripts are different from the mature polysomal mRNA emerged in the early 1960's due in part to the observation that the majority of the radioactivity, after pulse labelling of RNA in mammalian cell cultures, remained in the nucleus and was never transferred to the cytoplasm (Georgiev et al, 1963). Early analysis of this nuclear fraction revealed a heterogeneous sedimentation profile and the RNA, which became known as heterogeneous nuclear RNA (hnRNA), included molecules very much larger than the cytoplasmic polysomal mRNA (Weinberg, 1973). In spite of the size difference, hnRNA had many characteristics similar to mRNA and distinct from pre-rRNA. These included a mRNA-like base composition (Scherrer et al, 1963), a polyadenylated 3' terminus (Adesnik et al, 1972), internal methylation and 5' capping (Wei & Moss, 1977). All of these similarities gave rise to the idea that the hnRNA was a mRNA precursor, and potential pre-mRNA species were detected in hnRNA (Imaizumi et al, 1973).

The greatest/

The greatest problem with the precursor-product hypothesis, however, was the several fold difference in size between the 2 classes of molecules. Various models put forward to account for this paradox included the possibility that an hnRNA might include more than one mRNA sequence or that the hnRNA might be cleaved and the non-coding sequences digested. The former solution did not account for the fact that there is no evidence for polycistronic mRNA in eukaryotes. The second model was difficult to reconcile with the apparent conservation of both the 5' and 3' end of hnRNA in the mature mRNA.

It was the discovery of introns, noncoding regions of DNA that interupt the coding portions of the genes, that solved the problem of how long hnRNA molecules could encode mRNA with the same ends. Introns were first discovered in the gene for rabbit β-globin when the techniques of restriction mapping and R-loop mapping showed that the gene contained at least one long sequence that was not present in the mature mRNA (Tilghman et al, 1978).

This phenomenon of non-colinearity between the gene and its transcription product now seems to be a common one in eukaryotes, as introns, have been detected in numerous systems. Some of the first of these were the mouse β globin gene (Tilghman et al, 1978), chick ovalbumin gene (Mandel et al, 1978), human δ and β globin genes (Lawn et al, 1978), insulin genes (Lomendico et al, 1979), yeast tRNA genes (Knapp, 1978), immunoglobulin light chain genes (Cochet et al, 1979), and avian globin genes (Imaizumi et al, 1973). There seem/

seem to be no general rules concerning the sizes and the number of introns in different genes. They vary from no introns in most histone genes (Schaffner et al., 1976) and the gene for polypeptide IX in Adenovirus 2 (Alestrom et al., 1980) to about 50 introns in the collagen gene (Wozney et al., 1981).

The discovery of introns led to speculation that they were transcribed into pre-mRNA species and were removed during mRNA maturation. That this was the case was first demonstrated when Tilghman et al., (1978) followed up their discovery of the intron in rabbit β globin, with the demonstration that a 15S globin mRNA precursor contained a transcript of the intron. This was followed by the demonstration by Tsai et al., (1980) that the largest pre-mRNA for avian ovomucoid contained transcripts of all seven of the gene introns and that these were removed in a preferred but not necessarily obligatory order. The sequential maturation of an initial transcript into mature mRNA by the stepwise removal of intron nucleotides has also been demonstrated with precursors of ovalbumin mRNA (Tsai et al, 1980), globin mRNA (Kinniburgh & Ross, 1979) and the precursor of many other genes.

1.2 SnRNA

By 1970 it was known that low molecular weight RNA species occur in the nucleus. These include tRNA and 5.8S rRNA destined for transport to the cytoplasm, and a group of RNA species collectively known as small nuclear RNA (snRNA) found only in the nucleus (Frederiksen et al., 1981). These snRNA species have been designated 'U'RNA due to/

to their high content of uridylic acid and can be separated according to size into 6 major species (U1-U6) some of which have structural variants (Table 1). Of these U3 and its variants occur only in the nucleolus (Zieve & Penman, 1976), while others are nucleoplasmic in origin. They contain 90-220 bases (Lerner et al., 1980), are metabolically stable (Weinberg, 1973; Frederiksen et al., 1974), range in size from 4-8S (Weinberg, 1973; Lerner & Steitz, 1979) and are notable for their modified nucleotides which include a 'cap-like' 2,2,7-trimethylguanine at the 5' end (Reddy et al., 1979; Epstein et al., 1980), together with internal methylated residues and pseudouridine (Wise & Weiner, 1981).

Many of these snRNA molecules have been sequenced (Sri-Widada et al., 1981) including U2 (Reddy et al., 1981) and U3 from hepatoma cells (Reddy et al., 1979), U1 from Drosophila (Mount and Steitz, 1981) and rat brain (Gallinaro et al., 1981), U6 from hepatoma cells (Epstein et al., 1980) and some of their secondary structures have also been investigated in chicken, rat and human tissues (Branlant et al., 1981; Krol & Branlant, 1981). The sequence of the snRNA's are highly conserved between species, i.e. in Drosophila, U1 shows a 72% agreement with human U1 (Mount & Steitz, 1981). They are structurally similar in lower eukaryotes (i.e. slime moulds) and rats (Takeishi & Kaneda, 1981; Wise & Weiner, 1981) and therefore appear to be functionally analogous. U1, U2, U4, U5 and U6 also each contain their own distinctive structural features that have been conserved in the evolution of eukaryotes (Myslinski et al., 1984).

Although/

RNA SPECIES	NUMBER OF SUBSPECIES	NUMBER OF NUCLEOTIDES	ABUNDANCE (COPIES/CELL)	SUBCELLULAR LOCATION	NATURE OF 5' TERMINUS	ANTIGENIC AFFINITY
U1 or D	2	165	1×10^6	Nucleoplasm hnRNP		RNP Sm
U2 or C		188-189	5×10^5	Nucleoplasm hnRNP		
U3 or A	3	210-214	3×10^5	Nucleolus	2'2' dimethyl	
U4 or F		142-146	1×10^5	Nucleoplasm	7 methyl guanosine	Sm
U5 or G^1	2	116-118	2×10^5	Nucleoplasm		
U6 or H		107-108	3×10^5	Perichromatin granules		
7S or L		280	5×10^5	Nucleus and Polysomes		

TABLE 1 : CHARACTERISATION OF snRNAs

Although earlier studies concluded that U snRNA's of different tissues were similar, Krol et al, (1981), using chicken, rat and human snRNA's, have shown that there may be minor differences in the sequences of snRNA's of different tissues. Forbes et al, (1984) have shown that there are 7 species of U1 in amphibian cells which are expressed at different stages of development.

The first indication that snRNA molecules might be involved in the processing of high molecular weight RNA in the nucleus came from Prestayko et al, (1970) who found U3 hydrogen bonded to the precursors of 28S rRNA but not to mature sized rRNA. Sass & Pederson (1984) have shown that the amount of snRNA's increases as genes are activated and U4 has been implicated in polyadenylation. However, it is the possible involvement of U1 snRNA in pre-mRNA maturation which has attracted most attention. This role is considered in Section 2.1.

SnRNA has frequently been detected in ribonucleoprotein particles isolated from cell nuclei. These particles (snRNP) have a density of 1.43g/cm^3 in $CsCl_2$ and a sedimentation coefficient of 11-12S (Liautard et al, 1981; Sri-Widada et al, 1981; Brunel et al, 1981). 'U' series of snRNA were first isolated in particulate form after fractionation of nuclear extracts by gel filtration and on sucrose density gradients. Brunel et al, (1981) isolated purified snRNP's on $CsCl_2$ gradients containing 0.5% sarkosyl (Lauryl sarcosine - an anionic detergent). The purified U1 snRNP from He La cells contained 4-5 polypeptides with molecular weights of 10000-14000 (Brunel et al, 1981: Sri-Widada et al, 1982) but these must be regarded /...

regarded as the minimum components rather than the complete protein complement due to the drastic and severely denaturing purification procedure.

Lerner & Steitz (1979) have demonstrated that some patients with systemic erythromatosis, an autoimmune rheumatic disease, produce antibodies which specifically complex with snRNP particles.

Immunoprecipitates of snRNP particles have been shown to contain polypeptides ranging in molecular weight from 12000 to 35000. These polypeptides are unrelated to the proteins associated with hnRNA or to histones. However, U3, which is confined to the nucleolus is not present in these snRNP particles.

Lerner & Steitz (1979) have suggested that different subsets of particles vary in their snRNA composition but contain the same 7 protein components, i.e. each snRNA exists as a separate particle with an indistinguishable set of 7 polypeptides. Lerner & Steitz (1981) present evidence to show that the U1 antigenic determinant is conserved from insects to man since the human antibody is capable of precipitating U1 containing snRNP's from every intermediate species examined.

The antibodies against snRNP particles can be divided into 2 groups:- anti-Sm and anti RNP antibodies. Anti-Sm antibodies recognise antigens on U1, U2, U4, U5 and U6 containing particles, while anti-RNP antibodies recognise antigens which are specific to U1 snRNP (Lerner et al., 1981; Lerner & Steitz, 1981; Lenk et al., 1982; Liautard et al., 1982). Anti-Sm antibodies recognise U1 snRNP's at a/

at a site physically distinct from the determinant bound by anti-U1 RNP (Lerner & Steitz, 1981). Takano et al., 1981) have purified RNP and Sm antigens from calf thymus and shown that purified RNP antigens exhibit Sm activity. However, RNase treated RNP was immunologically inactive as a RNP antigen but still maintained Sm activity. They have shown that Sm and RNP antigens have the same 8 polypeptide components but the Sm antigen devoid of RNP activity only has 4 polypeptides of molecular weight 12000-13000, thus showning that RNP and Sm are distinct determinant sites on the same nuclear complex. This agrees with the results of Assens et al., 1982) who found that in HeLa cells the Sm antigenic determinants were among 4-5 proteins of molecular weight 9000-14000 which are tightly associated with snRNA's.

The RNA from the RNP-Sm complex is required only for RNP and not for Sm antigenicity although the role played by the RNA in specifying RNP antigenicity in conjunction with the protein is not known (Takano et al., 1981)

Expansion of work in this field has not led to a consensus of opinion on the number or molecular weight of proteins in the particles. Possible causes for these variations have been reviewed by MacGillivray et al., (1982) and include:-

1. Differences in methods of detection, e.g.
   a) gradient centrifugation (Assens et al., 1983)
   b) ion exchange, hydrophobic or ligand specific chromatography (Hinterberger et al., 1983)
   c) /

c) immunoaffinity chromatography (Bringman et al, 1983)

2. Differences in antisera used by different groups. A protein may be detected in one analysis and missed in another due to the partial sharing of antigenic sites and/or variations in antisera.

3. Tissue source or treatment: Although Lerner & Steitz (1981) have presented evidence for conservation across species of snRNP particle proteins, there is some evidence for tissue and species specificity (Hinterberger et al, 1983).

The purification of snRNP's is complicated because there is no assay for them other than their immunoreactivity. As snRNP's are multicomponent assemblies, held together by noncovalent interactions, antibody recognition of any component for the particle results in the immunoprecipitation of the entire particle.

There is now a general consensus that there is a common core in snRNP composed of polypeptides D (13,000), E (11,000), F (10,000) and G (8,000). In addition there are polypeptides uniquely associated with particular RNA species although there is not universal agreement as to their number or size (Hinterberger et al, 1983; Kinlaw et al, 1983; Billings et al, 1984; Lin & Pederson, 1984; Mattaj et al, 1985).

Liautard et al, (1982) have looked at protein-RNA interactions, in snRNP particles from HeLa cells, using nuclease digestion and have found that the more strongly bound proteins of molecular weight (D, E, F, G) 9000-14000, are associated with single stranded regions of/

of 23-35 nucleotides which are present in U1, U2, U4 and U5. This single stranded region includes the sequence A-$(U)_n$-G bordered by double stranded stems and is located near the 3' end of the snRNA.

Using deletion/substitution mutants (in cloned Xenopus U2 snRNA genes) Mattaj et al., (1985) have shown that a 12 nucleotide region containing sequences conserved in other UsnRNA's (Branlant et al., 1982) is essential for interaction with proteins recognised by anti-Sm antibodies. Xenopus oocyte snRNP proteins have the capacity to assemble snRNA from Drosophila and mouse into snRNP suggesting that the snRNP proteins recognise conserved secondary structures (Forbes et al., 1983).

U6 snRNA has no 2,2,7-trimethylguanosine cap (Branlant et al., 1982) but when snRNP's are fractionated on affinity columns prepared with an antibody for this cap structure (Anti m_3G), U6 is bound and co-chromatographs with U4 (Bringman et al., 1984). This suggests that either discrete U4 and U6 snRNP particles are associated or that they are organised in one RNP particle. Bringman et al., (1984) suggest that U4 and U6 are associated by base-pairing as U6 co-precipitates with U4 using anti m_3G following phenolisation at 0°C but at 65°C they dissociate. Hashimoto & Steitz (1984) have shown that U4 and U6 possess extensive intermolecular sequence complementarity and therefore have the potential to base pair.

Until recently no specific enzyme activities had been found associated with snRNP particles. This has been due to the methods used/

used in preparation which lead to protein denaturation. However, Hinterberger et al., (1983) have devised a method to prepare purified snRNP's which should allow analysis of enzyme activities associated with snRNP's as it involves a combination of ion-exchange and hydrophobic ion-exchange chromatography which does not denature the proteins. Bachman et al., (1984) have found an acidic endonuclease associated with 12S RNP from calf thymus and L5178y cells. U1 snRNA has been identified as a component of these 12S RNP. This endonuclease degrades poly (U) and poly (C) but is inhibited by poly (A). It is distinguished from pancreatic ribonuclease and endoribonuclease VII by its resistance to thiol reagents, inhibition by EDTA, Mg^{2+} requirement, pI (4.1) and pH optimum (pH 6.2).

Although there is experimental evidence for the role of snRNA's in processing of hnRNA (Lerner et al., 1980) their exact role is still conjectural. The question still to be answered is whether snRNA's are active as naked molecules or as protein complexes. If snRNP particles actually represent the natural state of snRNA's it should be possible to separate them from structures containing hnRNA. Experimental evidence seems to show that snRNP particles purified from hnRNP particles, and snRNP particles purified using antibodies, have the same RNA and protein compositions (Liautard et al., 1981; Sri-Widada et al., 1982).

U5 RNP's associated with hnRNP were found to be completely protected when these complexes were digested extensively by micrococcal/

micrococcal nuclease (Sri-Widada et al, 1983), suggesting that the U5 RNP is localised inside the hnRNP complex and may play a role in the packaging of hnRNP. U2 is also further protected by hnRNP suggesting that one third of U2 is localised inside hnRNP. The other fragments of UsnRNA's protected in hnRNP correspond to those protected in core snRNP.

Seytono & Pederson (1984) have investigated intermolecular base pairing in hnRNA and U1 in HeLa cells by psoralen cross linking in situ. Results indicate that when U1 base pairs with hnRNA it retains its RNP structure but this doesn't rule out the possiblity that the RNP structure or the conformation of the U1 RNP may change.

2. Processing of Eukaryotic Nuclear Pre-mRNA

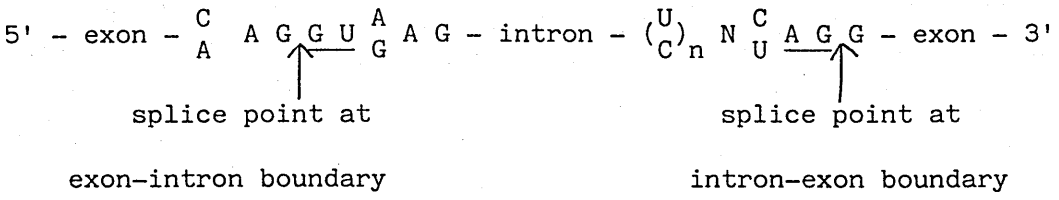
Processing of pre-mRNA occurs within the nucleus and involves cutting out the introns and joining the ends of the coding sequences (splicing), capping of the 5' end, polyadenylation of the 3' end, and internal modifications of the nucleotides (Fig. 1).

2.1 Splicing

Splicing is the process by which the noncoding parts of the RNA (introns) are removed,and the coding regions brought together and ligated. Although introns differ considerably in length and sequence, even for the same gene in different species, there is remarkable conservation of regions flanking the coding sequences.

Mount (1982) has catalogued the sequences of 139 exon-intron boundaries, and 130 intron-exon boundaries, and has derived a consensus/

consensus sequence which gives rise to the following transcript:-



The sequence immediately to the 5' side of the intron-exon boundary is always pyrimidine rich and free of the dinucleotide AG. The most invariant aspect of the consensus sequence is the GU at the beginning of the intron transcript and the AG at its end. Sequences similar to the consensus sequence occur throughout the gene but only those at known intron-exon boundaries are apparently recognised as splice points; therefore there must be additonal components which are involved in the specificity of splicing (Woo et al., 1981).

The role of the consensus sequence in splicing has been demonstrated in many systems and is particularly well exemplified in the β-thalassaemias, a group of hereditary anaemias. Several examples of these are known in which lack of β-globin production is due to a mutation in the DNA encoding the splice points of the large intron of the gene (Baird et al., 1981; Treisman et al., 1982).

In another example, a single G → A mutation created the sequence CTATTAG within the intron which closely resembled the intron-exon boundary sequence CCGTTAG and competed with it as a splice point (Busslinger et al., 1981).

A possible mechanism of splicing was independently suggested by Lerner et al., (1980) and Rogers & Wall (1980). They noticed that the 5' end of U1 snRNA showed extensive complementarity to the consensus sequence/

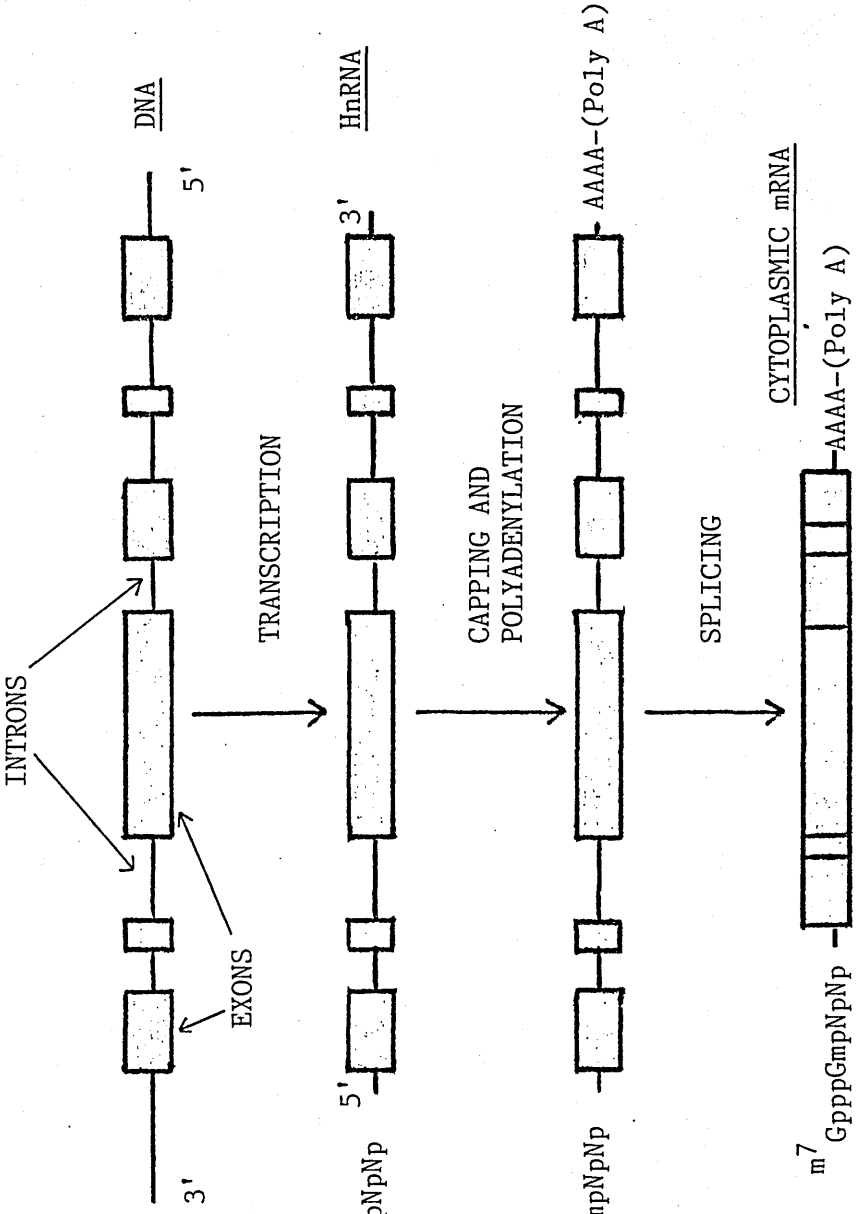


FIGURE 1: Proposed model for production of mRNA from DNA containing intervening sequences (introns).

sequence of the splice points (Lerner et al., 1980; Mount & Steitz, 1981) and suggested that the snRNA could base-pair with both ends of the intron causing the remainder of the intron to loop out and bringing the 2 exons into alignment for ligation. Figure 2A illustrates the proposed model of splicing and Figure 2B shows a variant model (Ohshima et al., 1981) conceived as taking the secondary structure of U1 snRNA into account.

A number of findings have supported the model of Figure 2 and implied that snRNP particles played a crucial role in the maturation of hnRNA. SnRNP particles were shown to associate with hnRNP, but when the 5' end of U1 was removed, the particles containing U1 no longer associated with the hnRNP (Lerner et al., 1980). Splicing was inhibited when antibodies against U1 snRNP's were added (Yang et al., 1981; Lenk et al., 1981) and Mount et al., (1983) have demonstrated that U1 snRNP's, but not protein free U1, bound to DNA transcripts which contained sequences corresponding to the small intron and flanking exons of the mouse β globin gene. In doing so, they protected a 15-17 nucleotide region containing the 5' splice site.

Extension of these results had to await the development of in vitro splicing systems (Goldenberg and Raskas, 1981) which demonstrated that U1 snRNA plus the proteins carrying the anti-Sm determinants copurified with splicing activity (Di Maria et al., 1985) and that removal of 5' sequence of U1 (Kramer et al., 1984) or the addition of anti-Sm or RNP antibodies (Bossoni et al., 1984; Konarska et al., 1985) inhibited the in vitro splicing system.

Recent/

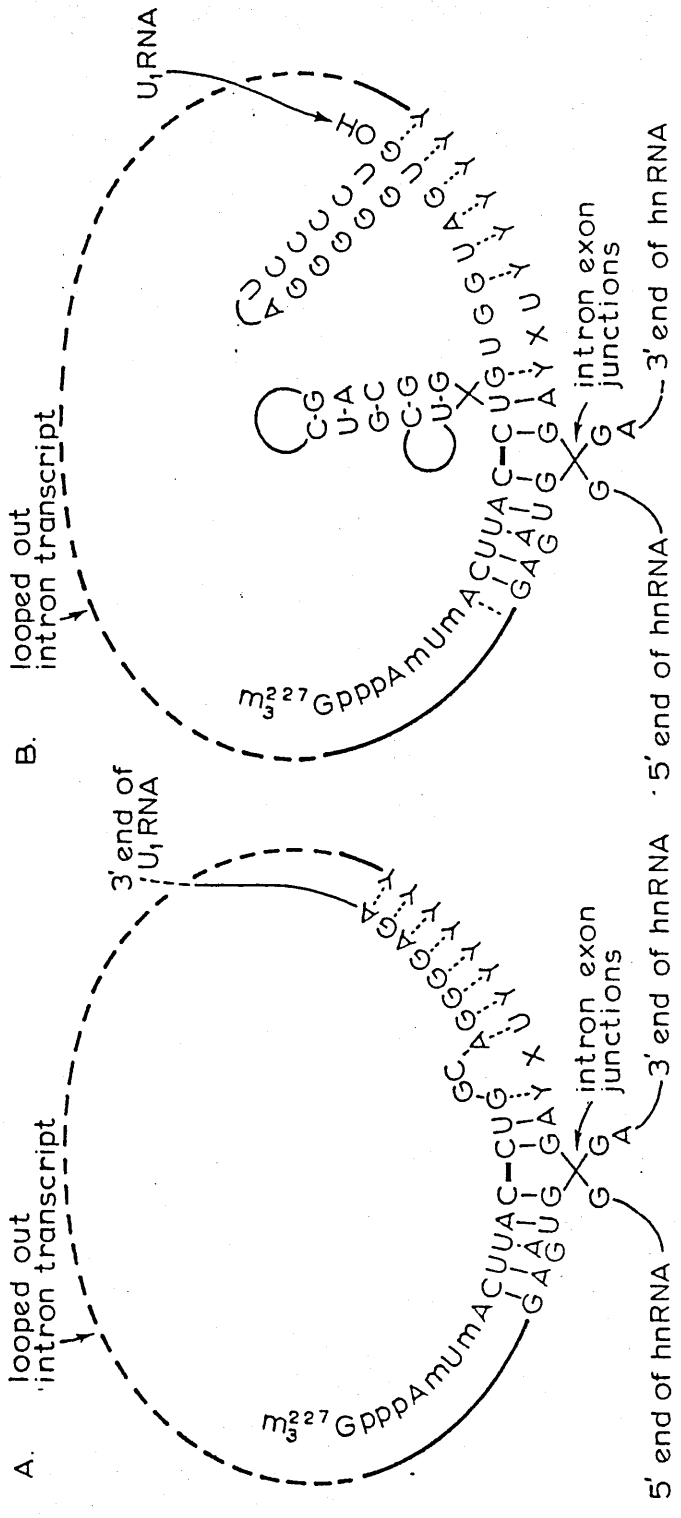


FIGURE 2: Possible base-pairing between the intron-exon junction of hnRNA and U1 snRNA. The models are based on those of Lerner et al (80) and Ohshima et al (81). (Y indicates pyrimidines, X is a variable base.)

Recent experiments have suggested that hnRNP proteins may bind preferentially to intron-exon junctions which are the presumed sites of U1 RNP binding (Mayrand & Pederson, 1983). Splicing may be initiated by hnRNP proteins aligning or folding the splice sites, then U1RNP might bind causing the displacement of the hnRNP proteins. This idea is compatible with experiments indicating that hnRNP particles are more nuclease sensitive after mRNA splicing (Pederson & Munroe, 1981).

It is of importance for future investigation of splicing to identify intermediates, and acceptance that an RNA species is an intermediate requires that the structure is consistent with the established pathway. The recent development of *in vitro* splicing systems has allowed the detection of proposed intermediates. The accumulation of excised introns (Grabowski *et al*., 1984) proves that splicing is not mediated by processive degradation of the intron but by cleavage at the 2 splice sites plus ligation of the exons. The excised introns plus the intermediate RNA species have the unusual configuration of a circle with a tail containing a branch point and are referred to as "Lariats" (Konarska *et al*., 1985; Grabowski *et al*., 1984; Keller, 1984; Zeitlin & Efstratiadis, 1984). In the Lariat, the 5' terminal guanosine of the intron is attached to an internal adenine residue by a 2'-5' phosphodiester bond (Grabowski *et al*., 1984). If biologically significant these branched structures should be detected *in vivo*. Wallace & Edmonds (1983) have observed that HeLa cell nuclear RNA contained branched/

branched nucleotides. These findings have led to a modification of the previous model for splicing (Figure 3).

This new model, based on the in vitro splicing of human β globin pre-mRNA and the Adenovirus system, starts with the formation of a lariat which is stabilized by base-pairing, followed by the formation of a 2'-5' pyrophosphate branch point (Konarska et al., 1985; Keller, 1984). The overall reaction seems to take place in two kinetically distinct phases. Phase one involves cleavage at the 5' splice site plus the branch formation, while in phase two cleavage at the 3' splice site plus ligation of the exons takes place. One problem with this model, when considering the base pairing interaction between the branch site and the 5' splice site, is the role of U1 snRNP. Little is also known about the order of intron excision from pre-mRNA's with multiple introns, or what governs the selection of alternative splice sites in differential splicing.

2.2 Capping

Eukaryotic and viral mRNA and its precursors are modified at their 5' end by the addition of methylated caps. This general cap structure (Figure 4) is a 7 methylguanosine linked to the 5' end of the transcript by a 5'-5' pyrophosphate bond. This structure, known as Cap 0, was found in yeast (Sripati et al., 1976) and the RNA was found to be 75% m^7G-(5')-pppAp and 25% m^7G-(5')-pppGp. In animal cells a purine or pyrimidine nucleotide can be adjacent to the cap whereas in yeast it is always a purine/

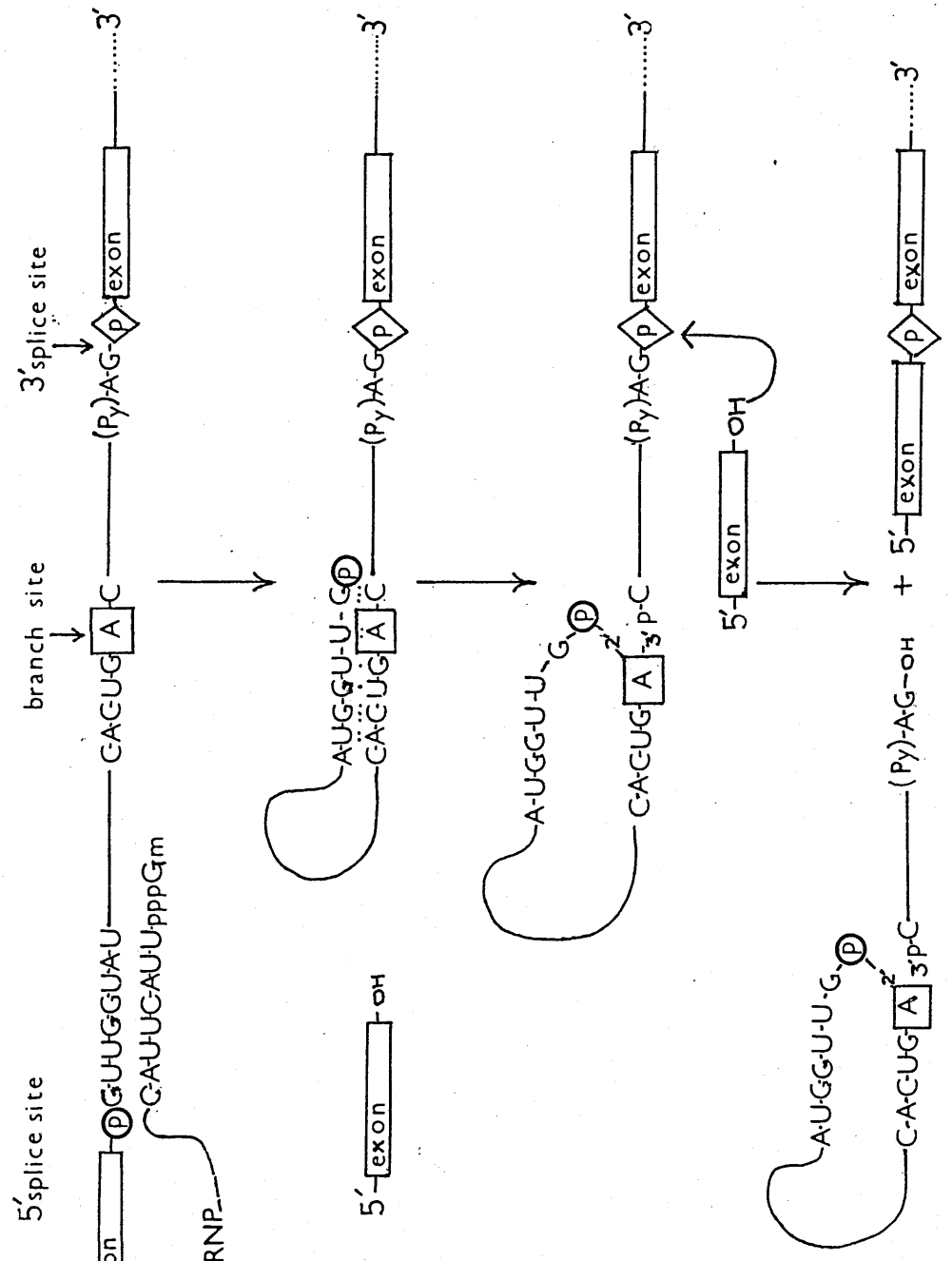


FIGURE 3 : Proposed pathway for processing of pre-mRNA involving the formation of a lariat structure.

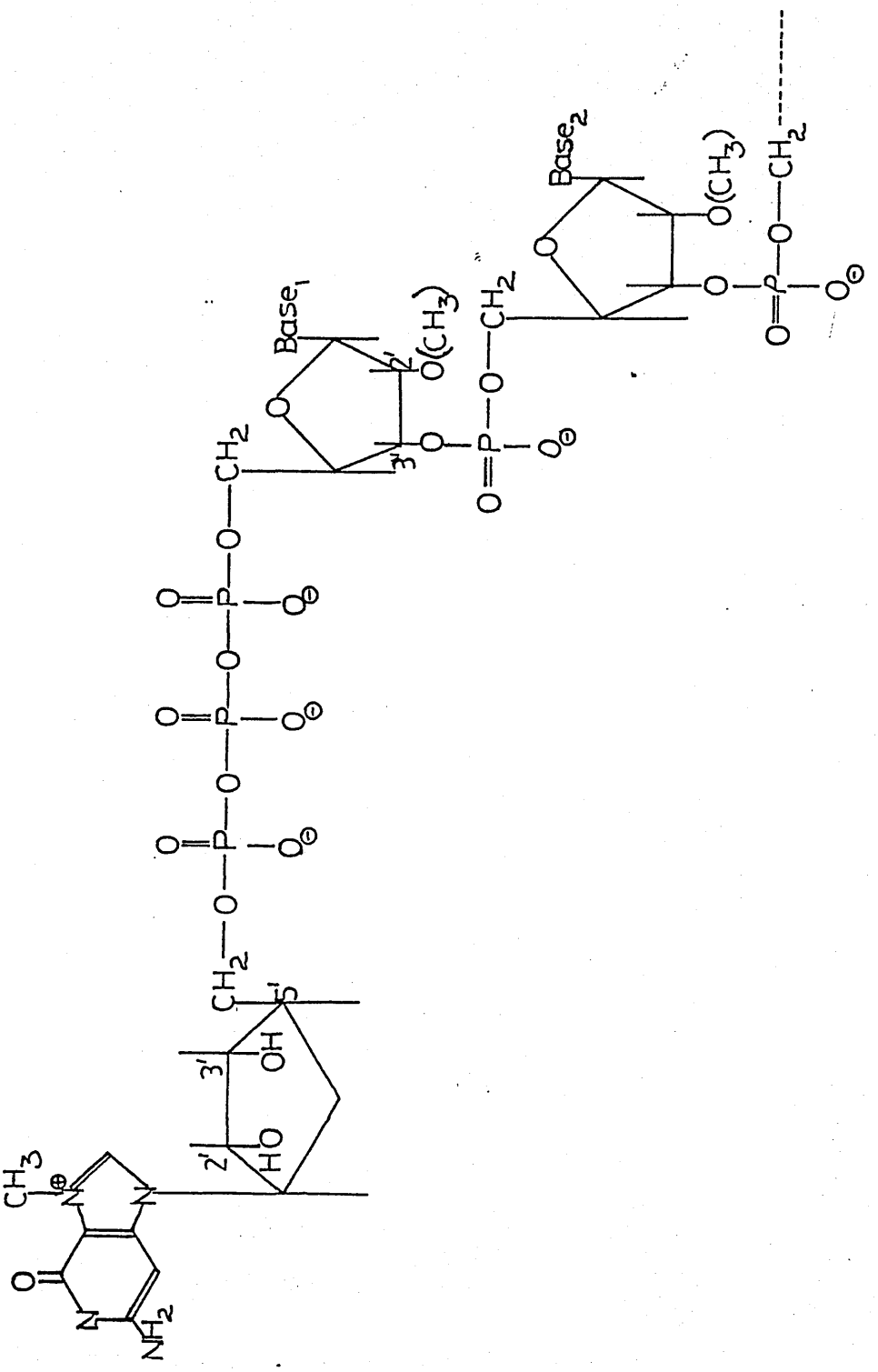


FIGURE 4: General cap structure at 5' end of hnRNA.

purine. Cap I is the structure m^7G (5')ppp(5')X^mpYp where the 7 methylguanosine is joined by a 5'-5' triphosphate linkage to a 2'-O-methylated nucleotide (Perry & Kelley, 1976). In animal cells Cap I is derived from the 5' cap of hnRNA. The third cap structure, Cap II, m^7G (5')ppp(5')$X^m pY^m$pZp is found in higher eukaryotes but is not detected in hnRNA. It arises by a second methylation of molecules bearing Cap I that occurs after the mRNA enters the cytoplasm (Perry & Kelley, 1976). Cap II is restricted to a particular subclass of mRNA with a high frequency of pyrimidine nucleotides at position Y, (Yang et al., 1976). The mRNA molecules with Cap II structures appear to be more abundant in polysomes, therefore either the Cap II has a higher affinity for ribosomes or the secondary methylation occurs after association with ribosomes.

Types I and II have been found in virus mRNA (Rose, 1975; Moss et al., 1976).

Three enzymes are involved in cap formation:-
RNA guanyltransferase transfers GMP from GTP to the 5' end of an unmethylated hnRNA containing at least 2 terminal phosphate groups, (Wei & Moss, 1977). RNA (guanine-7) methyl transferase methylates the transferred nucleotide and the 2'O position of the penultimate nucleotide (Ensinger et al., 1975) (Figure 5). Monroy et al., (1978) suggest that these 2 enzyme activities copurify. RNA (nucleoside-2) -methyltransferase methylates the Cap I structure at position Y (Keith et al., 1978).

Both/

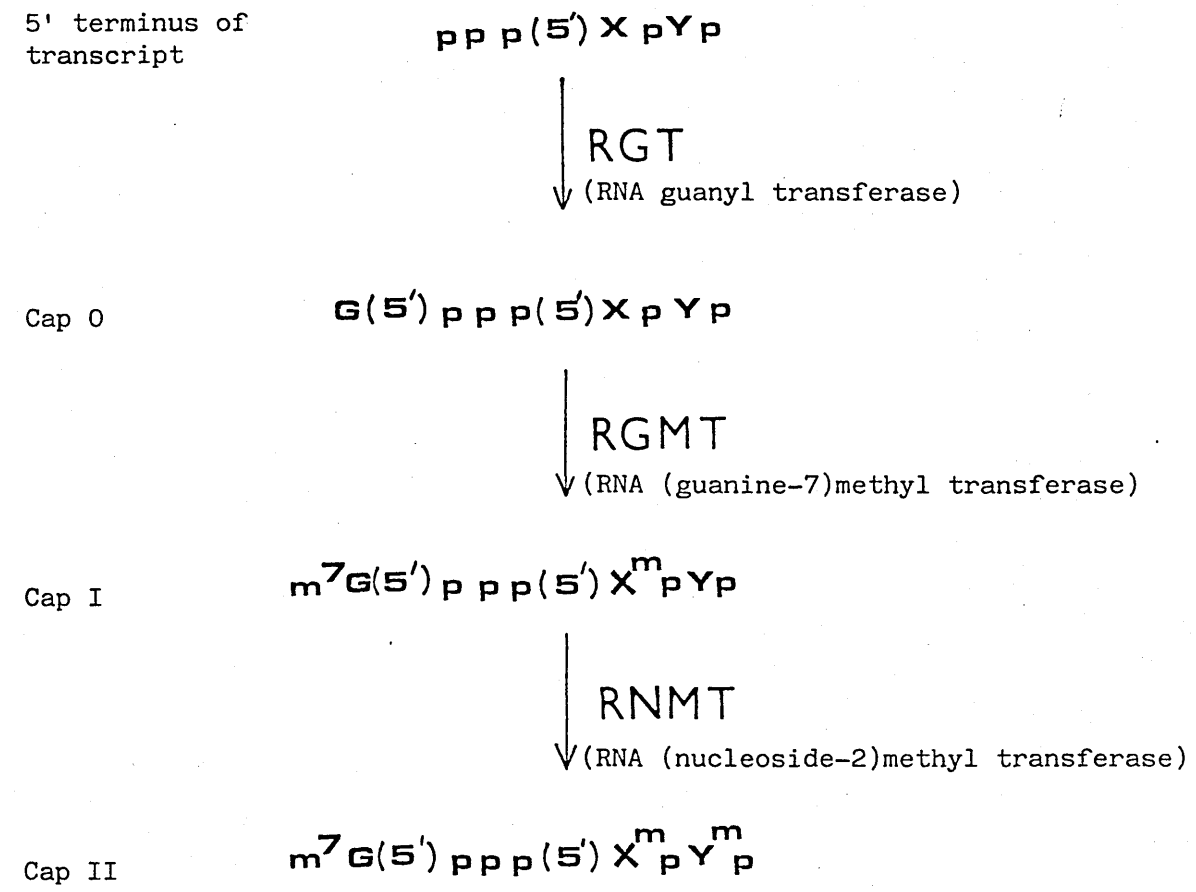


FIGURE 5: Enzymes involved in the production of cap structures.

Both et al., (1975) have shown that methylated mRNA molecules stimulated protein synthesis to a greater extent than unmethylated species, possibly due to the cap being involved in the formation of the initiation complex. This role of the cap in translation is backed up by the fact that capped mRNA's are more stable due to protection from 5' degradation (Furuichi et al., 1977). Pre-mRNA's injected in Xenopus oocytes are spliced only if the pre-mRNA is capped in vitro prior to injection, as the uncapped RNA is rapidly degraded (Green et al, 1983). Recent evidence has indicated that caps are required for splicing (Konarska et al., 1984).

The cap structure on pre-mRNA has been shown to be identical with that of mature mRNA (Roop et al., 1980) and the 5' termini of both mature mRNA and its precursors, map at the same point on the gene sequence.

2.3 Polyadenylation

Poly (A) sequences are present in 20-30% of all sizes of hnRNA (30-100S) and are conserved to a greater extent than the rest of the hnRNA (Jelinek et al., 1973). 70-90% of all cytoplasmic mRNA molecules contain poly (A) tracts (Brawerman, 1974). Non-polyadenylated mRNA appears to fall into 2 classes; those such as histone mRNA's which are usually non-polyadenylated (Isenberg, 1979) and a class of messengers for which there appears to be polyadenylated and non-polyadenylated molecules (Katinakis et al., 1980). The poly (A) tracts are resistant to T_1 RNase and consist of 200-250 nucleotides of adenosine, added sequentially to the 3' end of pre-mRNA as a post-transcriptional event.

The/

The enzyme involved in the formation of poly (A) sequences, poly (A) polymerase, is found free in the cell nuclei and bound to the chromatin (Jacob et al., 1976) and the chromatin bound enzyme is responsible for the initial polyadenylation (Rose et al., 1977). Poly (A) polymerase is a phosphoprotein (Rose & Jacob, 1979) of which the degree of phosphorylation varies in different tissues. Thus the poly (A) polymerase in rapidly growing hepatoma cells is twice as phosphorylated as the enzyme in normal liver cells (Rose & Jacob, 1979). This correlates with the finding that phosphorylation increases the activity of poly (A) polymerase (Rose & Jacobs, 1980).

Poly (A) polymerase catalyses the addition of AMP residues approximately 11-30 nucleotides after the base sequence AAUAAA near the 3' end of the gene transcript. This hexanucleotide or a close analogue has been found in all eukaryotic mRNA's so far examined (Proudfoot & Brownlee, 1976) and its spatial relationship to the site of polyadenylation is an essential feature of the addition mechanism (Fitzgerald & Shenk, 1981). A second recognition sequence element CAYUG has also been observed (Berget, 1984) either upstream or downstream from the poly (A) site. Moore & Sharp (1984) by using an in vitro polyadenylation system have shown that polyadenylation is coupled to active transcription. They suggest that part of the specificity of polyadenylation is established by in situ synthesis and that this specificity may involve snRNP particles as the reaction is inhibited by anti-Sm and anti-RNP antibodies.

Berget/

Berget (1984) has shown that the recognition sequences required for poly (A) addition are complementary to regions in U4 SnRNA, and suggests a model involving the hybridisation of U4 to AAUAAA as related to primary site selection, and hybridisation to CAYUG as related to cleavage site selection.

The balance of evidence suggests a role for poly (A) in extending the stability of mRNA (Gordon et al, 1973). Poly (A) may also modulate mRNA transport. There is some turnover of poly (A) in the nucleus and the addition of cordycepin, which inhibits the addition of poly (A) sequences to nuclear RNA, also inhibits the transport of mRNA to the cytoplasm, (Weinberg, 1973; Adesnik et al, 1972). Histone mRNA's, which are not polyadenylated, leave the nucleus faster than polyadenylated sequences. However, other non-polyadenylated mRNA's do not exhibit this differential rate of transport.

Brawerman & Diez (1975) have reported a process of elongation of pre-existing poly (A) which is distinct from de novo synthesis and it has been suggested that this extra polyadenylation may be necessary for mRNA protection (Diez & Brawerman, 1974).

2.4 Internal RNA Methylation

Very few methylated bases are found in hnRNA (4-8 m^6A, or m^6C) or mRNA (1-2) and they are limited to the eukaryotic system (Salditt-Georgieff et al., 1976). The function of these methylated bases is unknown but the modifications appear to be limited to the intron transcripts and may be involved in the recognition of the intron regions.

3. Heterogeneous Ribonucleoprotein Particles (hnRNP)

From a very early stage in its synthesis, hnRNA is associated with proteins (Schweiger & Hannig, 1971; McKnight & Miller, 1976; Sommerville, 1973) and it seems likely that these proteins will prove to have a role in mRNA maturation. Electron microscopy reveals protein associated with hnRNA as ribonucleoprotein particles (hnRNP) while the RNA is still being transcribed (McKnight & Miller, 1976) and while it is still attached to the chromatin (Sommerville, 1973).

In transcriptionally active regions of chromatin, ribonucleoprotein fibres are seen branching off the deoxyribonucleoprotein axis. Across the wide range of species, tissues and developmental stages there is some variation in the observed structure, but in general the nascent RNP chains appear to share many characteristics. They consist of 4 nm RNP fibrils, which, at apparently non-random intervals along their length, are complexed with or aggregated into RNP particles of diameter 25 nm (Malcolm & Sommerville, 1974). Pederson (1981) concludes that RNP particles, and not 'naked' RNA molecules, are the native vehicles for mRNA transport in the living cell.

Normally a gradient of increasing RNP fibril length is seen along the length of the transcription unit and is assumed to represent progression from initiation to termination. Some transcription units display abrupt changes in fibril length suggesting cleavage of RNA at specific sites on nascent transcripts (Miller/

(Miller & Hankalo, 1972). In oocyte nuclei, the gradient of RNP fibrils of increasing length from the point of initiation along the length of a transcription unit is often interrupted by abrupt discontinuities which appear to be caused by the removal of part of the transcript at a time when it is still being extended at its 3' end (Laird & Choo, 1976; Scheer et al., 1979). This is also seen by Beyer et al., (1981) in Drosophila and the results suggest that cleavage may occur within the growing RNP fibrils. Beyer et al., (1980) have shown that RNP particles assemble after synthesis of 500 nucleotides, and free hnRNA has not been detected in the cell.

The failure to generate hnRNP-like complexes by the addition of deproteinised hnRNA to cellular extracts (Pederson, 1981; 1974a), the fact that particles prepared in the presence of radioactively labelled proteins from other cellular pools failed to show significant contamination and the fact that crosslinking experiments (Van Eekelen et al, 1981a; Mayrand & Pederson, 1981; Karn et al., 1977) have shown that proteins found in isolated hnRNP can be crosslinked in vivo, all suggest that hnRNP particles are not artefacts formed during the isolation procedure.

3.1 Isolation of hnRNP Particles

There is no universal method for isolating hnRNP's which can be applied to all cell types. Methods available for the preparation of hnRNP particles have been reviewed by Van Venrooij & Jansen (1978) and/

and all start with the isolation of purified nuclei. The original method was devised by Samarina et al., (1968) for rat liver cells. It involves the extraction of nuclei with an isotonic buffer at pH 7.0 to remove preribosomes and ribosomes, followed by extraction of the hnRNP with a pH 8.0 buffer. This method gives good results with many tissues but does not give acceptable results with tissue culture cells where the higher temperature required to extract particles can lead to partial degradation. Other methods rely on nuclear disruption followed by fractionation of the lysate for hnRNP preparation. Nuclear lysis has been achieved by using hypertonic buffers (Moule & Chauveau, 1968), detergent (Stevenin & Jacob, 1972), sonication (Pederson, 1974a; Stevenin & Jacob, 1974), disintegration in the French Press (Parsons & McCarthy, 1968) or digestion with DNase (Penman et al., 1968).

Nuclear lysis methods give higher yields of hnRNP than extraction at pH 8.0, but are more likely to result in contamination. The DNase method leads to considerable contamination with chromatin components (Penman et al., 1968), while detergents appear to have deleterious affects on hnRNP particles and disrupt the chromatin and nuclear matrix which in turn contaminate the preparation. The use of the detergent sodium deoxycholate can cause dissociation of hnRNP (Faiferman et al., 1971; Stevenin & Jacob, 1974). Triton X 100 does not appear to have deleterious effects on either the protein composition or enzyme activities (Wilks & Knowler, 1981a) but it has/

has been suggested that it activates latent RNases and increases hnRNP degradation (Lund-Larsen, 1975).

The most widely used of the nucleolytic methods is that using sonic disruption. This method is used in cases where the endogenous RNase levels are low, and it is often the method of choice when isolating hnRNP particles from tissue culture cells as extraction at pH 8.0 from the nuclei of such cells requires an unacceptably high temperature. The particles isolated by the extraction method or by nuclear lysis are very similar (Pederson 1974a; Suria & Liew, 1979; Albrecht & Van Zyl, 1973).

Extraction at pH 8.0 yields monomeric particles which are widely believed to be identical to 25nm particles seen on nascent RNP. In the presence of RNase inhibitor (Roth, 1958), polymers can be extracted and in electronmicrographs they appear to be strings of particles arranged along an hnRNA backbone. Such polymeric hnRNP, of up to 300S is more readily produced by sonic disruption of purified nuclei (Stevenin & Jacob, 1972; Pederson, 1974a). The polymers are converted to monomers by low levels of ribonuclease (Samarina et al., 1968; Pederson, 1974a).

Kloetzal et al., (1982) have reported a gentle preparation of hnRNP from easily disrupted amphibian oocytes and this may well lead to the most native hnRNP, but Sommerville (1981) has suggested that hnRNP from oocyte and other germ line cells may differ markedly from those of somatic cells.

HnRNP/

HnRNP particles isolated by extraction or lysis are further purified by centrifugation on sucrose density gradients followed by isolation of the particle containing fractions (Samarina et al., 1976). Metrizamide has also been used (Karn et al., 1977) but has been shown to cause disruption of hnRNP (Gattoni et al., 1977).

3.2 Properties of HnRNP Particles

HnRNP monoparticles have a sedimentation constant of 30-40S (Samarina et al., 1968; Pederson, 1974a) and a molecular weight of 1×10^6 (Krichevskaya & Georgiev, 1969). The particles exhibit some heterogeneity in sucrose density gradients which may reflect differences in the size of the associated RNA. However, some workers have claimed to detect several distinct peaks on buoyant density gradients (Houssais, 1975; Faiferman et al., 1970) and Guialis et al., (1983) have identified two discrete classes of particle within the monomer population of rat liver hnRNP.

Notwithstanding these possibilities, however, hnRNP particles from a variety of tissues and cultured cells exhibit a remarkable uniformity in their physical and chemical properties (Brunel & Lelay, 1979) (Table 2).

The RNA from hnRNP particles will hybridise to cDNA made against total mRNA or specific mRNA species (Mantieva et al, 1969). The presence of mRNA sequences coding for cellular proteins has also been detected in hnRNP (Thomas et al, 1981; Gaitskhaki et al, 1981; Maundrell & Scherrer, 1979).

3.3/

SOURCE	SEDIMENTATION CONSTANT (S*)	(g/cm^3)	RNA (SEDIMENTATION CONSTANT) (S*)	PROTEINS NUMBER OF SPECIES	REFERENCE
Rat liver	38-200	1.39	8	45-50	Northeman et al., 1977
		1.39	15-20	45-50	Northeman et al., 1978
	30 -400	1.39-1.4	5-8		Niessing & Sekeris, 1971a
HeLa	40-200	1.43-1.45	20-30	12-25	Pederson, 1974a
Slime mould	55	1.41-1.43	15		Firtel & Pederson, 1975
Wheat embyro		1.4	15-30		Ajtkhozhun et al., 1975
Sea urchin embryo		1.4-1.55	10-30		Ajtkhozhun et al., 1975
Drosophila	30-80	1.4	-		Szabo et al., 1981
Chick oviduct	30-350	1.4	93		Thomas et al., 1981

TABLE 2: Properties of hnRNP particles.

* The wide range of S values and RNA size reflects the differing extents to which different preparations preserve polymeric rather than monomeric hnRNP

3.3 Protein Components of HnRNP Particles

A number of detailed studies on the polypeptides of hnRNP particles from a variety of sources have been performed and have shown that proteins comprise about 80% of the total mass of the particle. Considerable controversy has existed over the precise number of proteins which are associated with the particles. Early analyses produced protein compositions varying from one in rat liver hnRNP (Samarina et al., 1968) to 90 polypeptides in hnRNP from avian erythroblasts (Maundrell & Scherrer, 1979). However, these variations were largely due to differences in the purity of the product and the sensitivity of the protein fractionations employed (Van Venrooij & Jansen 1978; Heinrich et al., 1978). Virtually all investigators are now agreed that the proteins are heterogeneous, with a dominant group of proteins collectively known as the core proteins. Small differences have been detected in the minor polypeptide components of hnRNP's of differenct tissues (Gallinaro-Matringe et al., 1977), different species (Beyer et al., 1977; Wilks & Knowler, 1980), different stages of development (Maxwell & Fisher, 1979) and after viral infection (Gattoni et al., 1980). These differences occur mainly in the protein components of molecular weight 45000-150000, although there are some species differences in the smaller core proteins.

3.3.1 Major Core Proteins: This group of proteins forms the dominant part of the protein constituent of hnRNP and on one-dimensional gels are seen as 4-6 species of molecular weight 30000-45000/

30000-45000 (Pederson, 1974a; Karn et al., 1977; Beyer et al., 1977; Stevenin & Jacob, 1979; Northeman & Heinrich, 1979; Wilks & Knowler, 1980). Rat liver has four species while Beyer et al., (1977) have suggested that the 6 major core proteins in HeLa cells are present as 3 groups of doublets (A, B and C). The C proteins (molecular weight 42000 and 44000) interact directly with the RNA, while the A proteins (32000 and 34000 daltons) comprise 60% of the particle mass and apparently function structurally in the packaging and stabilization of the hnRNA in a manner analogous to histones in chromatin nucleosomes (Kornberg, 1974).

There is disagreement over the proportion of core proteins in hnRNP particles and estimates range from 25% (Pederson,1974a) to 70% of the total mass (Karn et al., 1977; Brunel & Lelay, 1979). A considerable amount of circumstantial evidence indicates that the core proteins are the structural proteins of the particles. They are stable and abundant proteins that are universally present and conserved between species. Antibodies raised against the core proteins of one species cross react with those of a wide range of mammalian, avian and amphibian species (Martin et al.,1981; Hugle et al., 1982; Leser et al., 1984; Choi & Dreyfuss, 1984a).

In parallel with other nucleic acid binding proteins, i.e. histones and ribosomal proteins, the core proteins are basic with pI 8-10 (Suria & Liew, 1979; Wilks & Knowler, 1980), and have low turnover rates (Martin et al., 1979). They also have a high glycine content (Karn et al., 1977; Beyer et al., 1977) and have unusual (Fig.6) methylated/

methylated arginine residues - N^9N^9 dimethylarginine (Karn et al., 1977; Fuchs et al., 1980). The abundance of glycine in the composition of core proteins has led Le Stourgeon et al., (1978) to suggest that they contain a high percentage of β sheet in their structure, and as such might be admirably suited for intercalating with double stranded portions of RNA in the hnRNP particles. It has also been suggested that dimethylarginine may be involved in protein-RNA interactions (Beyer et al., 1977; Le Stourgeon et al., 1979). The core proteins have a high affinity for RNA and they will reconstitute with RNA in vitro to form particles indistinguishable from non-dissociated controls (Le Stourgeon et al., 1979).

By dissociating hnRNP particles and looking at their reassembly on sucrose density gradients, Pullman & Martin (1983) have suggested that the core proteins determine the basic structural properties of hnRNP particles. Wilk et al., (1983), by dissociating hnRNP from HeLa cells with micrococcal nuclease, have shown that particles which resemble native hnRNP reform when a variety of exogeneous RNA's are added. Therefore the hnRNP core proteins appear to have the intrinsic capacity to associate with single-stranded RNA irrespective of its nucleotide sequence. This intrinisic ability to form complexes has also been shown in brine shrimp Artemia salina (Thomas et al., 1983). The HD40 (molecular weight 40000) is the major protein of 30S hnRNP in the shrimp and forms structures, very similar to hnRNP, with exogeneous/

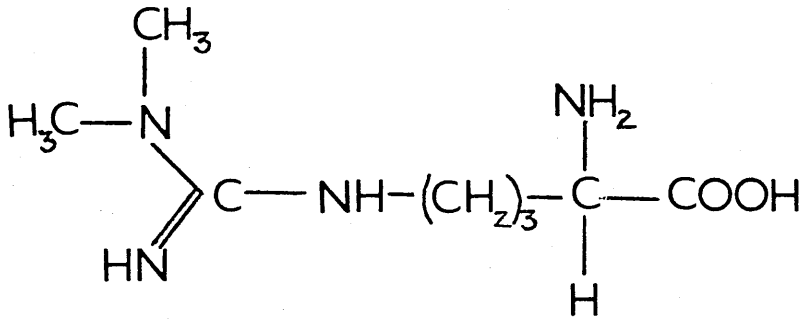


FIGURE 6: $N^{9}N^{9}$ dimethylarginine

exogeneous poly (A) strands. It should be mentioned however, that the evidence that reconstituted hnRNP particles are the same as isolated particles depends on the currently available methods by which particle structure is analysed. These are not very rigorous.

On 2-dimensional gel fractionation systems the major core proteins exhibit charge heterogeneity (Suria & Liew, 1979; Maundrell & Scherrer, 1979; Wilks & Knowler, 1980). Each of the bands detected on one-dimensional SDS-polyacrylamide gel electrophoresis, can be resolved into a string of stainable spots. In at least one case this heterogeneity appears to result from phosphorylation (Wilks & Knowler, 1981a). Tryptic peptide mapping has been employed to analyse the structural relationship of the core protein chain of spots (Wilks & Knowler, 1981b; Fuchs et al., 1980). Wilks & Knowler (1981b), using 2-dimensional gels, showed that the most abundant core proteins in rat liver hnRNP comprised 4 species. Polypeptides in the same group appear to be structurally related whereas there is little resemblance between the different groups. This has been interpreted as evidence for post-translational modification of the core proteins but does not disprove the possibility that they may be products of multiple gene families. Fuchs et al., (1980) isolated individual basic core polypeptides from one-dimensional gels, and concluded that there may be only 4 major basic proteins. Le Stourgeon et al., (1978) concluded that there were/

were 4 basic and 2 acidic core proteins in HeLa cell hnRNP. Wilk et al., (1985) by looking at the amino acid sequence and tryptic peptide maps of the core proteins from HeLa cell hnRNP have suggested that there are 3 groups of core proteins - 1) basic proteins (A1,A2,B1a,B1b,B2,C1), 2) slightly acidic (B1c,C2), 3) acidic (C3).

The proteins associated with hnRNA have been shown to be subject to post-translational modification including phosphorylation (Karn et al., 1977; Blanchard et al., 1978; Choi & Dreyfuss, 1984a), methylation (Karn et al., 1977; Beyer et al., 1977; Blanchard et al, 1978), ADP-ribosylation (Kostka & Schweiger, 1982) and glycosylation (Jacob et al., 1981). In all cases of phosphorylation reported the covalent linkage of the phosphate group is via serine and threonine ester linkages.

Modifications of core proteins might permit variation in the degree of interaction between the proteins and the RNA in the same way that modifications of histones affects their association with the DNA. The finding that phosphorylated particle proteins are more tightly associated with the RNA than nonphosphorylated species (Gallinaro-Matringe et al., 1975) and the fact that hnRNP particles contain an endogenous protein kinase (Karn et al.,1977; Blanchard et al., 1977) has led to the suggestion that phosphorylation of hnRNP particle proteins might control pre-mRNA processing.

3.3.2 /

3.3.2 Minor hnRNP Proteins: These minor proteins are mostly neutral or acidic with pI values of 4.9-6.5 (Pederson, 1974a). They appear to vary between different species (Karn et al., 1977; Brunel & Lelay, 1979) and very few of them have been ascribed specific functions. One polypeptide of molecular weight 110000 is found to be common to hnRNP particles and to cytoplasmic messenger RNP complexes (mRNP) (Schweiger & Kostka, 1980). They suggest that this protein may be involved in the transport of the RNA to the cytoplasm.

3.3.3 HnRNP Proteins Associated with Poly (A) Sequences: There is good evidence that the 3' poly (A) tail of hnRNA forms a RNP particle which differs in size and composition from the remaining hnRNP complex. After treatment of hnRNP with mild RNase, poly (A) is found in separate particles which sediment at 15S (Samarina et al., 1968; Samarina et al., 1973). They contain at least 60% of the nuclear poly (A) in lengths of up to 230 nucleotides (Kish & Pederson, 1975) and their buoyant density is 1.36 g/cm^3 (Quinlan et al., 1977) suggesting a higher percentage of protein than the 40S hnRNP. The 15S particles do not contain core proteins. Their major protein is a polypeptide of molecular weight 73-78000 (Kish/

(Kish & Pederson, 1975). A polypeptide of similar molecular weight is also found associated with the poly (A) component of mRNA (Blobel, 1973; Morel et al., 1973) and it has been suggested that a single polypeptide is common to the poly (A) segment of hnRNP and mRNP. However, Seytono & Greenberg (1981)suggest that while the polypeptide associated with poly (A) in cytoplasmic mRNP's has a molecular weight of 78000, that associated with hnRNA has a molecular weight of 60000 (p60A). Furthermore, the two polypeptides differ in their partial peptide maps. They suggest that this p60A may be the poly (A) polymerase activity present in hnRNP as it is the same size as the poly (A) polymerase described by Rose et al., (1979).

Tomescanyi et al., (1983) suggest that the 15S poly (A) RNP are composed of 90% protein and that almost the entire poly (A) is located on the surface of the particles. This agrees with the results of Quinlan et al., (1977) who found that 15S RNP particles were composed of 80% protein.

3.4 Models for HnRNP Particle Structure

The earliest model of hnRNP structure was based on the interaction of the hnRNA with 20-40 identical polypeptides of molecular weight/

weight 40000 (informatin), (Samarina et al., 1968; Krichevskaya & Georgiev, 1969; Lucanidin et al, 1972). This model was a polysome like structure of proteinaceous beads around which the RNA was wound in a manner analogous to the way in which DNA is thought to coil round the nucleosome (Figure 7). Martin et al., (1977) supported this model with a slight modification in that they say that the RNA linker between particles was double stranded. Acceptance of the heterogeneous nature of hnRNP proteins and investigation of the effects of increasing concentrations of NaCl, detergents and RNase digestion (Stevenin & Jacob, 1972, 1974; Le Stourgeon et al, 1979) has provided evidence for an interior location of the hnRNA. Munroe & Pederson (1981) show that messenger homologous sequences in hnRNP are protected by proteins from nuclease attack.

Sekeris & Niessing (1975) have suggested a model for hnRNP complexes which locates RNA externally as well as internally (see Figure 7b). This model consists of rapidly labelled hnRNA, responsible for formation of the polymeric structures, and a structural low molecular weight RNA (snRNA) on which the proteins are assembled to form the monomeric unit.

Heinrich & Northeman (1981) have proposed a model in which the snRNA plus the core proteins form a 'core' complex (Figure 8), while Stevenin et al, (1977) have suggested the presence of 2 classes of hnRNP particles with densities 1.18 g/cm^3 and 1.28-1.3 g/cm^3. In truth, however, all of these models are based on very limited evidence. There is a need to raise specific antibodies, study crosslinking and topographical locations before any model can be taken seriously.

3.5/

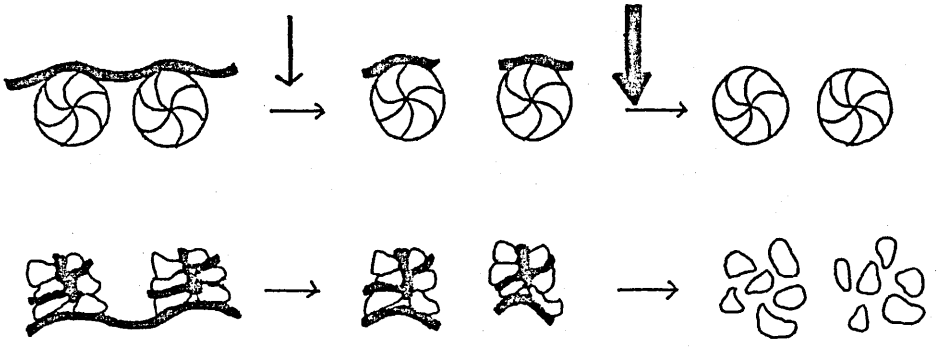


FIGURE 7: Models of hnRNP particles.

(a) Model of Samarina et al (1968):one main polypeptide of molecular weight 40000.

(b) Model of Sekeris & Niessing (1975): 2 types of RNA.

(↓ : treatment with low levels of RNase

⬇ : treatment with high levels of RNase).

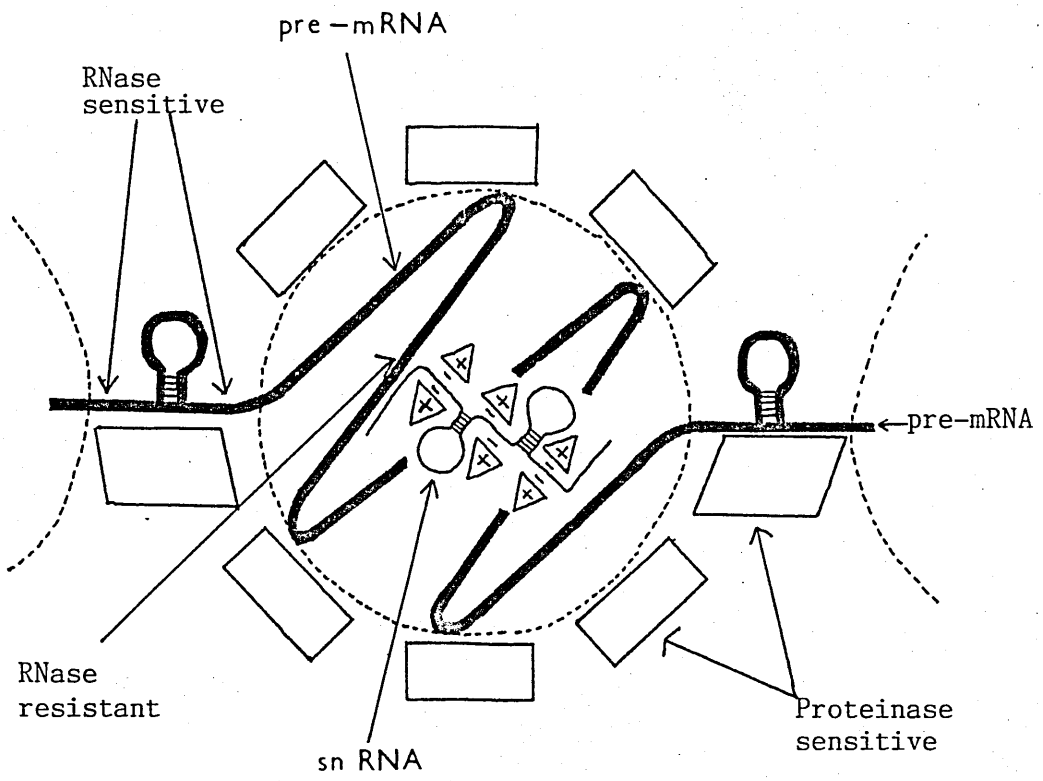


FIGURE 8: Model of hnRNP structure (Heinrich & Northeman, 1981). Basic proteins (molecular weight 30000-45000) (△), bound to snRNA by ionic interactions. Proteins of molecular weight > 40000 (▭) and of 115000 (▱) complexed with pre-mRNA.

3.5 Enzyme Activities Associated with HnRNP Particles

Care must be taken in defining any activities as true hnRNP proteins, as it is very easy to contaminate subcellular fractions.

Three capping enzymes, guanyltransferase, N^7-methyltransferase and 2-O methyltransferase have been found bound to hnRNP particles (Bajszar et al., 1978).

Jeanteur (1981) has also described an hnRNP associated methyl-transferase activity which is capable of transferring the methyl group of S-adenosyl-methionine to the synthetic substrate GpppG. The capping enzymes do not appear to be strongly or uniquely bound to hnRNP. Enzyme activities would have great difficulty in performing their catalytic function if they were a rigid and integral part of the particle, and might best serve their function if able to move from transcript to transcript.

Several RNase activities have been detected in hnRNP particles, including a sequence dependent endonuclease (Niessing & Sekeris, 1970) and a 5' exonuclease activity (Molnar et al., 1978), and an RNase H activity able to digest the RNA strand of a DNA: RNA hybrid (Kish & Pederson, 1975). Double stranded RNA specific RNases have also been described in the hnRNP of HeLa cells (Rech et al., 1979) and rat liver (Molnar et al., 1978). Double stranded regions, in hnRNA, with a hairpin-like structure have long been recognised (Ryskov et al., 1973), as have branched structures (Wallace & Edmonds, 1983). The significance of such findings has greater meaning since the discovery of Lariat structures in pre-mRNA processing intermediates (see Section 2.1). The comigration, in density gradients, of hnRNP particles/

particles and these enzyme activities may reflect a functional approximation of the processing enzymes and their substrates (i.e. hnRNA). Bachman et al., (1984) have identified an endoribonuclease VII associated with 45S hnRNP. This enzyme has a pH optimum of 7.2, pI 8.5 and Bachman et al., (1984) have suggested that it is one of the hnRNP proteins of molecular weight 74k.

Other RNA processing activities found associated with the hnRNA in hnRNP particles (Table 3) includes poly (A) polymerase (Niessing & Sekeris, 1972) and other homoribopolymerases capable of synthesising guanosine, cytosine or adenosine polymers (Niessing & Sekeris, 1973). Niessing & Sekeris (1973) have presented evidence for 2 separate poly (A) polymerase activities - one dependent on Mg^{2+} and the other on Mn^{2+}. Much of the poly (A) polymerase is only loosely associated with hnRNP. From its amino acid composition (Rose & Jacob, 1976) and antigenicity (Rose et al., 1979), it has been suggested that the 73-78K protein may be the poly (A) polymerase (Jeanteur, 1981).

Protein kinase activity has been detected in hnRNP particles (Blanchard et al., 1977; Karn et al., 1977; Periasamy et al., 1979; Wilks & Knowler, 1981a; Holocomb & Friedman, 1984) and it may be a possible regulatory enzyme (Blanchard et al., 1977, 1978). Partial purification of the kinase activity (Periasamy et al., 1979) revealed similarities to the nuclear kinase NK1 of rat liver. The kinase activity detected by Blanchard et al., (1977) and Karn et al., (1977) had a pH optimum at pH 8.5 and was unresponsive to cAMP, cGMP or calmodulin. Wilks & Knowler (1981a) also detected a kinase activity associated/

associated with hnRNP particles, but it had an optimum activity at pH 6.5 and was stimulated by cAMP and polyamines. The kinase activity detected by Holocomb & Friedman (1984) was cAMP and Ca^{2+} independent but was stimulated by polyamines. A molecular weight of 48000 for the protein kinase has been determined from chromatography on Sephadex, while SDS-polyacrylamide gel electrophoresis suggested that the protein was a dimer with 25000 and 28000 molecular weight subunits (Periasamy et al., (1979).

The kinase has been shown to auto-phosphorylate hnRNP particle proteins (Blanchard et al., 1978; Wilks & Knowler, 1981a). Autoradiographs of 2 dimensional polyacrylamide gels of hnRNP particles show the core proteins as strings of (^{32}P) spots (Brunel & Lelay, 1979; Wilks & Knowler, 1981a). The proteins labelled in vivo and in vitro are similar, and peptide mapping reveals that some of the tryptic peptides are phosphorylated (Brunel & Lelay, 1979).

Histones are able to be phosphorylated and changes in their level of phosphorylation have been implicated in changes in the condensation of chromatin (Louie & Dixon, 1973). It is therefore tempting to speculate that the phosphorylation of core proteins may control the extent to which hnRNA is available to the enzymes of maturation or degradation. Some support for this idea comes from the findings of Gallinaro-Matringe et al., (1975), who showed that phosphorylated hnRNP particle proteins are more tightly bound to the particles than non-phosphorylated species of similar molecular weights. The protein kinase may also play a role by modifying the activities of some of the other hnRNP particle enzymes. For example,/

ACTIVITY	SOURCE	REFERENCE
Poly (A) polymerase	rat liver	Niessing & Sekeris (1972)
capping	rat liver	Bajszar et al., (1978)
RNase	rat liver	Niessing & Sekeris (1970)
		Molnar et al., (1978)
double stranded specific RNase	rat liver	Molnar et al., (1978)
	HeLa	Rech et al., (1979)
Protein Kinase	rat liver	Schweiger & Mazur (1975)
		Karn et al., (1977)
	HeLa	Blanchard et al., (1977)
		Periasamy et al., (1979)
Phosphoprotein phosphatase	HeLa	Periasamy et al., (1977)

TABLE 3: Enzyme activities associated with hnRNP particles.

For example, nuclear poly (A) polymerase is known to be phosphorylated, and phosphorylation has been shown to exert a positive control on enzymatic activity (Rose & Jacob, 1979).

The protein kinase activity in hnRNP particles is balanced by the presence of phospho-protein phosphatase activity (Periasamy et al, 1977) which is distinct from the nonspecific alkaline phosphatase found in nucleoli.

3.6 Association of HnRNP Particles With the Nuclear Matrix

When nuclei are lysed by methods which do not involve shear forces, native hnRNA is not seen as free hnRNP particles, but is intimately associated with the nuclear matrix (Faiferman & Pogo, 1975). The nuclear matrix is a recently identified nuclear entity, left behind when nuclei are sequentially extracted with high salt concentrations and detergents and are then treated with DNase and RNase (Faiferman & Pogo, 1975). It consists of a network of proteinaceous fibres together with nucleolar structures, nuclear pores and connecting lamina. The matrix is bordered by an outer nuclear lamina connected to the cytoskeletal framework as well as the inner filaments. The filaments are organised in a three dimensional network in which nucleoli are enmeshed (Capco et al, 1982). The matrix is composed of 97% protein (Peters & Cumings, 1980), and is almost devoid of DNA.

If the RNase step is omitted during the isolation, the matrix is strongly associated with hnRNA or hnRNP (Maundrell et al, 1981). Results show a 200-300Å granular component superimposed on the 50Å fibrous network. The hnRNA is not an integral part of the filaments since/

since RNases can digest the majority of the hnRNA without affecting the matrix architecture (Miller et al, 1978). Protein-protein and protein-RNA are important interactions which maintain the RNP complexes attached to the nuclear skeleton (Miller et al, 1978). Van Eekelen & van Venrooij (1981) have shown, by crosslinking and RNase studies, that the hnRNP particles of HeLa cells are attached to the nuclear matrix via two proteins of molecular weights 41500 and 43000 which correspond to the C group proteins (Beyer et al, 1977). Maudrell et al, (1981) have suggested that the core proteins are not involved in association with the matrix, as in a high salt extraction of the nuclear matrix no core proteins remain associated with the hnRNA: matrix complex. Only 2 proteins remained associated with the hnRNA when the matrix was isolated in this way. One was a 43000 molecular weight protein and the other, of molecular weight 73000, migrated with the poly (A) binding protein. This, plus the evidence that poly (A) sequences are left in the matrix of HeLa cells after RNase digestion (Herman et al, 1978), has led to the suggestion that hnRNP might be bound to the matrix via a poly (A) ribonucleo-protein complex (Maundrell et al, 1981).

There is a growing body of evidence that the nuclear matrix may be closely associated with both transcriptional and post-transcriptional processes.

In HeLa cells infected with Adenovirus 2, where the virus specific hnRNA associates with the nuclear matrix, crosslinking with U.V. light shows that all viral poly (A) mRNA molecules (i.e. precursors,/

precursors, products and processing intermediates) are crosslinked to 2 host cell proteins of molecular weight 41500 and 43000 (Mariman et al, 1982). This supports the concept that the nuclear matrix may function in the localisation and structural organisation of hnRNA during processing. Gallinaro et al, (1983) have compared the nuclear matrix and hnRNP particles to establish whether pre-mRNA is associated with the same constituent in both structures. They propose that the results are compatible with the idea that the salt resistant complexes of hnRNP constitute the fibrils associated with pre-mRNA in the nucleoplasmic matrix. They suggest that the fibrils may be the basic unit of splicing and their organisation in the matrix might provide the spatial configuration necessary for regulation. DNA is thought to be arranged in supercoiled loops anchored to the matrix and actively transcribed genes such as the ovalbumin gene of chicken oviduct cells are preferentially associated with the nuclear matrix, (Robinson et al, 1982). Ciejek et al, (1982) have shown that all precursors to ovalbumin and ovomucoid mRNA's in chick oviduct cells are associated with the nuclear matrix and have suggested that the matrix may be the structural site for RNA processing.

Busch & Reddy (1982) proposed that snRNP molecules are polymerised on to moving elements of the nuclear matrix. The sites proposed are assembly points for U-series snRNP-hnRNP complexes which then migrate to the envelope chromatin where processing occurs. As a/

As a result of processing the mRNP particles are released into the cytoplasm and the snRNP particles are recycled.

3.7 Regulation of HnRNP Particle Protein Synthesis

The major core proteins, which are thought to be the structural proteins of the particles, are highly conserved between species (Karn et al., 1977; Pederson, 1974a) while some of the other hnRNP particle proteins (molecular weights 40000-120000) appear to be tissue specific (Karn et al., 1977; Brunel & Lelay, 1979).

Pederson (1974b) has shown that the administration of hydrocortisone caused an increased incorporation of (^{3}H) orotate into rat liver hnRNP particle hnRNA, accompanied by an increased synthesis of acidic hnRNP particle proteins. The proteins which respond are likely to be concerned with the post-transcriptional event, namely assembly and processing of hnRNP particles. Similar increased synthesis of proteins has been observed by Stanton & Holoubek (1977) in human amnion cells infected with poliovirus, and in stimulated human lymphocytes (Henniki, 1975). In HeLa cells and HeLa cells infected with Adenovirus 2, the hnRNP particles show the same general characteristics except that in infected cells the hnRNP particles contain 6 additional polypeptides (Gattoni et al., 1980).

Rats fed the carcinogenic dye 3' methyl-4-dimethylaminoazobenzene, have altered protein compositions in 30S particles, and lose one major protein (Patel & Holoubek, 1976). In comparing normal liver nuclei and hepatoma nuclei hnRNP particles, a 60-70% decrease/

decrease in the relative amount of a polypeptide of molecular weight 125000 was found in hepatoma cells (Albrecht & Van Zyl, 1973).

HnRNP particles from adrenalectomised rats show a decrease in protein kinase activity and many of the proteins are no longer phosphorylated (Knowler & Wilks, 1980). Administration of dexamethasone, a synthetic glucocorticoid, to adrenalectomised rats, restores their pattern of phosphorylation to that of control animals.

HnRNP particles isolated from Drosophila cells heat shocked at 37°C, show an altered protein composition (Kloetzel & Bautz, 1983). HnRNA synthesis continues at a normal rate but hnRNP assembly is incomplete (Mayrand & Pederson, 1983). Mayrand & Pederson suggest that this incomplete assembly leads to an abortive processing of some precursors and favours processing of others whose maturation is less dependent on normal RNP structure. It is clear that the protein component of hnRNP particles can vary with the cellular environment thus strengthening the suggestion that they play a role in post-transcriptional processing of mRNA and its regulation.

4. Protein Kinases

The results section describes an analysis of protein kinase activity in hnRNP particles of rat liver. Because it is possible that such enzymes have a role in control of mRNA maturation, the introduction concludes with a short review of the known precedents for protein kinases having a role in the control of cellular events.

Protein/

Protein phosphorylation is now recognised to be a general mechanism by which intracellular events in mammalian tissues are controlled by external stimuli, and the idea that different cellular functions are controlled by common protein kinases and protein phosphatases is gaining acceptance.

For protein phosphorylation-dephosphorylation (Figure 9) reactions to function in regulation, it is apparent that appropriate signals should bring about changes in the relative concentrations of the phosphorylated and nonphosphorylated forms of the protein substrates. This could occur through control of the protein kinase step, the phosphoprotein phosphatase step, or through the simultaneous regulation of both reactions. These controls could involve rapid or immediate responses, due to fluctuations in the levels of effector molecules (i.e. cAMP, cGMP, calcium) or they might be mediated by adaptive changes that alter the ratio of protein kinase to phosphoprotein phosphatase.

A number of protein kinases are regulated through their direct interaction with specific regulatory agents.

Cyclic AMP dependent protein kinase (cAMP-PrK) is a multisubunit enzyme that normally exists as an inactive complex in the absence of cAMP. There are at least 2 classes but all have the same general subunit structure and obey the same general mechanism of activation. The inactive holoenzyme is a tetramer containing 2 regulatory (R), and 2 catalytic (C) subunits (Rubin & Rosen, 1975).

$$R_2C_2 + 4\ cAMP \rightleftharpoons R_2 - (cAMP)_4 + 2C$$

INACTIVE HOLOENZYME — ACTIVE CATALYTIC SUBUNIT

cAMP/

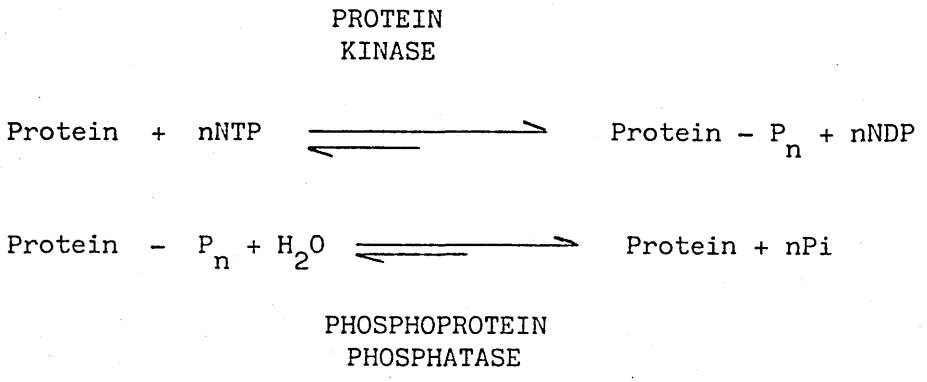


FIGURE 9: Phosphorylation-dephosphorylation reactions.

cAMP promotes dissociation of the holoenzyme producing a fully active C subunit. cAMP-PrK Type I is found predominantly in skeletal muscle while Type II is found predominantly in cardiac muscle (Rubin et al., 1972). The differences between the 2 types lie in the R subunits while the C subunits are identical (Hofman et al., 1975).

cGMP dependent protein kinase (cGMP-PrK) is made up of 2 identical subunits and the cGMP binding and catalytic activity reside on a single polypeptide chain (Gill et al., 1977). cGMP-PrK has similar but not identical structural determinants of specificity to cAMP-PrK (Lincoln & Corbin, 1978). Lincoln & Corbin (1978) suggest that the 2 kinases have overlapping substrate specificities in vitro.

The amino acid residues to which the phosphoryl group is transferred is usually serine or threonine but recently phosphotryosine has also been detected (Eckhart et al., 1979) in the transforming proteins of tumour viruses. Tyrosine protein kinase activity has been found in rat spleen and other tissues (Swarup et al., 1983) and a primary effect of 2 cellular growth factors is to activate intracellular tyrosine protein kinases (Ushiro et al., 1980).

It is important to consider criteria that have to be satisfied to establish that a given enzyme undergoes physiologically significant phosphorylation-dephosphorylation:-

1. Demonstration that the enzyme can be phosphorylated in vitro at a significant rate by an appropriate protein kinase and dephosphorylated by a phosphoprotein phosphatase.

2./

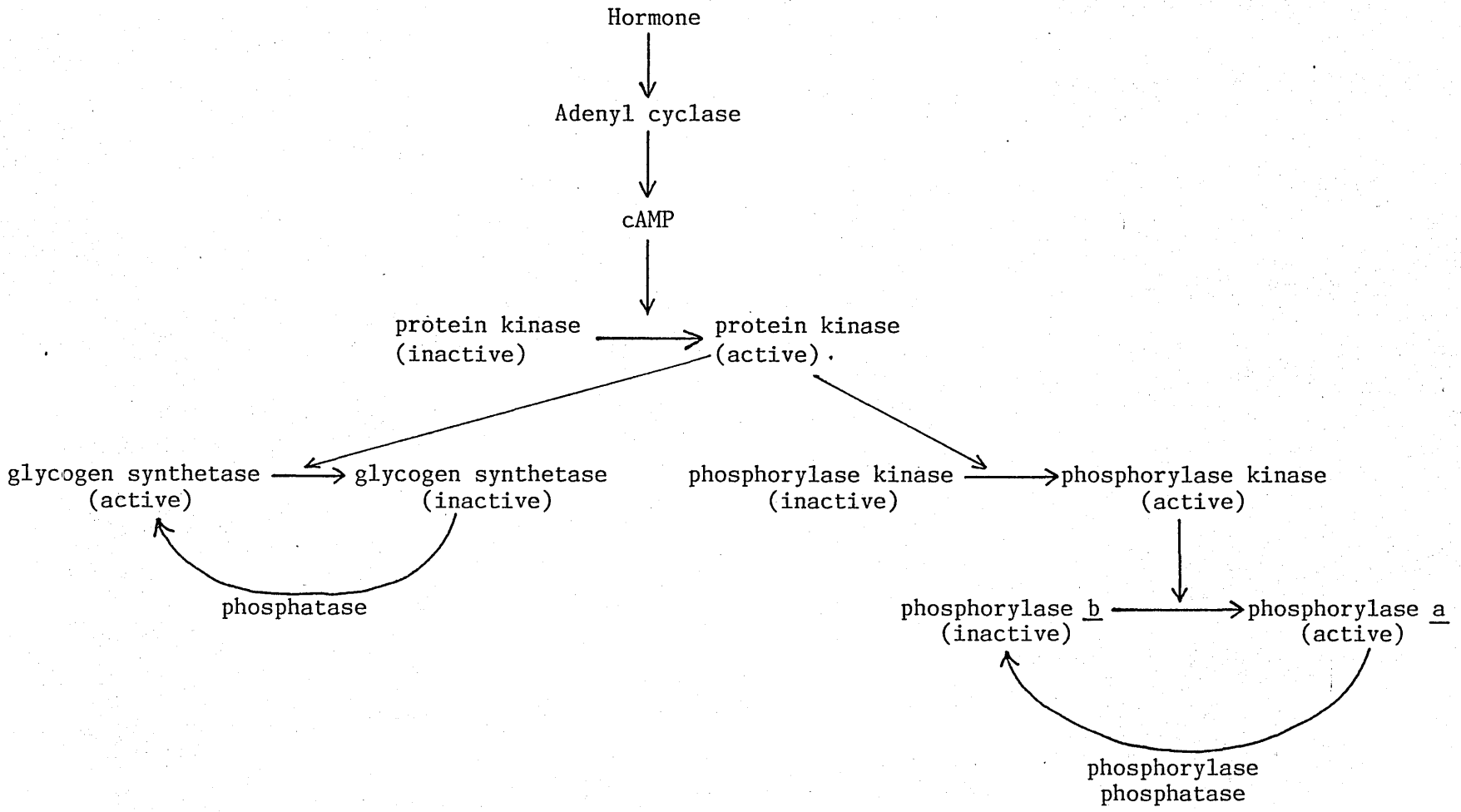
2. Demonstration that the functional properties of the enzyme undergo meaningful changes that correlate with the degree of phosphorylation.
3. Demonstration that the enzyme can be phosphorylated and dephosphorylated *in vivo* or in the intact cell and that this accompanies functional changes.

Discovery of enzyme regulation by reversible phosphorylation stems from the discovery that neural and hormonal control of glycogen metabolism in skeletal muscle was mediated by changes in the phosphorylation state of glycogen phosphorylase (Krebs *et al*, 1956), phosphorylase kinase (Krebs *et al*, 1959) and glycogen synthetase (Friedman *et al*, 1963).

Hormones bind to receptors on the cell membrane activating formation of cAMP which in turn activates the protein kinase (Figure 10). The cAMP-PrK then catalyses the phosphorylation of phosphorylase kinase converting it into its active form. The active phosphorylase kinase then phosphorylates phosphorylase *b*, the modified enzyme being the active phosphorylase *a* which is responsible for glycogen breakdown. At the same time that cAMP turns on phosphorylase *a* through this cascade mechanism, it activates the cAMP-PrK to phosphorylate the active form of glycogen synthetase but in this case phosphorylation causes inactivation thus inhibiting glycogen synthesis. Thus the 2 opposing pathways of glycogenolysis and glycogen synthesis may be regulated in an synchronous manner.

A number of other enzymes are regulated by cAMP-PrK *in vitro* and/

FIGURE 10: Role of cAMP-Pr in glycogen metabolism.



and are likely to be physiological substrates for this enzyme. cAMP-PrK is a highly specific enzyme and phosphorylates few proteins at significant rates.

The activation of triglyceride lipase (Steinberg, 1976) and the inactivation of glycerol-phosphate acyltransferase (Nimmo, 1980) by phosphorylation by cAMP-PrK may coordinate triglyceride breakdown and synthesis in adipose tissue in response to Adrenalin. The activation of cholesterol esterase increases the pool of cholesterol in the adrenal cortex in response to ACTH (Boyd et al., 1980). Phosphorylation of acetyl CoA Carboxylase decreases its activity (Hardie et al., 1980), while phosphorylation also inhibits pyruvate kinase and increases the K_m for phosphoenolpyruvate and makes it more sensitive to inhibitors (Engstrom, 1980).

Phosphorylation of troponin I in cardiac muscle fibres decreases the affinity of troponin-C for Ca^{2+} and may contribute to the adrenalin induced relaxation of cardiac muscle (England, 1980). Phosphorylation of phospholamban in cardiac sarcoplasmic reticulum is associated with activation of the sarcoplasmic reticulum ATPase and increases Ca^{2+} uptake (England, 1980).

It was perhaps fortunate that the first example of enzyme regulation by reversible phosphorylation (activation of glycogen phosphorylase) should involve phosphorylation at a single site by a single protein kinase since it greatly facilitated elucidation of the complex effects of covalent modifications on the kinetic properties. This situation is relatively uncommon however and 'multi-site' phosphorylation, which increases the regulatory potential/

potential of enzymes, is turning out to be the rule rather than the exception. Phosphorylation at one site may amplify or even antagonise effects of phosphorylation at other sites or alter the rates at which they are phosphorylated or dephosphorylated. Phosphorylation at different sites by different protein kinases enables enzymes to respond to several physiological stimuli and in such situations interactions between phosphorylation sites may represent the mechanism by which one stimulus influences another.

Phosphorylase kinase is composed of 4 subunits $\alpha\beta\gamma\delta$ and has the overall structure $(\alpha\beta\gamma\delta)_4$. Phosphorylase kinase is phosphorylated at 2 sites by cAMP-PrK, one on the α subunit and one on the β subunit and activation correlates with the phosphorylation of the β subunit (Cohen, 1973).

Glycogen synthetase is phosphorylated by 5 different kinases. The general effect is to decrease activity but different phosphorylation sites have different activities. Phosphorylation increases the Km for substrate, decreases the Km for inhibitors and increases the Ka for activators (Roach, 1982). Multiple site phosphorylation produces cooperative interactions among the phosphorylation sites (De Paoli-Roach et al, 1983).

Pyruvate dehydrogenase is inhibited by phosphorylation by a cAMP independent protein kinase. Pyruvate dehydrogenase is a multienzyme complex and is phosphorylated on the α subunit of the pyruvate decarboxylase component causing inactivation. Phosphorylation also occurs at other sites but their significance is not known (Nimmo & Cohen, 1977).

Phosphofructokinase/

Phosphofructokinase is activated via phosphorylation by a cAMP independent protein kinase (Brand & Soling, 1975). Histone H1 is phosphorylated by cAMP-PrK (Langan, 1969) and is also phosphorylated by another kinase at distinct sites although the role of the phosphorylation is not very clear.

Calcium ions can also influence the activity of protein kinases. Many of the biological actions of Ca^{2+} are mediated by the Ca-binding protein calmodulin. The ability of calmodulin to function as a Ca-dependent regulator of enzyme activity is a consequence of conformational changes following its binding of Ca^{2+} which leads to the formation of specific interaction domains.

In mammalian cells there is only one catalytic subunit of cAMP-PrK that phosphorylates a variety of proteins, whereas many different protein kinases, with restricted substrate specificities, are activated by calmodulin. The cAMP and calmodulin pathways are closely interlinked and it has been suggested that cAMP and Ca^{2+} - calmodulin dependent protein kinases phosphorylate the same proteins, although at distinct sites, e.g. glycogen synthetase (Payne & Soderling, 1980) and phospholamban (Le Peuch et al., 1979).

The action of many hormones and neurotransmitters depends on the hydrolysis of membrane phosphoinositides (Nestler & Greenberg, 1983). The phosphoinositides breakdown rapidly in response to receptor mediated activation (Majerus et al., 1984) to give diacylglycerol and inositol -1, 4, 5 triphosphate ($1P_3$) which function as second messengers to activate two independent but parallel signal pathways in a synergistic manner. These in turn stimulate a wide variety of cellular/

cellular processes. Diacylglycerol functions within the plane of the membrane to increase protein phosphorylation via activation of Ca-dependent protein kinase C. It does this by increasing its affinity for Ca^{2+} (Nishizuka, 1984), while IP_3 is released into the cytosol to mobilise Ca^{2+} from intracellular stores (Berridge, 1984; Nishujuha, 1984). This synergistic role is found in catecholamine release from adrenal medullary cells (Knight & Baker, 1983), aldosterone secretion from adrenal glomerulosa cells (Kojima et al., 1983) and insulin release from the pancreas (Zawalich et al., 1983).

Tumour promoting esters such as TPA (tetradecanoyl phorbol acetate) have been shown to stimulate protein kinase C in the same way as diacylglycerol (Majerus et al., 1984) and the results suggest that the tumour promoters are intercalated into the membrane to modify the microenvironment thus leading to activation of the kinase (Nishijuha, 1984).

Kinase activity has also been found associated with the receptors for growth hormone and insulin (Strosberg, 1984). These receptors possess an intrinsic tyrosine specific protein kinase which may mediate the activity of the peptide factors (Carpenter & Cohen, 1984). The ligand binds on the outside of the cell membrane while the kinase domain is on the inside (Hunter, 1984), therefore the kinase is probably activated by a conformational change in the receptor. The binding of epidermal growth factor (EGF) to its receptor results in the phosphorylation of a number of endogenous membrane/

membrane proteins including EGF receptor (Cohen et al., 1980) (Figure 11). Tumour promoters block this hormonal stimulated tryrosine phosphorylation of the EGF receptor although they do not block EGF binding to the receptor. This again suggests a role for protein kinase C (Friedman et al., 1984) especially as recent evidence suggests that the major receptor for tumour promoting agents and the protein kinase C copurify (Ashendel et al., 1983; Hunter 1984).

Transforming genes of retroviruses display homologies with a class of cellular genes that are normally concerned with regulating cell growth. Evidence suggests that some of these oncogenes code for growth factors or their receptors. Growth factor receptor and viral transforming proteins both have tyrosine kinase activity, and in both cases this tyrosine kinase activity is associated with cell proliferation. The mechanism by which the signal is transmitted from the receptor into the cell is unknown but is likely to involve tyrosine kinase phosphorylation of cytoplasmic proteins. Barkerkow & Bauer (1984) have demonstrated the differential expression of the src gene product (the normal cellular analogue of the transforming protein of Rous sarcoma virus) and its phosphokinase activity by showing an age and tissue dependent difference.

New examples of enzymes undergoing phosphorylation-dephosphorylation reactions are being revealed at a steady rate. The recent increase in knowledge concerning these enzymes and their substrates is leading to a more detailed understanding of the regulation of many/

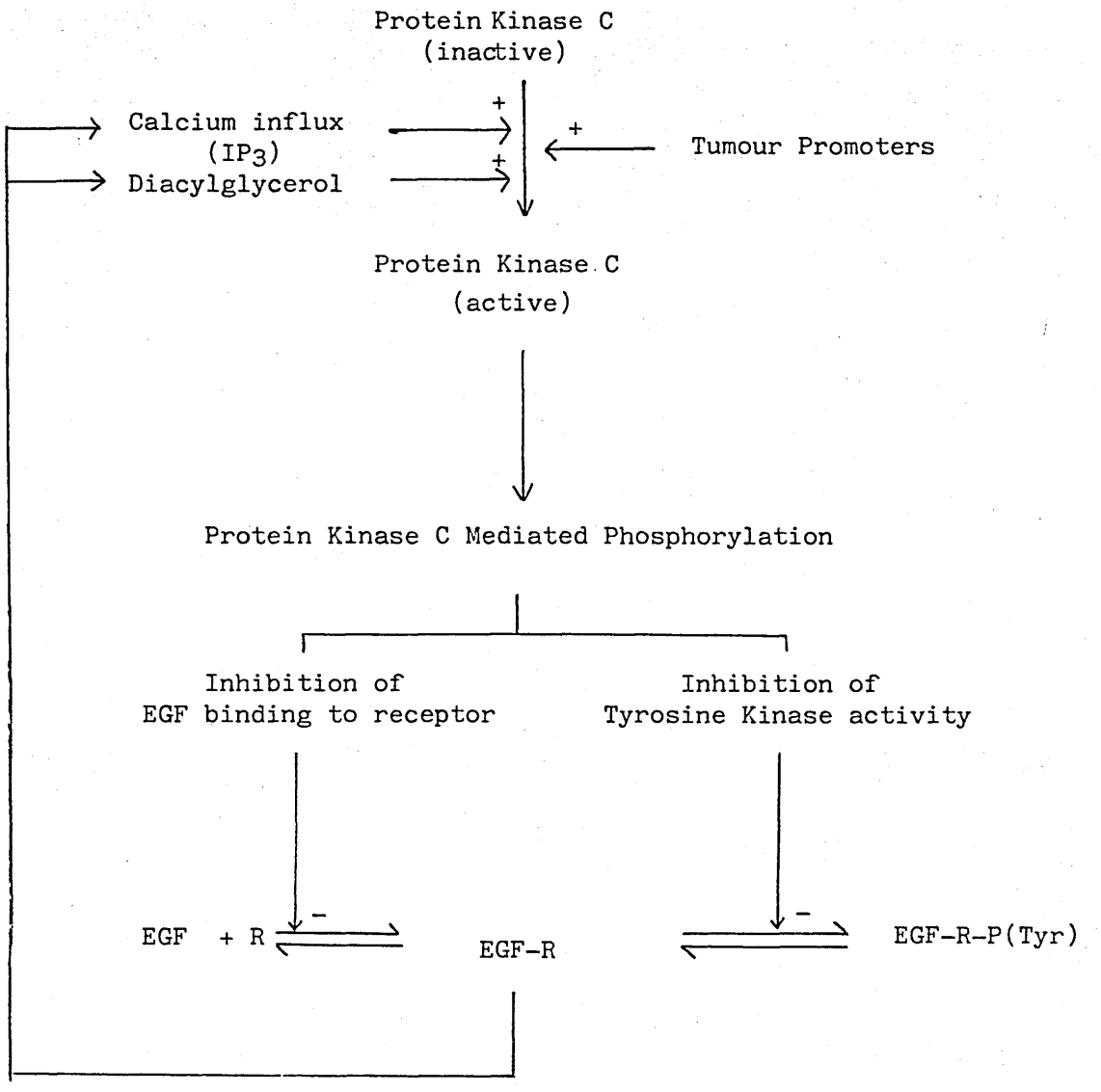


FIGURE 11: Proposed model for role of EGF-receptors in the activation of Protein Kinase C.

(EGF : epidermal growth factor

R : membrane receptor

EGF-R-P(Tyr) : Tyrosine phosphorylated EGF Receptor

+ : stimulation

− : inhibition)

many physiologically important processes. Current evidence suggests that there is an integrated network of regulatory pathways, mediated by reversible phosphorylation, that allows diverse cellular events to be controlled by neural and hormonal stimuli.

MATERIALS AND METHODS

MATERIALS.

1. Suppliers

Unless otherwise specified, all chemicals used were, as far as possible, AnalaR Grade supplied by B.D.H. Chemicals Ltd. Where chemicals and equipment were obtained from other sources, this is indicated in the text and a list of the names and addresses of the suppliers is given below.

B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

Beckman Spinco Ltd., Pala Alto, California, U.S.A.

Bio-Rad Laboratories Ltd., Watford, Herts., U.K.

Corning Glass Works, New York, U.S.A.

Eastman-Kodak Co., Rochester, New York, U.S.A.

Jencons Ltd., Hemel Hempstead, Herts., U.K.

Koch-Light Laboratories, Colnbrook, Bucks., U.K.

L.K.B. Instruments Ltd., South Croydon, Surrey, U.K.

Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Amersham International plc, Amersham, Bucks., U.K.

P.L. Biochemical Inc., Wisconsin, U.S.A.

Schleicher & Schull, Dassel, W. Germany.

Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

Whatman, Cambridge, England, U.K.

2. Experimental Animals

Male albino rats, derived from the Wistar Strain, were bred in the departmental animal house. All animals were 200-250g in weight and/

and were starved overnight prior to death.

3. Glassware

Glass homogenisers with motor driven Teflon pestles were purchased from Jencons Ltd., and Corex glass centrifuge tubes were obtained from Corning Glass Works. Pipettes, glass centrifuge tubes, test tubes and other glass items were sterilized at 160°C for at least 6 hours before use. Plastic tubes and most solutions were autoclaved.

Cellulose nitrate tubes for ultracentrifugation were obtained from Beckman Spinco Ltd., and were rinsed in sterile distilled H_2O before use.

4. Radioisotopes and Materials for Liquid Scintillation Counting

A. Amersham International plc, Amersham:

$[\gamma - {}^{32}P]$ ATP

$[{}^{32}P]$ orthophosphate

B. Koch-Light Laboratories Ltd.

2,5 diphenyloxazole (PPO) - Scintillation Grade

Toluene - AnalaR Grade

5. Electrophoresis

A. B.D.H. Chemicals Ltd.

Acrylamide - electrophoresis grade

Bisacrylamide - electrophoresis grade

Coomassie Blue R250

Urea - AristaR Grade

SDS/

SDS - electrophoresis grade

Ammonium persulphate - AnalaR Grade

B. Koch-Light Laboratories Ltd.

Bromophenol Blue

TEMED

Mercaptoethanol

C. Pharmacia

Ampholines - 40% solution pH range 3.5 - 10

6. Metabolites

A. Sigma Chemical Co.

cyclic-3'-5'-AMP

cyclic-3'-5'-GMP

B. P-L Biochemicals Inc.

ATP - sodium salt

7. Gel Exclusion Chromatography

A. Bio-Rad Laboratories

Bio Gel A - 0.5m

B. Pharmacia

Sephadex G-100 - fine

8. Ion Exchange Chromatography : Whatman

Phosphocellulose - P11

DEAE-Cellulose - DE52

CM-Cellulose - CM52

9./

9. Protein Kinase Activity

A. Schleicher & Schull

Nitrocellulose filters - 25mm, 0.45μ

B. Sigma Chemical Co.

Casein - hydrolysed and partially dephosphorylated

Histone - Type II (Calf thymus)

METHODS.

1. Preparation of Nuclei

Rat liver nuclei were prepared by modification of the method of Chauveau et al., 1956. Livers of rats (200-250g) were finely chopped with dissecting scissors at 4°C and then homogenised at 20ml 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 4 layers of muslin and the filtrate layered over 7ml cushions of 2.3M sucrose, 10mM $MgCl_2$ in SW27 centrifuge tubes. The homogenate was then centrifuged at 40000 x g_{av} for 1 hour in a Beckman SW27 rotor. The supernatant was removed leaving a nuclear pellet.

2. Preparation of hnRNP Particles

The extraction method of Samarina et al., 1968 was employed. The nuclear pellet was resuspended in STM7 buffer (100mM NaCl, 10mM Tris HCl pH 7.0, 1mM $MgCl_2$). The nuclear suspension was gently stirred at 0°C for 15 minutes and the nuclei recovered by centrifugation at 6000 x g_{av} for 10 minutes in a Sorval HB4 rotor. The supernatant was discarded and the residual nuclei were suspended in the same buffer at pH 8.0 (STM8), stirred at 0°C for 1 hour and centrifuged to recover the nuclei. This was repeated twice more, pooling the pH 8.0 extracts. This extract was then applied to the top of 38ml 15-30% sucrose density gradients, which were then centrifuged at 78000 x g_{av} in a Beckman SW27 rotor for 15-17 hours. Completed gradients were then harvested from the bottom by suction through a Sigma peristaltic pump via a narrow tube introduced from the/

the top of the gradient. OD_{260nm} was continuously monitored by passing the eluate through the flow cell of a Gilford 240 spectrophotometer. The portions of the gradients containing hnRNP particles were collected for further analysis.

3. Fractionation of HnRNP Particle Proteins

Many chromatographic fractionation techniques were tried. Some of these are described in the relevant section of results. The methods presented here are limited to those that constitute standard methods employed.

3.1 Gel Exclusion Chromatography

This was done using either BioGel A - 0.5m (Bio Rad Ltd.) or Sephadex G-100. For gel filtration of hnRNP particles on BioGel A - 0.5m, an all glass Pharmacia column of internal diameter 3cm, was filled to a height of 30cm with degassed BioGel A - 0.5m, pre-equilibrated at 4°C with STM8 (100mM NaCl, 10mM Tris HCl pH 8.0, 1mM $MgCl_2$). The column was arranged with the entry port within 0.5cm of the surface of the gel and a speed flow of $10cm^3$/hour set on a peristaltic pump which was placed at the exit port. HnRNP particles, suspended at 1mg/ml in STM8, were applied to the surface of the column.

HnRNP particles treated with 1M NaCl were subjected to gel filtration on Sephadex G100. An all glass column of internal diameter 2.5cm was filled to a height of 85cm with degassed Sephadex G-100, pre-equilibrated with 10mM Tris HCl pH7.5, 1mM EDTA, 1M NaCl, 6mM β-mercaptoethanol. The column was run at a speed of $10cm^3$/hour set on a peristaltic pump placed at the exit port.

On/

On both columns the void volume of the column was determined by using blue dextran and fractions were collected in a LKB automatic fraction collector.

3.2 CM - Cellulose Fractionation

This was done by disrupting purified hnRNP particles with 1M NaCl and adding a known amount of protein to CM-cellulose in dialysis tubing and dialysing to reduce the salt concentration. The slurry was collected in a test tube, spun at 2500rpm for 10 minutes and the 'supernatant' collected. Sequential elution of the proteins was carried out by bringing the slurry to 250 mM with respect to NaCl, mixing for 5 minutes and collecting the supernatant. This was repeated with 500mM, 750mM, 1M, 2M NaCl and 6M urea.

4. Techniques used in the Analysis of hnRNP Particles

4.1 SDS-Polyacrylamide Gradient Gel Electrophoresis

This was performed using modifications of the method of Jeppeson (1974). An all glass apparatus as described by O'Farrell (1975) was used, and consisted of 2 glass plates (one notched and the other plain), two 1mm thick perspex spacers, one piece of compressible silicon tubing and 4 large bulldog clips. A second rubber tube was placed inside the sealing tube, through which the polymerising mixture was poured from a perspex gradient maker. 17mls of a 5% w/v acrylamide solution (acrylamide: bis-acrylamide ratio of 30:1 in 0.375M Tris HCl pH8.8, 20% w/v sucrose, 1.2% w/v SDS), plus 15 µl 10% v/v TEMED was placed in the chamber nearest the exit. 17mls of a 15% w/v acrylamide solution (acrylamide:bis-acrylamide ratio of 30:1 in 0.375M Tris HCl pH8.8, 10% w/v sucrose, 1.2% w/v /

w/v SDS) plus 10µl 10% v/v TEMED was placed in the other chamber. 20µl 10% w/v $(NH_4)_2$ SO_8 was added to each chamber. The gel was then overlaid with isobutanol. A 3% w/v stacker in 0.12M Tris HCl pH6.8, 0.1% w/v SDS was allowed to set round a teflon well-forming comb. Electrophoresis was performed using SDS-gel running buffer (25mM Tris, 192mM glycine pH8.8, 10% w/v SDS) at 35mA until the bromophenol blue marker was 1cm from the bottom of the gel. 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 in 50% v/v methanol, 10% v/v acetic acid until the background was clear. The gels were then dried on to 3mm filter paper under vacuum on a Bio Rad gel drier.

4.2 Two-Deminsional Gel Electrophoresis

4.2.1 NEPHGE: Non-equilibrated pH gradient electrophoresis gels were prepared as described by O'Farrell et al., (1977). Pyrex tubes of internal diameter 3.5mm and length 12cm were prepared for electrophoresis by washing with Decon followed by thorough washing in water and air drying. One end of each tube was sealed with a rubber cap and the tubes were arranged in a vertical position.

The gel solution was prepared by mixing 5.5g AristaR urea, 1.33ml of a mixture of 28.38% w/v acrylamide and 1.62% w/v bis-acrylamide, 2mls 10% w/v N-P40 (Nonidet P40), 2ml H_2O and 0.5ml 40% w/v ampholines (pH3.5-10). The gel mix was degassed and 15µl 10% w/v $(NH_4)_2S_2O_8$ and 10µl TEMED added. The polymerising mixture was then poured into the gel tubes to a height of 10cm via a teflon cannula. Gels were overlaid with 20µl distilled water/

water and left for 2 hours.

NEPHGE gels were loaded anodically and electrophoresis was performed using a disc electrophoresis apparatus. 10mM phosphoric acid was placed in the upper chamber (anode) and 20mM NaOH in the lower. Electrophoresis was carried out for 4 hours at 500 volts (2000 v.hr). Protein preparation (either lyophilisates or acetone, ethanol or TCA precipitates) were dissolved at 5-15mg/ml in lysis buffer (9.5M urea, 5% v/v mercaptoethanol, 2% w/v N-P40, 2% w/v ampholines pH3.5-10). Between 100-500µg of protein was applied to the gel. After electrophoresis the gels were incubated for 10 minutes in SDS sample buffer (2.3% w/v SDS, 5% v/v mercaptoethanol, 10% v/v glycerol, 62.5mM Tris HCl pH6.8, 0.01% w/v bromphenol blue). If the gels were not to be run in the second dimension immediately, they were stored in sample buffer at -20°C.

4.2.2 Second dimension fractionation: SDS polyacrylamide gel electrophoresis was performed essentially as described by Le Stourgeon & Beyer (1978). An all glass slab gel apparatus as described by O'Farrell (1975) was used and consisted of 2 glass plates (one notched, the other plain), two 1mm thick perspex spacers, one piece of compressible silicon tubing and 4 large bulldog clips.

For an 8.75% w/v polyacrylamide gel, 18ml distilled H_2O, 10ml resolving gel buffer (1.48M Tris HCl pH8.8, 0.25% w/v SDS), 11.4mls acrylamide solution (30% w/v acrylamide, 0.8% w/v bisacrylamide) were mixed, degassed and polymerisation initiated by the addition of 150µl 10% w/v $(NH_4)_2S_2O_8$ and 10µl TEMED. The mixture was then rapidly pipetted into the assembled apparatus. Gels were overlaid with/

with isobutanol to provide a level gel surface. Pre-incubated first dimension gels were applied to the polymerised slab gel by squeezing them into direct contact with the gel surface (Garrels, 1979). Electrophoresis was performed using SDS gel running buffer (25mM Tris, 192mM glycine pH8.8, 10% w/v SDS) at 35mA/gel until the bromophenol blue marker was 1cm from the bottom of the gel. Completed gels were stained, destained and dried as for the SDS gradient gels.

4.3 SDS-Polyacrylamide Gel Electrophoresis

This was performed using the slab apparatus as described by O'Farrell (1975). An 8.75% w/v polyacrylamide gel was prepared as for the second dimension gels (See Methods 4.2.2). A 3% w/v stacking gel was prepared by mixing 6.5ml disilled H_2O, 2.5ml stacking gel buffer (0.536M Tris HCl pH6.8, 0.25%w/v SDS), 1ml acrylamide (30% w/v acrylamide, 0.8% w/v bisacrylamide). The mixture was degassed and polymerisation initiated by adding 10µl 10% w/v $(NH_4)_2S_2O_8$ and 10µl TEMED and was poured round a teflon well-forming comb. Protein samples were dissolved in sample buffer (0.1M sucrose, 0.1% w/v SDS, 1% v/v mercaptoethanol, 0.2M Na_2HPO_4, 0.2M NaH_2PO_4, 0.05% w/v bromophenol blue).

4.4 Non-denaturing Polyacrylamide Gel Electrophoresis

The apparatus used was the same as that used for the second dimension gels (4.2.2). For a 7% w/v polyacrylamide gel, 6mls of solution A (36.3g Tris, 48ml 1M HCl, 300µl TEMED), 12mls solution B (28% w/v acrylamide, 0.74% w/v bisacrylamide) and 6mls distilled H_2O were mixed and degassed. Polymerisation was initiated by adding/

adding 24mls of a $(NH_4)_2S_2O_8$ solution (1.4mg/ml). Gels were overlaid with isobutanol and left for one hour to set. 1% w/v agarose stacker in 10.7mM Tris was then poured. Protein samples were brought to 10% w/v glycerol before being loaded. Electrophoresis was performed using 10.7mM Tris, 76.8mM glycine, 0.1% v/v mercaptoethanol at 10mA/gel. Gels were fixed and stained in 50% v/v methanol, 10% v/v acetic acid containing 0.1% w/v Coomassie blue R250 for one hour at 40°C. The gels were then destained in 10% v/v methanol, 10% v/v acetic acid.

4.5 Protein Kinase Assay

This was performed using a modification of the method of Kish and Kleinsmith (1974). The assay volume of 500µl contained 7.5µmoles Mg Acetate, 400µl sample in 50mM MOPS pH6.5, 100µg casein and 0.1nmoles $[\gamma-^{32}P]$ ATP at 100mCi/mmol. All tubes were held on ice prior to initiation of the reaction with addition of Mg Acetate. The assay was incubated at 30°C for 10 minutes and was terminated by the addition of 3mls of 1mM ATP to 150µl of the assay volume. Then 3mls of ice cold 10% w/v trichloroacetic acid, 3% w/v Na pyrophosphate was added and the mixture filtered through nitrocellulose membrane filters (0.45µm) presoaked in 1mM ATP. Each filter was washed twice with 5ml of 5% w/v trichloroacetic acid, 1.5% w/v Na pyrophosphate. The filters were oven-dried and counted by liquid scintillation counting in 5mls of toluene/PPO.

4.6 Kinase Detection in Non-denaturing Polyacrylamide Gels

Non-denaturing gels with 1% w/v agarose stackers were prepared and run as described in Methods (4.4). Protein kinase activity was assayed/

assayed in the gel by the method of McClung & Kletzien (1981). After electrophoresis the gels were soaked in 20mM Hepes pH7.2 at 30°C for 30 minutes with 2 changes of buffer. The buffer was removed and the gels were soaked in 20mM Hepes pH7.2 containing Histone or casein at 3-7.5mg/ml. After 30 minutes at 30°C the gels were incubated at 37°C for 20 minutes in 20mM Hepes pH7.2, 10mM $MgCl_2$, 1mM EGTA, 1mM dithiothreitol, and 1µM ATP (approximately) 0.15mCi $[\gamma\text{-}^{32}P]$ ATP per gel per 60mls buffer). Gels were rinsed in 5% w/v ice cold trichloroacetic acid and left overnight ir 1L 5% w/v trichloroacetic acid with gentle agitation, washed for 7-8 hours in 5% w/v trichloroacetic acid and left overnight in 10% w/v acetic acid. The gels were dried on to 3mm filter paper under vacuum on a Bio Rad gel drier and autoradiographed using Kodak X-Omat H film.

5. Protein Estimation

5.1 Bradford Protein Assay

This assay is based on the binding of Coomassie Blue G250 to protein and the resulting spectral shift. The assay solution was prepared by dissolving 100mg G250 in 50mls 95% w/v ethanol. 100mls 85% w/v phosphoric acid was added and the solution filtered to remove any insoluble material and then made up to 1L. This solution is stable for 5-10 weeks if stored at 4°C. The assay consisted of 1ml of solution plus 20µl of the sample to be tested. The O.D. was measured at 595nm and the concentration of protein read directly from a standard curve which was constructed using BSA.

5.2 Bramhall Protein Assay

This assay was used when the protein samples were in buffer containing/

containing high concentrations of NaCl. Samples were blotted on to 2.5cm Whatman No. 1 filter discs, dried, washed in cold 7.5% w/v trichloroacetic acid and heated to 80°C for 30 minutes in 7.5% w/v trichloroacetic acid. The filters were then washed in 2 changes of 7.5% w/v trichloroacetic acid and stained in 10mg/ml Xylene Brilliant Cyanin G in 7% v/v acetic acid at 50°C for 15 minutes. Excess dye was removed with several washings with hot (50°C) 7% v/v acetic acid until the background was almost white. The filters were then washed in 50% v/v methanol, 10% v/v ether, then washed in ether, dried and each filter added to 5mls of destaining fluid (66mls methanol, 34mls H_2O, 1ml 0.88% ammonia). The O.D. of the solution was then measured at 610nm, and the concentration of protein read directly from a standard curve which was constructed using BSA.

6. Microdialysis

For dialysis of very small volumes (less than 200µl), where conventional dialysis is inadequate, the sample was placed in an Eppendorff reaction tube which has had the centre of its cap removed. A piece of dialysis tubing was placed over the top of the tube and it was recapped so that the semi-permeable membrane replaced the cut out cap. The Eppendorffs were briefly centrifuged in an inverted position in order to bring the sample into contact with the membrane. The inverted tubes were then placed in a polystyrene holder and allowed to float on dialysis buffer, making sure that there were no air bubbles between the membrane and the buffer.

RESULTS

1. Isolation and Characterisation of Rat Liver HnRNP Particles

1.1 Fractionation Sucrose Density Gradients

There is considerable ultrastructural data showing that nascent transcripts of eukaryotic structural genes become complexed with protein at a very early stage in their existence (Miller & Hamkalo, 1972; Malcolm & Sommerville, 1974, 1977). However the assumption that isolated hnRNP particles are the same as the particles observed *in vivo* is difficult to prove. Section 3 of the introduction discussed the approaches to this problem employed by other workers and some of these are repeated in the present study.

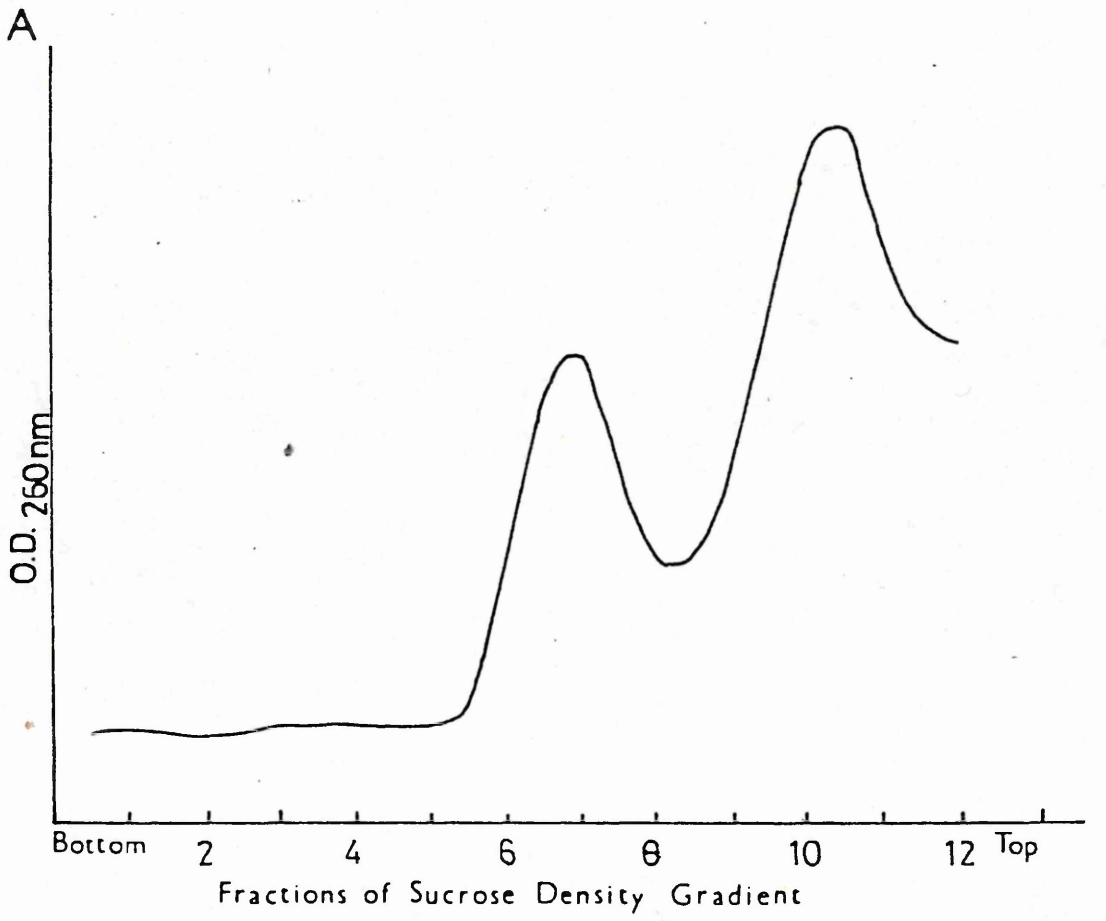
Rat liver nuclei were prepared as described in the methods section and examined by phase contrast microscopy to ensure that they were free from gross contamination with cytoplasmic debris. They were then extracted at pH7.0, a procedure which according to Samarina *et al.*, (1967) removed peri-nuclear ribosomes and nucleoplasmic pre-ribosomal particles, before being re-extracted three times at pH8.0 to recover hnRNP. When this extract was fractionated on sucrose density gradients, a single peak of hnRNP (Figure 12), the so called 40S peak was recovered as described by many other workers (Karn *et al.*, 1977; Beyer *et al.*, 1977; Wilks and Knowler, 1980). Furthermore, the protein profile of this peak (Figure 13) was characteristic of hnRNP and was dominated by the so-called proteins of molecular mass 26000-42000 and arrowed on Figure 13. Neither the 40S peak, the initial pH8.0 extract, nor even the pH7.0 extract, contained clearly/

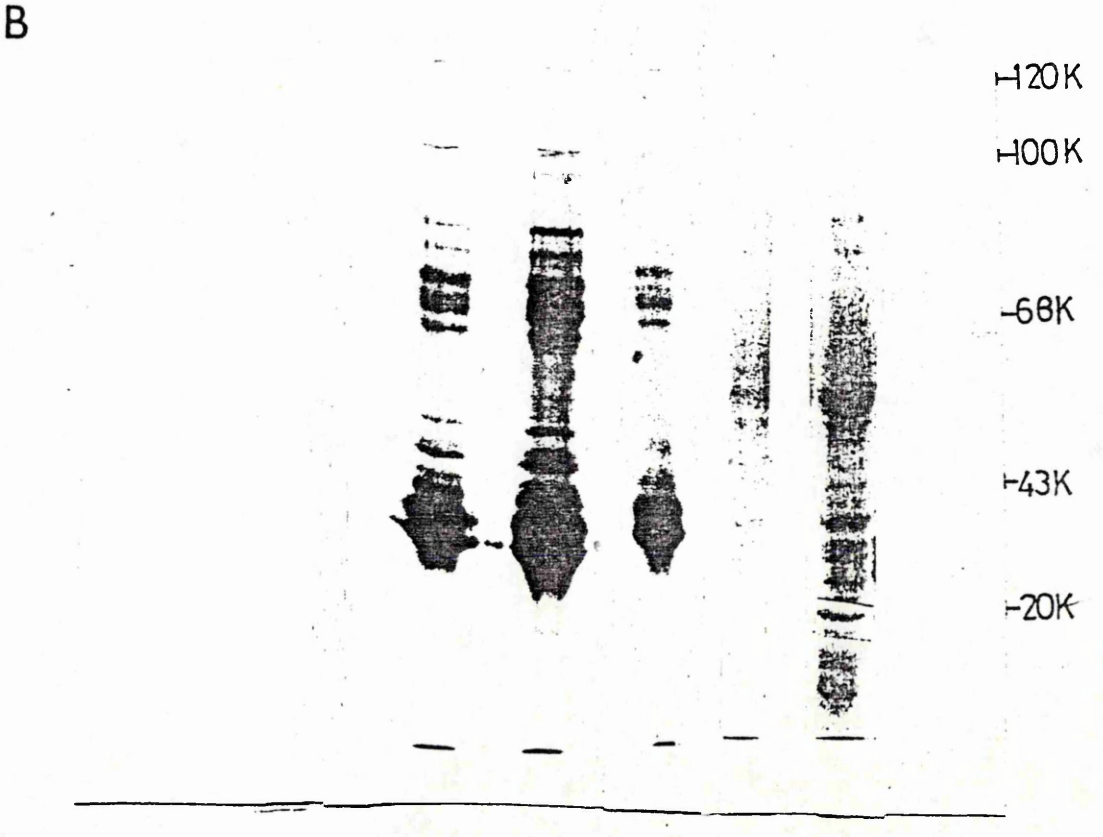
FIGURE 12: Analysis of the Protein Components of Each Fraction of a Sucrose Density Gradient

The pH8.0 extract of purified nuclei was layered onto 15-30% sucrose density gradients containing 100mM NaCl, 10mM Tris HCl. pH 8.0, 1mM $MgCl_2$ and centrifuged at 78000 x g_{av} for 15-17 hours in a SW27 rotor. The gradients were then scanned at 260nm by passing through a flowcell attachment of a Gilford 260 Spectrophotometer.

The 3 ml fractions collected from the gradient were dialysed against H_2O, precipitated with 3 volumes of ethanol, redissolved in SDS sample buffer and analysed on a 5-15% SDS polyacrylamide gradient gel as described in Methods (4.1).

The stained pattern (B) has been arranged below the $O.D._{260nm}$ trace of the sucrose density gradient (A) so that the relative positions correspond.


A
O.D. 260nm
Bottom
2
4
6
8
10
12
Top
Fractions of Sucrose Density Gradient


B
120K
100K
68K
43K
20K

clearly identifiable levels of ribosomal proteins or histones (Figure 13). The initial pH 7.0 extract contained no particulate matter detectable on sucrose density gradients (results not shown) nor did it contain core proteins. Some of its proteins were apparently identical to those of the pH 8.0 extract although most of these were non-particulate and remained at the top of the sucrose density gradient (compare Lanes 4, 5, 6 and 7 of Figure 13 and Figure 12). Those proteins which remained at the top of the sucrose density gradients included very little core protein. However some of the higher molecular mass bands appeared to be approximately equally partitioned between the particles and this non-particulate part of the pH 8.0 extract (compare Lanes 6 and 7 of Figure 13). This could be interpreted as contamination of the particles with non-particlate polypeptides. Alternatively, it might reflect the weak association of some hnRNP proteins and their normal partitioning between hnRNP and the nucleoplasm. As will be seen, the kinase activities associated with the particles showed a similar partitioning.

1.2 Characterisation on BioGel A-0.5m

The particlate nature of the hnRNP isolated from sucrose density gradients was further demonstrated by taking the peak from a sucrose density gradient, dialysing away most of the sucrose and running it on a second gradient to recover the same peak. Alternatively the 40S peak from a sucrose density gradient could/

FIGURE 13: Comparison of HnRNP Particle Proteins with Histones and Ribosomal Proteins

Samples of the pH 7.0 and pH 8.0 extracts of purified nuclei, 40S hnRNP, and fractions from the top of sucrose density gradients loaded with pH 8 extracts, and centrifuged at 78000 x g_{av} for 15-17 hours, were dialysed against H_2O, precipitated with 3 volumes of ethanol, redissolved in SDS sample buffer and analysed on 5-15% polyacrylamide gradient gels as described in Methods (4.1). The proteins present were compared with histones and ribosomal proteins.

Gel Lane No.	
1, 11	Markers
2, 10	Histones
3, 9	Ribosomal proteins
4	STM 7
5	STM 8
6	Top of sucrose density gradient
7	40S HnRNP

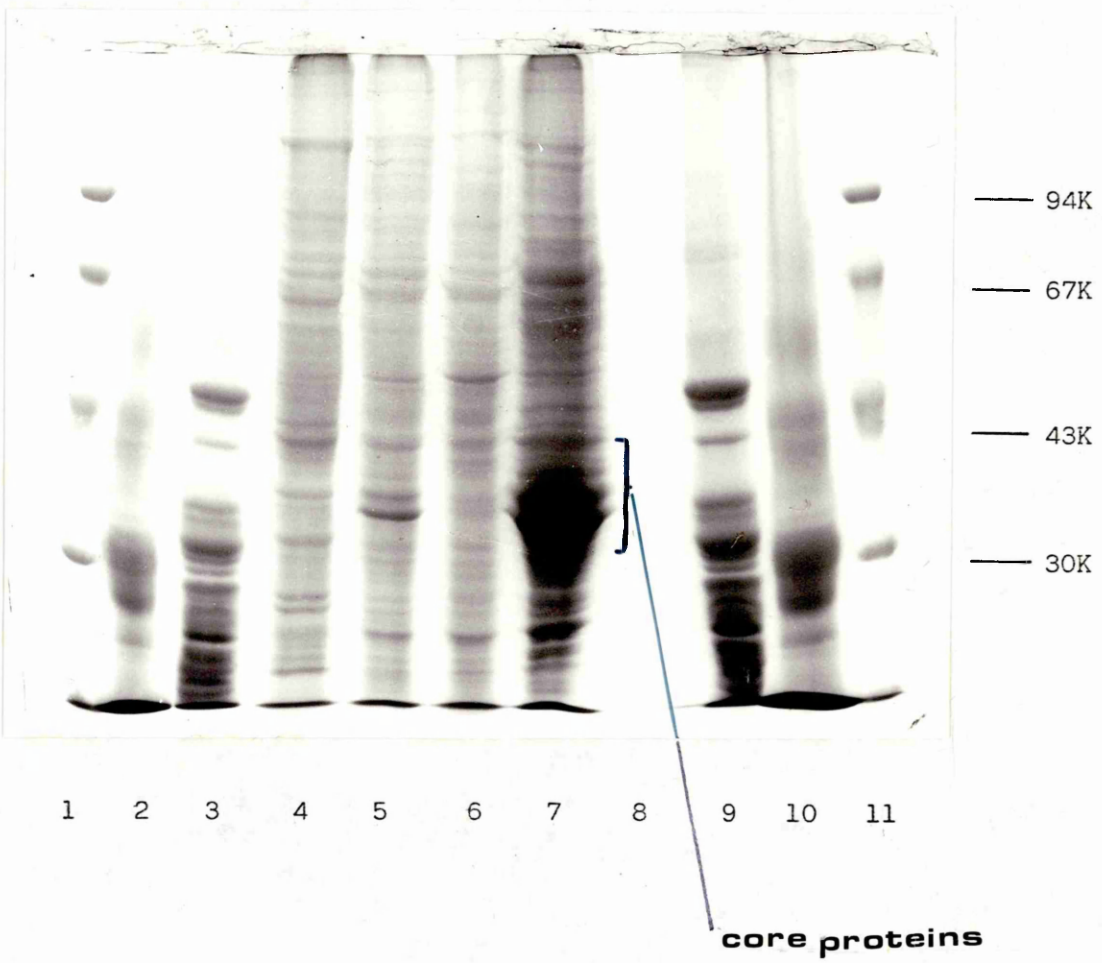
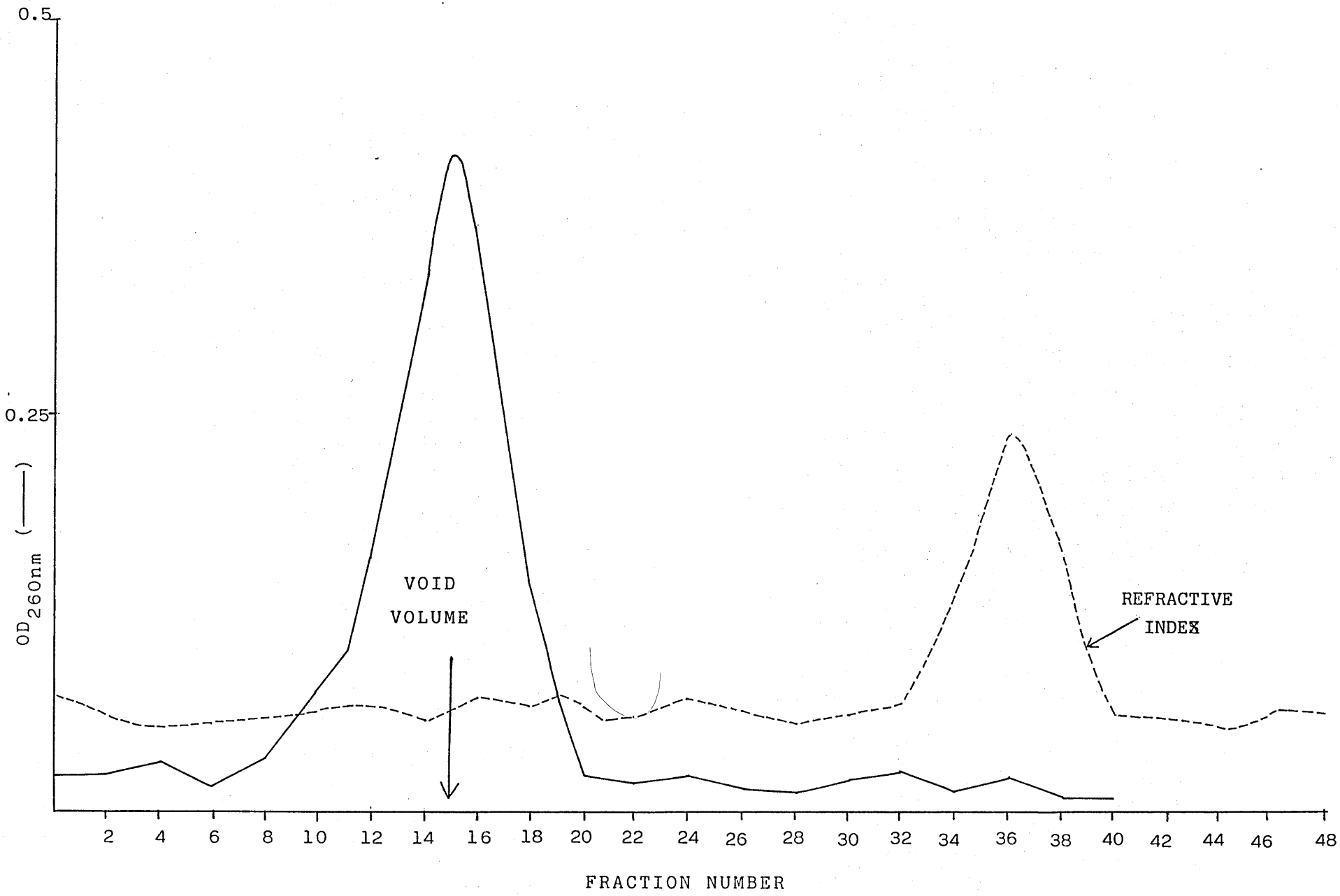

94K
67K
43K
30K
1 2 3 4 5 6 7 8 9 10 11
core proteins

FIGURE 14: Purification of hnRNP on Bio Gel

HnRNP particles isolated from 15-30% w/v sucrose density gradients, as described in Methods (2) were loaded onto a Bio Gel A-0.5m column as described in Methods (3.1). 5 mls fractions were collected and analysed for protein and refractive index to demonstrate separation of the hnRNP particles from the sucrose.


0.5
0.25
OD 260nm (——)
VOID
VOLUME
REFRACTIVE
INDEX
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
FRACTION NUMBER

could be placed directly onto a Bio Gel A-0.5m gel filtration column. The hnRNP particles were eluted with the void volume (Figure 14). For a while this appeared to be a better method than dialysis to remove the sucrose from hnRNP but yields were poor and aggregation appeared to be a problem.

1.3 Characterisation on One-Dimensional Polyacrylamide Gels

The protein profile of the 40S peak from sucrose density gradients showed considerable complexity on SDS polyacrylamide gradient gels (Figure 12), being dominated by 5-7 polypeptides of molecular weight 25000-45000, known as the core proteins, with 2 main bands and up to 5 minor bands. Early investigations of hnRNP's which used urea gels resolved a single polypeptide of molecular weight 40000 which was designated "informatin" (Samarina et al, 1968) but it has been shown (Niessing & Sekeris, 1971b; Gallinara-Matringe et al, 1975; Northeman et al, 1978; Suria & Liew, 1979) that this observation of a single polypeptide was an artefact of the urea based gel fractionation system employed. Subsequent analysis using SDS polyacrylamide gel electrophoresis has revealed between 14 (Niessing & Sekeris, 1971b) and 45 protein components (Gallinaro-Matringe et al, 1975).

Apart from core proteins other proteins are also present including those of molecular weight 120000, 100000, 70000 and 68000 which are seen in the 40S hnRNP profile (Figure 13) and which have all been seen by other workers (Karn et al, 1977; Beyer et al, 1977).

1.4/

1.4 Characterisation on Two-Dimensional Polyacrylamide Gels

Two dimensional electrophoretic fractionation is a powerful technique for analysis of a complex protein mixture. HnRNP particle proteins have been analysed in this way by a number of workers (Suria & Liew, 1979; Brunel & Lelay, 1979; Wilks & Knowler, 1980).

The more usual isoelectric focusing (IEF) coupled to polyacrylamide gel electrophoresis (O'Farrell, 1975) caused the loss of the basic core proteins (Wilks & Knowler, 1980) even if a basic range of ampholines (pH9-11) was employed in the first dimension or if the protein was added by anodic loading. The non-equilibrated pH gradient in the first dimension, which produced a pH gradient of pH 3.9-9.1 (Figure 15), gave a satisfactory fractionation of the complex proteins of hnRNP particles. It was used here to demonstrate that the particles isolated had the protein components described by others, and was used in subsequent experiments to analyse the proteins which could be phosphorylated. The two-dimensional fractionation of hnRNP particles and pH 7.0 and pH 8.0 extracts revealed that the pH 7.0 extract differed from the other two (Figure 16), although it is clear in this experiment that the pH 7.0 extract does contain some core protein.

On two-dimensional gels some of the core proteins fractionated into several polypeptides which exhibited heterogeneity in their charge (Figure 16A). The hnRNP particles also contained a large number of minor acidic polypeptides with molecular weights of greater than 45,000. The overall protein profile on these gels is in agreement/

FIGURE 15: pH Gradient of NEPHGE Gels

NEPHGE gels were prepared and run as described in methods (4.2.1). The gels were then cut into 0.9cm lengths, shaken gently for 5-10 minutes in 1ml 0.01M KCl and the pH of the eluate then measured.

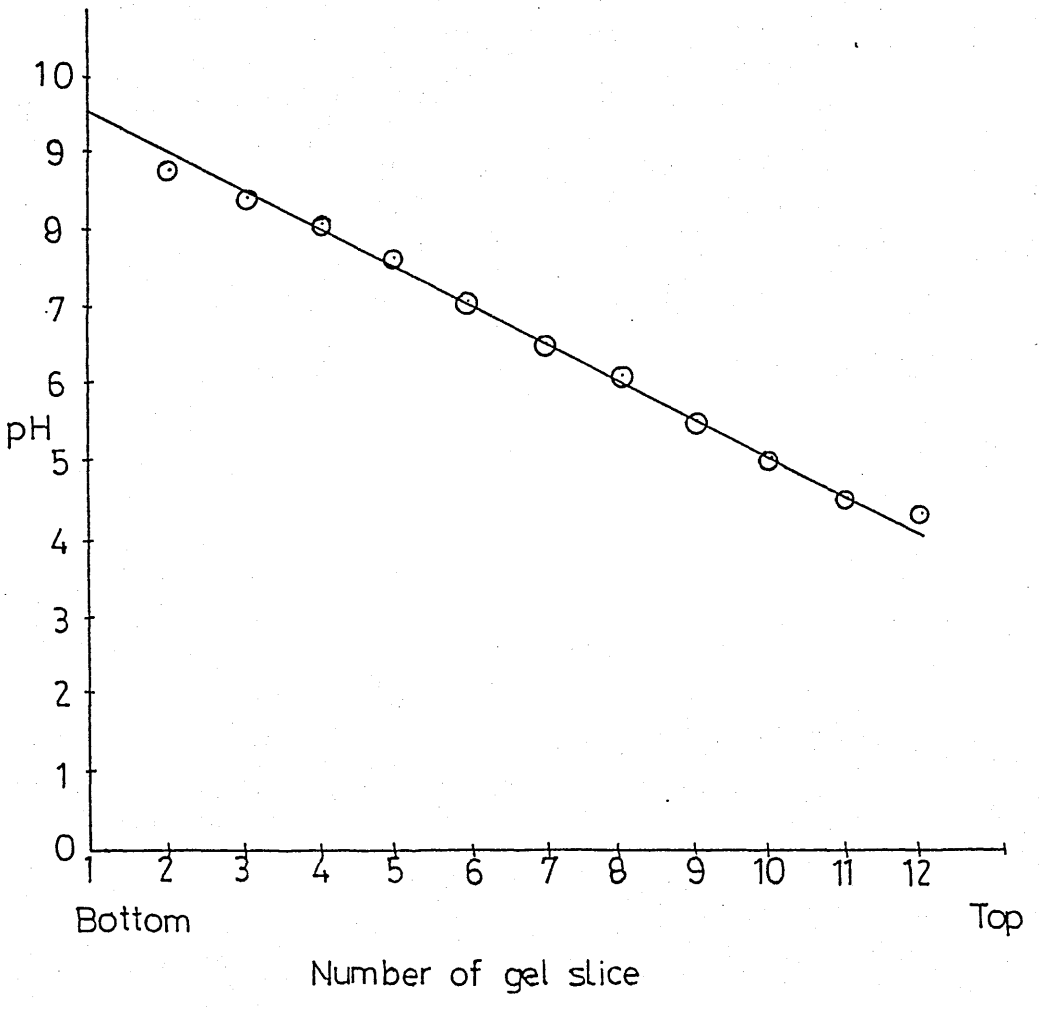

10
9
8
7
6
pH
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
12
Bottom
Top
Number of gel slice

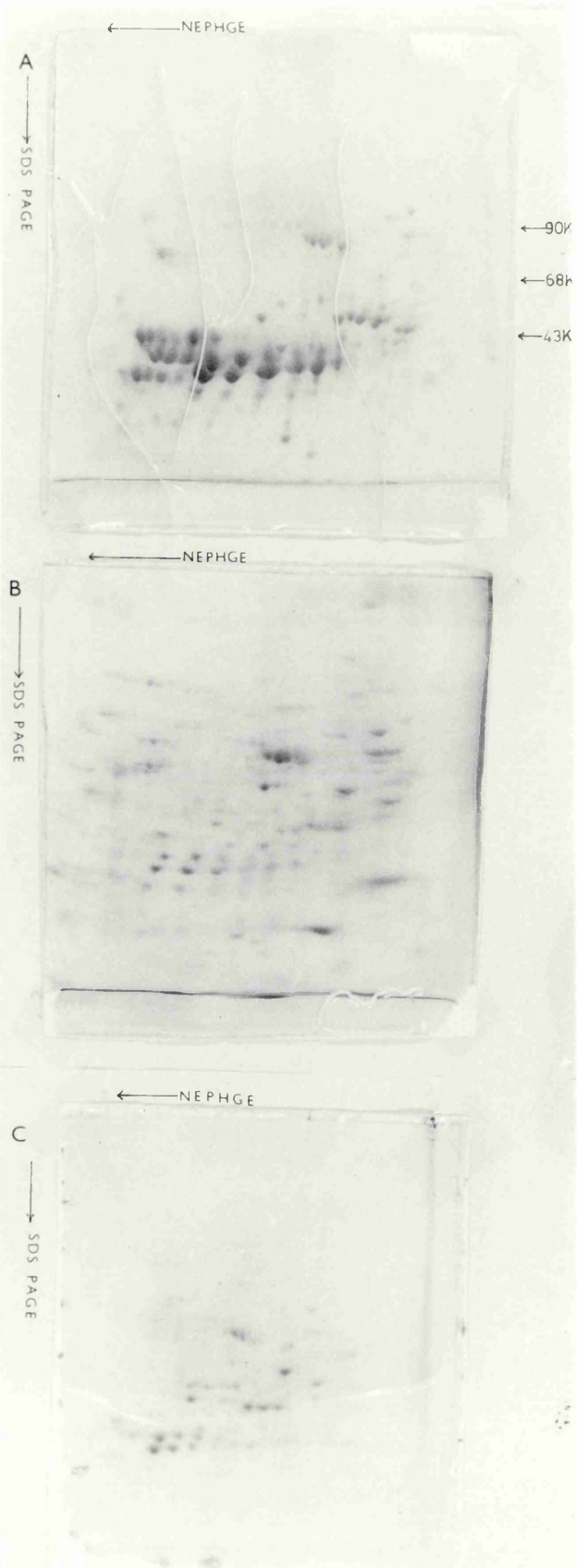
FIGURE 16: Two Dimensional Fractionation

Two dimensional gels were prepared and run as described in Methods (4.2) using NEPHGE gels in the first dimension and SDS Polyacrylamide gels in the second. HnRNP particles isolated from sucrose density gradients as described in methods (2), and STM7 and STM8 extracts of purified nuclei were dialysed against H_2O, precipitated with 2-3 volumes of ethanol and resuspended in lysis buffer (9.5M urea, 5% v/v mercaptoethanol, 2% w/v NP-40, 2% w/v pH 3.5-10 ampholines), before being loaded onto the NEPHGE gels.

A: 400µg hnRNP particles

B: 300µg pH7·0 extract of purified nuclei

C: 150µg pH8·0 extract of purifed nuclei


NEPHGE
A
SDS PAGE
90K
68K
43K
NEPHGE
B
SDS PAGE
NEPHGE
C
SDS PAGE

agreement with the results of other workers, (Suria & Liew, 1979; Brunel & Lelay 1979; Maundrell & Scherrer, 1979; Wilks & Knowler, 1980; Peters & Cummings, 1981). Strangely, the probable core protein aggregates described in Section 1.2 were not seen on two-dimensional gels.

1.5 Characterisation on Non-Denaturing Polyacrylamide Gels

As a further test of the particulate nature of the hnRNP particles, they were fractionated on non-denaturing gels prepared as described in the Methods section 4.4.

HnRNP particles were found as a band at the top of the resolving gel. They appeared to move towards the anode and therefore had an overall negative charge at the pH at which the gel was run (pH9.2) (Figure 17A). Flat bed gels were also used, the hnRNP sample being loaded in the centre of the gel. The hnRNP particles still moved towards the anode.

The sucrose density gradients, BioGel separations and electrophoretic analyses described in sections 1.1 to 1.4 showed that particulate matter had been isolated from nuclei which showed the characteristics of hnRNP described by many other workers. These particles formed the material for subsequent analysis of endogenous protein kinases and their potential hnRNP particle substrates.

2. HnRNP Particle Kinase Activity

Autophosphorylation of hnRNP's was first noted by Schweiger & Schmidt (1974) and similar kinase activity has been detected by other/

FIGURE 17: Non-Denaturing Gels

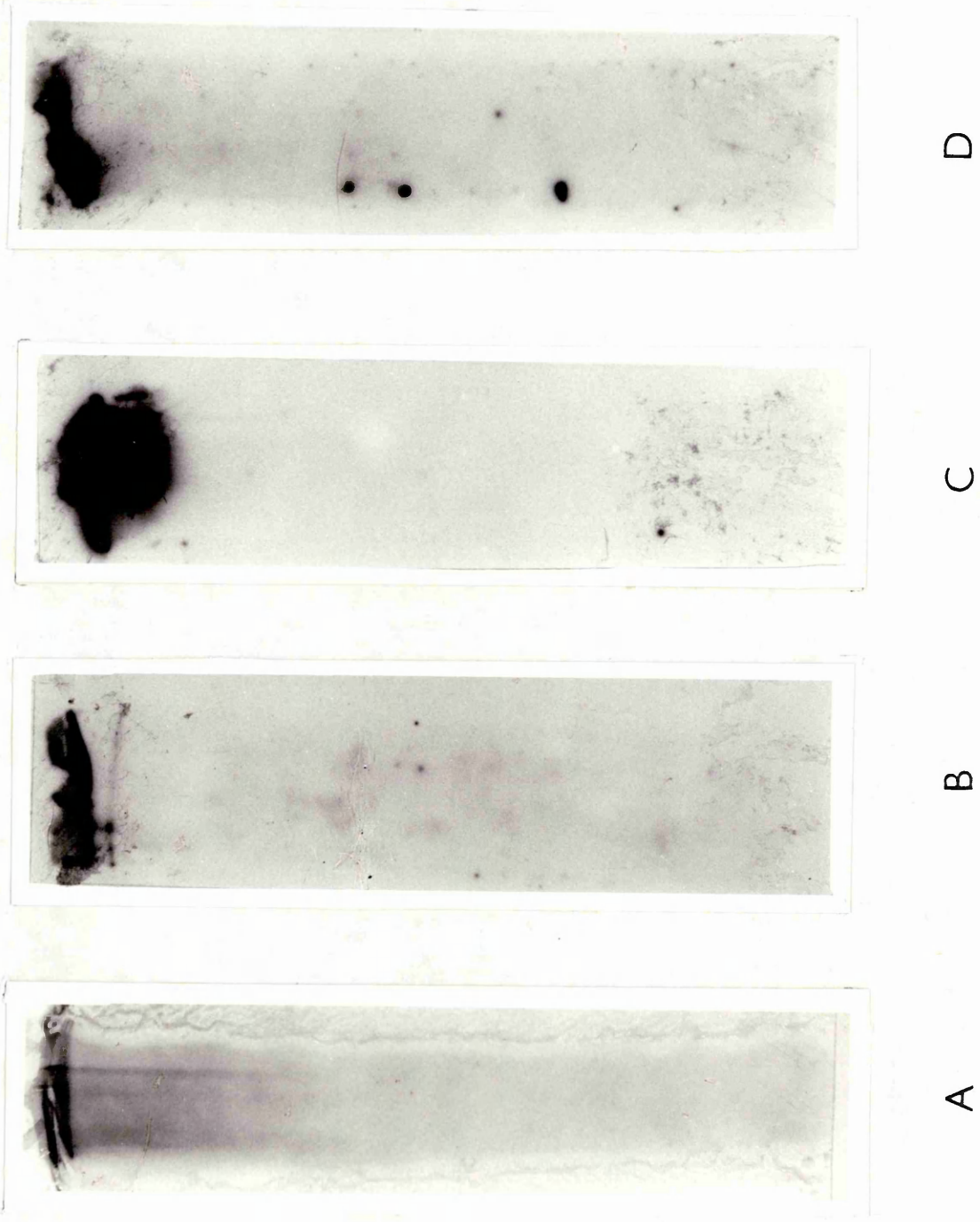
HnRNP particles isolated from sucrose density gradients as described in Methods (2) were run on non-denaturing gels as described in Methods (4.4). The gels were then tested for kinase activity as described in Methods (4.6) using casein or histone as exogeneous substrates.

A: stained gel

B: autoradiograph of gel : showing endogenous phosphorylation

C: autoradiograph of gel with casein added as exogeneous substrate

D: autoradiograph of gel with histone added as exogeneous substrate


D
C
B
A
Direction of Gel

other workers (Blanchard et al, 1975, 1979; Periasamy et al, 1979; Wilks & Knowler, 1981a).

2.1 Association of Kinase with 40S hnRNP

Several lines of evidence have supported the association of the kinase with rat liver hnRNP particles. The first support came from the analysis of each fraction of a sucrose density gradient which showed a peak of kinase activity coinciding with the 40S peak (Figure 18). A second peak of kinase activity was found at the top of the gradient and represented kinase activity not bound to the hnRNP particles.

When particles, isolated from one gradient, were re-run on a second sucrose density gradient, kinase activity was still found associated with the hnRNP particles although there was a 60% loss of activity. Patel & Holoubeck (1977) found that centrifugation of hnRNP's on sucrose density gradients caused loss of minor high molecular weight polypeptides. Repeated recentrifugation resulted in progressive loss until the predominant core proteins were virtually the only polypeptide components observed.

Clearly, the minor proteins, including the kinase activity, could be contaminants or they could be weakly bound but important proteins. These alternatives are discussed in detail later, and it will suffice here to state that at least some protein kinase activity was sufficiently bound to hnRNP to remain associated after a second sucrose density gradient, after exclusion chromatography on BioGel A-0.5m (Figure 19) or sephacryl, and after fractionation on non-denaturing gels (Figure 17).

2.2/

FIGURE 18: The *in vitro* incorporation of phosphate into fractions from sucrose density gradients

The pH 8 extract from purified nuclei was loaded onto a 15-30% w/v sucrose density gradient and spun at 78000 x g_{av} for 17 hours as described in Methods (2). 2.5ml fractions from the gradient were dialysed against 50mM MOPS, 10mM $MgCl_2$, pH 6.5 and assayed for kinase activity as described in methods (4.5).

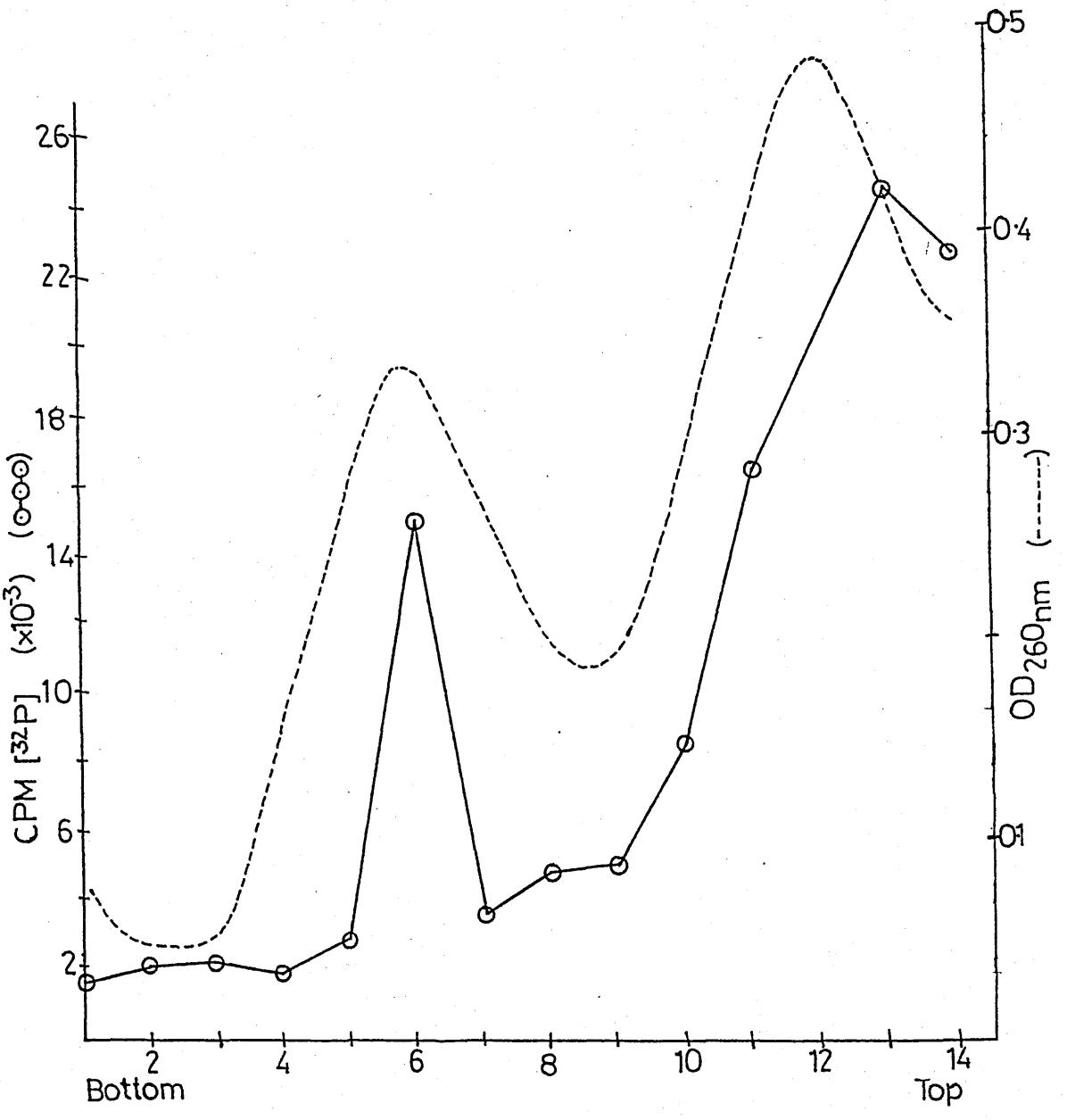
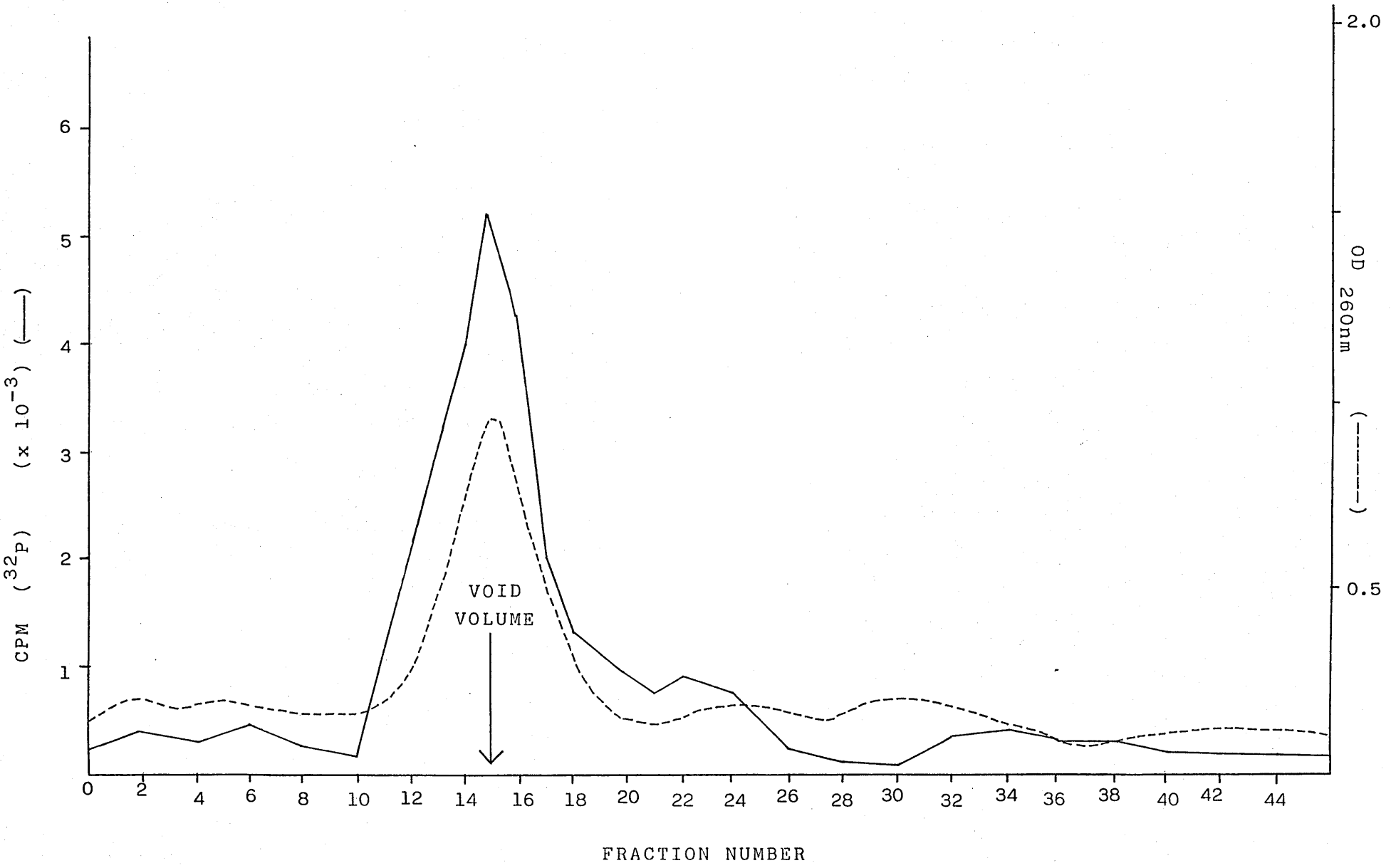

26
22
18
14
10
6
2
CPM [32P] (x10-3) (o-o-o)
0·5
0·4
0·3
0·1
OD260nm (----)
2
4
6
8
10
12
14
Bottom
Top
Fraction of gradient

FIGURE 19: The in vitro incorporation of phosphate into fractions from A Bio Gel A-0.5m column

HnRNP particles isolated from sucrose density gradients as described in Methods (2) were fractionated by gel exclusion chromatography on Bio Gel A-0.5m as described in Methods (3.1). 5 ml fractions were assayed for protein, and microdialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5 before being assayed for kinase activity as described in Methods (4.5).


CPM (^{32}P) (x 10^{-3}) (——)
OD 260nm (- - - - -)
VOID VOLUME
FRACTION NUMBER
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
1
2
3
4
5
6
0.5
2.0

2.2 Optimisation of Kinase Assay Conditions

The time course for *in vitro* phosphorylation showed an increase in phosphate incorporation for up to 30 minutes after which it levelled off and remained stable for at least one hour (Figure 20.1). This differed from the time course of the kinase activity detected in rat liver by Wilks & Knowler (1981a) which showed a linear increase for 10-15 minutes followed by rapid decrease in phosphate incorporation.

The level of phosphate incorporated was proportional to the amount of hnRNP particle protein added to the assay (Figure 20.2).

Rat liver hnRNP kinase activity showed a pH optimum at pH 6.5 whereas the kinase detected in HeLa cell hnRNP particles (Blanchard *et al.*, 1977) had a pH optimum of 8.5. The pH optimum of rat liver hnRNP's kinase activity detected here agrees with that detected by Wilks &Knowler (1981a). (For characterisation of pH and temperature effects see Results Section 4.1).

Phosphorylation was dependent on Mg^{2+}, for which the optimal concentration was 15-20mM (Figure 21.2), and the reaction was totally inhibited by EDTA. This agreed with the results of Blanchard *et al*, (1977) and Wilks &Knowler (1981a). Mn^{2+} ions also exhibited a stimulatory effect on kinase activity but to a lesser extent than Mg^{2+}.

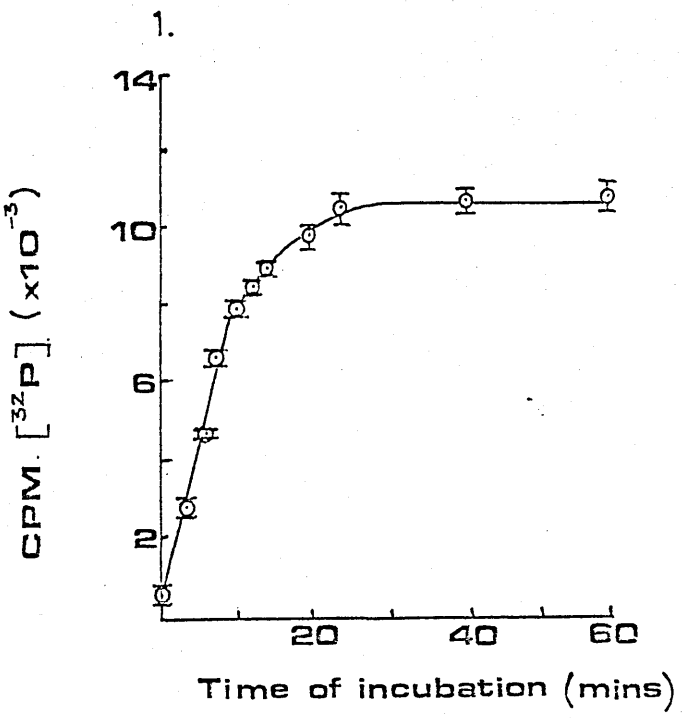
The purification of the kinase activity, which was a major objective of this work, required dissociation of the kinase from the/

FIGURE 20: Optimisation of *in vitro* kinase assay conditions

1. Time: HnRNP particles isolated from 15-30% w/v sucrose density gradients as described in Methods (2), were dialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5, then assayed for kinase activity as described in Methods (4.5) after incubating at 30°C for various time intervals.

2. Amount of protein: HnRNP particles isolated from 15-30% w/v sucrose density gradients as described in Methods (2) were dialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5, then assayed for kinase activity as described in Methods (4.5) after adding various amounts of hnRNP particle proteins to the assay.

(The error bars represent standard deviations derived from duplicate analyses).


1.
14
10
6
2
CPM. [32P] (x10-3)
20
40
60
Time of incubation (mins)

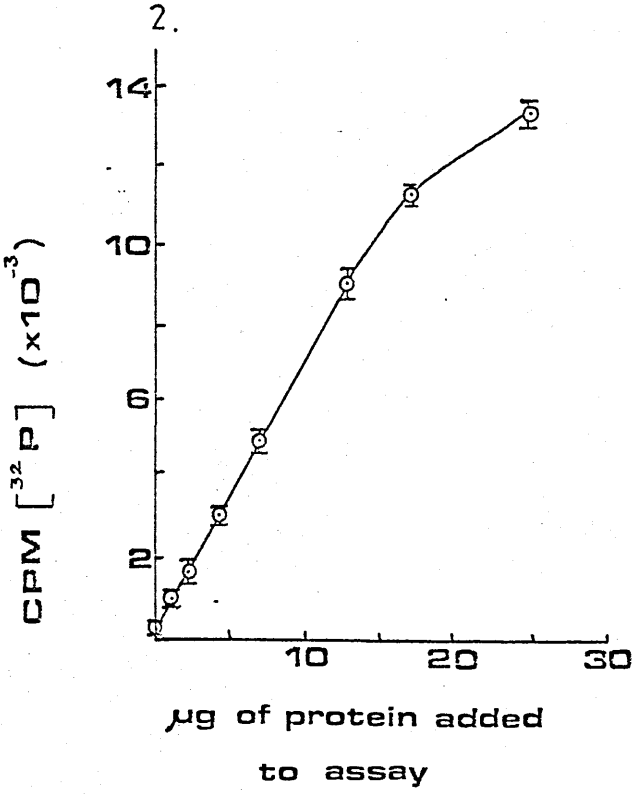

2.
14
10
6
2
CPM [32 P] (x10-3)
10
20
30
µg of protein added
to assay

FIGURE 21: Effects of Mg^{2+}, Mn^{2+}, Na^{+}, Triton-X100 and Urea on hnRNP particle kinase activity

HnRNP's isolated from sucrose density gradients as described in Methods (2) were dialysed against buffer to remove the sucrose and then treated as follows:-

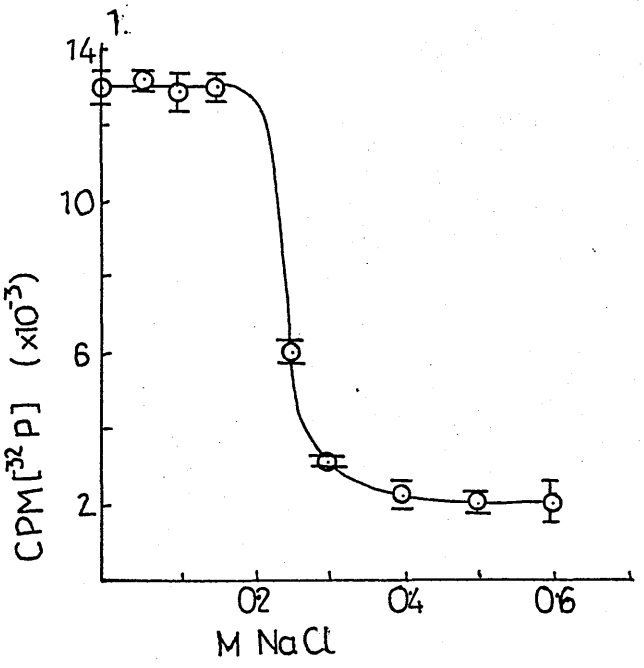
1: hnRNP were dialysed against 50mM MOPS, 10mM $MgCl_2$, pH 6.5 and then brought to various Na^{+} concentrations by addition of 5M NaCl. Kinase activity was then measured as described in Methods (4.5) using casein as exogeneous substrates.

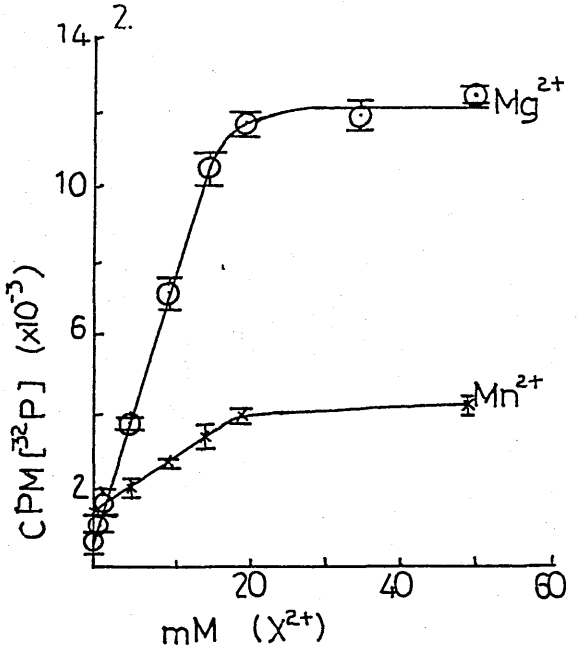
2: hnRNP dialysed against 50mM MOPS pH 6.5 were brought to various Mg^{2+} or Mn^{2+} concentrations by adding 250mM $MgCl_2$ or $MnCl_2$. Kinase activity was then measured as described in Methods (4.5) using casein as exogeneous substrates.

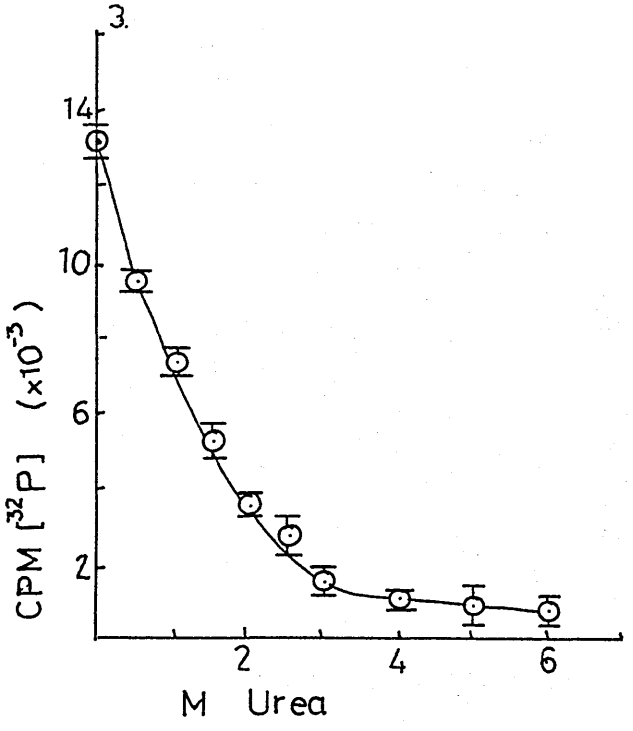
3: hnRNP dialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5 were brought to various urea concentrations by adding 12M urea. Kinase activity was then measured as described in Methods (4.5) using casein as exogeneous substrates.

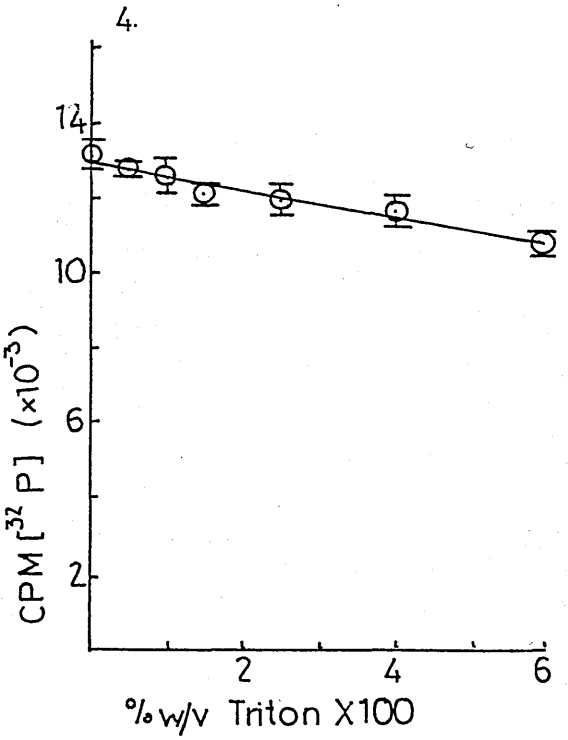
4: hnRNP dialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5 were brought to various Triton-X100 concentrations by adding 12% v/v Triton-X100. Kinase activity was then measured as described in Methods (4.5) using casein as exogeneous substrates.

(The error bars represent standard deviations from duplicate analyses ; similar data to that presented was obtained when histone was used as a substrate).


1.
14
10
6
2
CPM [32P] (x10-3)
02
04
06
M NaCl


2.
14
10
6
2
CPM [32P] (x10-3)
Mg2+
Mn2+
20
40
60
mM (X2+)


3.
14
10
6
2
CPM [32P] (x10-3)
2
4
6
M Urea


4.
14
10
6
2
CPM [32P] (x10-3)
2
4
6
%w/v Triton X100

the particle without destroying kinase activity. Therefore the effects of a number of reagents of possible use in this respect were investigated.

Kinase activity fell off rapidly in NaCl concentrations above 0.2M (Figure 21.1) in agreement with the results of Beyer et al., (1977), Blanchard et al., (1977) and Wilks & Knowler (1981a). 250mM NaCl has been shown to initiate disaggregation of hnRNP particles (Blanchard et al., 1977).

Urea inhibited rat liver hnRNP particle kinase activity while Triton-X100 had only a slight inhibitory effect (up to 6% w/v Triton-X100). (Figure 21.3 and 21.4). HnRNP particles treated with either 1M NaCl, 2M NaCl, 10% w/v Triton-X100, or 5M urea showed inhibition of kinase activity varying from 100% inhibition by 5M urea, to 3% inhibition with 10% w/v Triton-X100. However dialysis of hnRNP particles treated with 1M NaCl, 2M NaCl, 10% w/v Triton-X100 or 5M urea restored some of the kinase activity although control samples showed that 10% of the activity was lost upon dialysis alone (Table 4).

β-mercaptoethanol (up to 10mM) had no effect on rat liver hnRNP particle kinase activity, whereas RNase at 2µg/ml (37°C for half an hour) decreased activity by 20%.

Cyclic AMP did not appear to have any effect on hnRNP particle kinase activity. This result agrees with the results of most workers using a variety of tissues, although Wilks and Knowler (1981a), using rat liver, and Ohtshuki et al., (1980) using mouse spleen have shown the presence of a cAMP dependent protein kinase associated with hnRNP particles.

2.3/

TABLE 4: Effect of treatment of NaCl, Triton-X100 and Urea followed by dialysis

HnRNP isolated from sucrose density gradients as described in Methods (2) were treated with either 1M NaCl, 2M NaCl, 5% Triton-X100 or 5M Urea. 200 µl of each was microdialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5, then all fractions were analysed for kinase activity as described in Methods (4.5).

(Background levels have been subtracted, and levels have been corrected for volume changes due to micro-dialysis).

(Each result is the average of 2 determinations).

	C.P.M/assay (before dialysis)	C.P.M/assay (after dialysis)
Control	10980 ± 80 = 100%	9880 ± 110 = 90%
10% Triton	10840 ± 150 = 97%	9550 ± 120 = 87%
5M Urea	Zero	3420 ± 180 = 31%
1M NaCl	3830 ± 20 = 35%	8150 ± 60 = 74%
2M NaCl	2770 ± 160 = 25%	7370 ± 120 = 67%

TABLE 4

2.3 Phosphorylation of hnRNP Particle Proteins

The endogeneous protein kinase activity of hnRNP particles could use added casein or histone as substrates, the casein being a better substrate than histone (Figure 17, and data to be presented) but the activity was also capable of phosphorylating hnRNP particle proteins (Figures 22 and 23). On 2-dimensional fractionation it was seen that some core proteins plus a number of other proteins were phosphorylated. Some of the phosphorylated proteins were sufficiently abundant to give stainable spots. Others were not detected by staining, but were strongly phosphorylated. This was particularly true of heterogeneous entities of molecular weight 32000, (Figures 22 and 23).

3. Attempts to Purify hnRNP Particle Kinase Activity

3.1 The Use of Ion-Exchange Chromatography

In order to determine more precisely the nature and number of protein kinases present, it was necessary to fractionate the particle proteins. As core proteins are basic while most of the minor proteins are acidic or neutral, it was decided to fractionate the core proteins using CM-Cellulose ion-exchange chromatography and in this way it was hoped to separate the kinase from the core proteins. HnRNP particles were loaded on to CM-Cellulose columns at a variety of ionic strengths and pH values. The columns were then washed with salt solutions to release any bound proteins. Samples from each wash were analysed/

FIGURE 22: Phosphorylation of HnRNP Particle Proteins

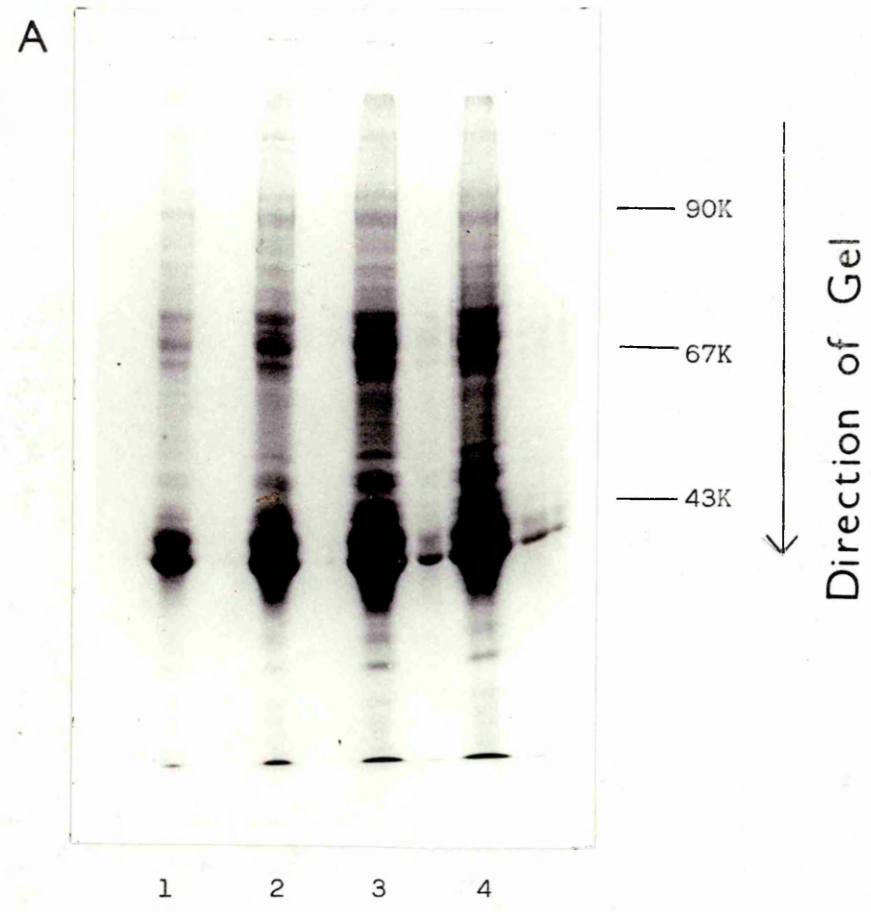
HnRNP particles isolated from sucrose density gradients as described in Methods (2), were dialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5. They were then incubated at 30°C for 10 minutes with (γ-^{32}P) ATP at 10m Ci/μmol using 50μ Ci/500 μg hnRNP, then precipitated with 2-3 volumes of ethanol and resuspended in SDS sample buffer. The precipitated proteins were then run on a SDS polyacrylamide gel as described in Methods (4.3).

The gel was stained, destained then dried and autoradiographed.

A: stained gel

B: autoradiograph of stained gel

Gel track	μg hnRNP
1.	20
2.	40
3.	60
4.	75


A
90K
67K
43K
Direction of Gel
1
2
3
4

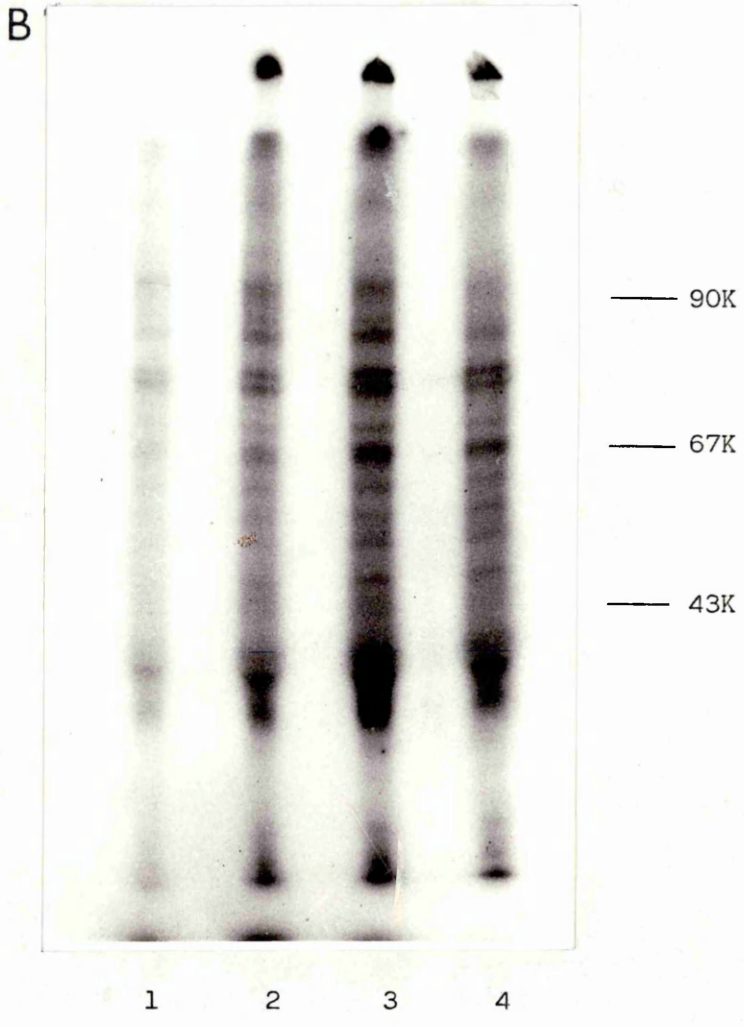

B
90K
67K
43K
1
2
3
4

FIGURE 23: 2-Dimensional Fractionation of Phosphorylated HnRNP Particle Proteins

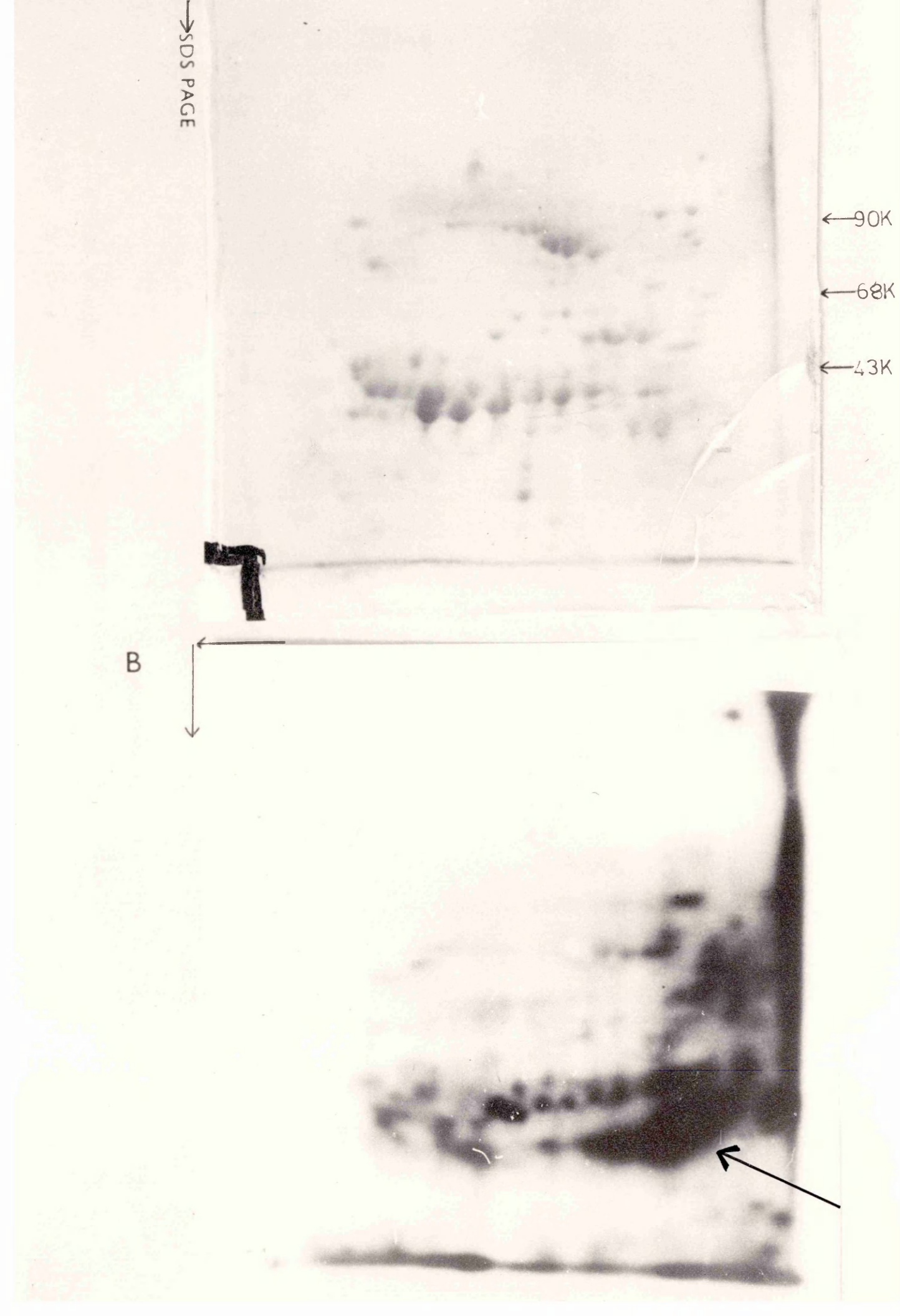
HnRNP particles isolated from sucrose density gradients as described in Methods (2) were dialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5. They were incubated at 30°C for 10 minutes with (γ-^{32}P)ATP at 10m Ci/μmol using 50μ Ci per 500 μg hnRNP . The proteins were then precipitated by adding 2-3 volumes of ethanol. The precipitated proteins were resuspended in lysis buffer before being loaded onto NEPHGE gels. The first and second dimension gels were run as described in Methods (4.2).

The gel was stained, destained, dried and auto-radiographed.

A: Stained gel (400 μg hnRNP)

B: autoradiograph of stained gel

(The arrow on B indicates the 32000 molecular weight heterogeneous entity).


SDS PAGE
90K
68K
43K
B

analysed for kinase activity and for any proteins present. It was seen that any fractions in which kinase activity could be detected also contained core proteins plus other particle proteins. These results may have been due to whole particles binding to the CM-Cellulose and all the proteins being eluted together. Therefore it was decided to disrupt the particles first before trying to fractionate the proteins.

In one type of experiment (Methods 3.2) hnRNP particles were dissociated in 1M NaCl which was then dialysed away with CM-Cellulose present in the dialysis tubing. The cellulose was then separated from any unbound protein by centrifugation, packed in columns and any bound protein eluted with increasing salt concentration. Very little protein was unbound but salt washes eluted all bound protein, including core proteins and kinase activity, virtually simultaneously, (data not shown).

Phosphocellulose is capable of functioning rather like an affinity chromatographic system for kinase enzymes, and at first appeared to give encouraging results. HnRNP particles were dissociated in 1M NaCl for 15 minutes at 0°C and then diluted to 0.1M NaCl immediately before application to a 3cm x 1cm column of phosphocellulose. Kinase activity could be eluted with 0.5M NaCl (Figure 24) but this activity was only 30% of control values. A small amount of activity was also detected in the 0.75M NaCl wash (10% of control hnRNP levels).

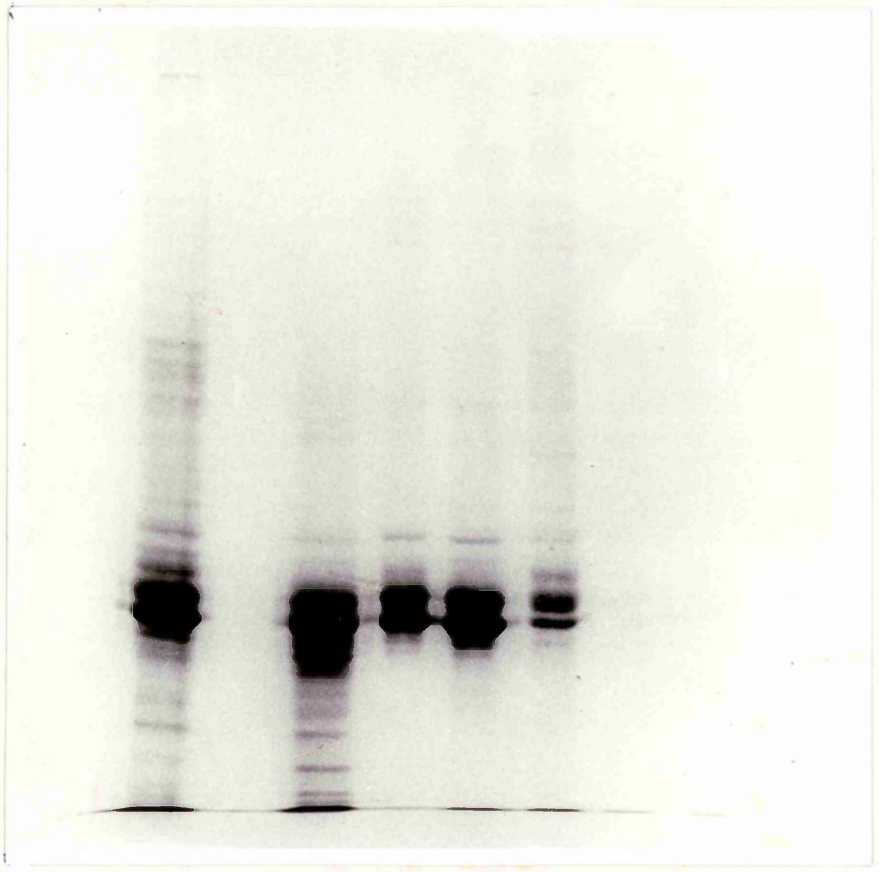
Attempts were then made to improve the specificity and completeness of the recovery of kinase activity from phospho-cellulose by eluting with buffers containing 5mM ATP as well as/

FIGURE 24: Fractionation of HnRNP Particle Kinase activity on Phosphocellulose

HnRNP particles were brought to 1M NaCl and stirred at 0°C for 15 minutes. The sample was then diluted to 0.1M NaCl with column buffer (10mM Tris HCl pH 7.9, 100mM NaCl, 1.5mM $MgCl_2$, 6mM β mercaptoethanol, 10% w/v glycerol), and 400-500μg protein (at 4000 cpm/μg protein) applied to a 3cm x 1 cm phosphocellulose column (Whatman No.11) equilibrated with column buffer. The column was then washed with increasing concentrations of NaCl. Some of each of the collected fractions was microdialysed and assayed for kinase activity as described in Methods (4.5) while the rest was dialysed against H_2O, ethanol precipitated and analysed on SDS polyacrylamide gels as described in Methods (4.3).

Gel Track No.	Kinase Activity
1. wash through eluate	-
2. 0.10M NaCl wash (4mls)	-
3. 0.25M NaCl wash (4mls)	-
4. 0.50M NaCl wash (4mls)	1200cpm/μg protein
5. 0.75M NaCl wash (4mls)	400cpm/μg protein
6. 1.00M NaCl wash (4mls)	-

(↑ Indicates fractions in which kinase activity was detected).


90K
67K
43K
1
2
3
4
5
6

as increasing concentrations of NaCl. Again the results seemed encouraging in that kinase activity was recovered in fractions containing relatively few other hnRNP proteins (Figure 25). However, yield was once again unacceptable being only 35% recovery.

Samarina et al,(1967) have shown that when the salt is removed from NaCl-dissociated hnRNP's, the particles are able to re-associate, and Stevenin et al, (1979) have shown that this occurs even after the digestion of the RNA component with ribonuclease. It seems very likely that reassociation into particles or other aggregates was a contributory factor to the poor results with ion-exchange chromatography and this was supported by continuing poor results when ribonuclease was employed, (data not shown).

Before trying any further fractionations it was decided to examine the efficiency of salt dissociation and the extent to which reassociation occurred after the salt was removed.

3.2 Effect of NaCl on hnRNP particles

pH8·0 extracts of isolated nuclei, prepared as described in Methods (2.), were treated with 1M NaCl then loaded on to 15-30% sucrose density gradients containing 1M NaCl. When the gradients were scanned in the usual way, no 40S hnRNP peak was seen, and all of the hnRNP particle proteins were found at the top of the gradient (Figure 26A). These results indicated that 1M NaCl disrupted hnRNP particles and caused the proteins to be found at the top of the gradient.

To/

FIGURE 25: ATP wash of Phosphocellulose Column

HnRNP particles were treated with 1M NaCl and stirred at 0°C for 15 minutes. The sample was then diluted to 0.1M NaCl with column buffer (10mM Tris pH 7.9, 50mM NaCl, 1.5mM $MgCl_2$, 6mM mercaptoethanol, 10% w/v glycerol) and then 400-500µg protein (kinase activity 4300cpm/µg protein) was applied to a 3cm x 1cm phosphocellulose column. The column was then eluted with 4ml aliquots of buffer with continually increasing concentrations of NaCl and ATP. The collected fractions were microdialysed and assayed for kinase activity as described in Methods (4.5) or dialysed against H_2O, precipitated with ethanol and analysed for protein on SDS polyacrylamide gels as described in Methods (4.3).

	Kinase Activity
1. wash through eluate	-
2. 0.1M NaCl wash	-
3. 0.25M NaCl wash	-
4. 0.3M NaCl wash	-
5. 0.3M NaCl/5mM ATP wash	500cpm/µg protein
6. 0.3M NaCl/5mM ATP wash	-
7. 0.3M NaCl wash	-
8. 0.5M NaCl/5mM ATP wash	1500cpm/µg protein
9. 0.5M NaCl/5mM ATP wash	-

(↑ indicates fractions in which kinase activity is detected).

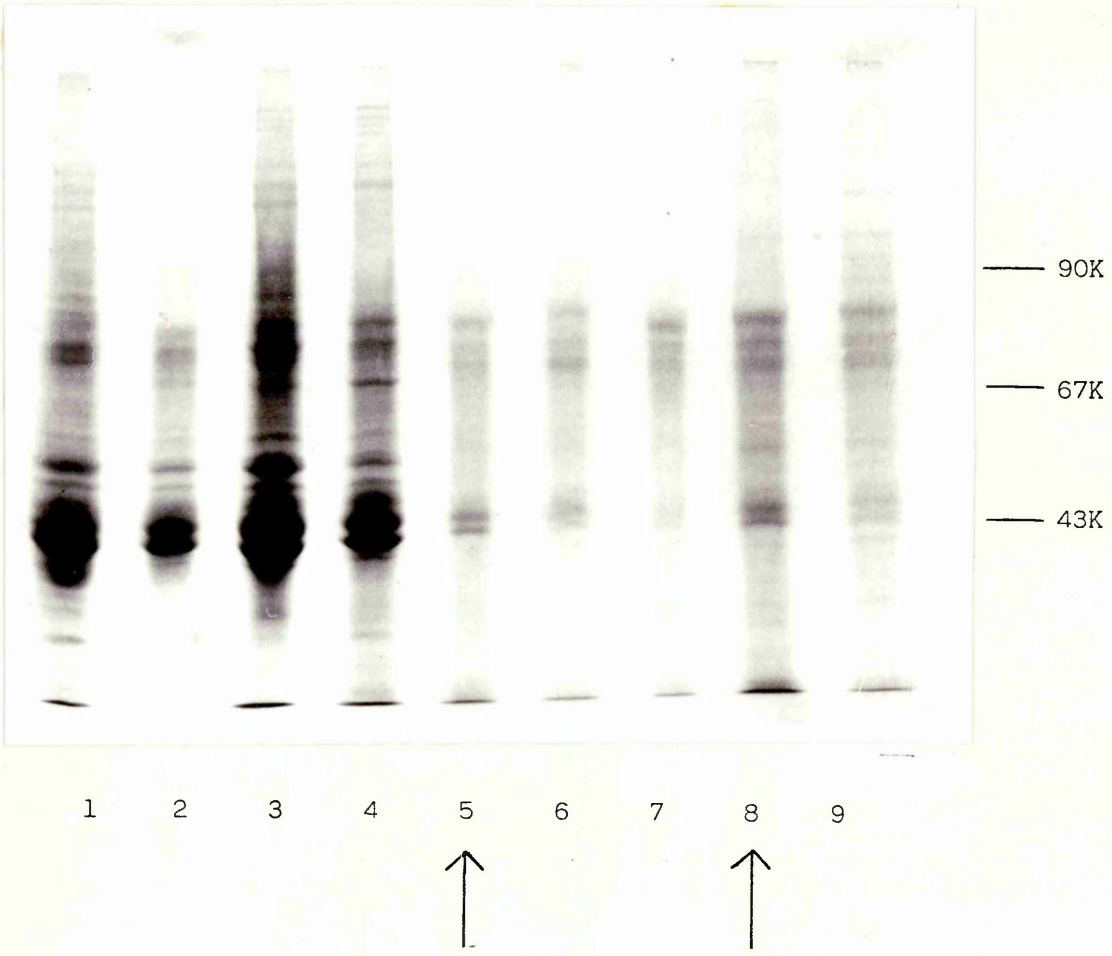

90K
67K
43K
1
2
3
4
5
6
7
8
9

FIGURE 26: Effect of NaCl on HnRNP Particles

A: STM8 extract of isolated nuclei was treated with 1M NaCl then some was loaded on to a 15-30% w/v sucrose density gradient containing 1M NaCl while the rest was dialysed against STM8 before being loaded onto a normal gradient. The gradients were spun at 78000x g_{av} for 17 hours in a Beckman SW27 rotor, then scanned as described in Methods (2). The results were compared to STM8 extract loaded onto gradients as per usual.

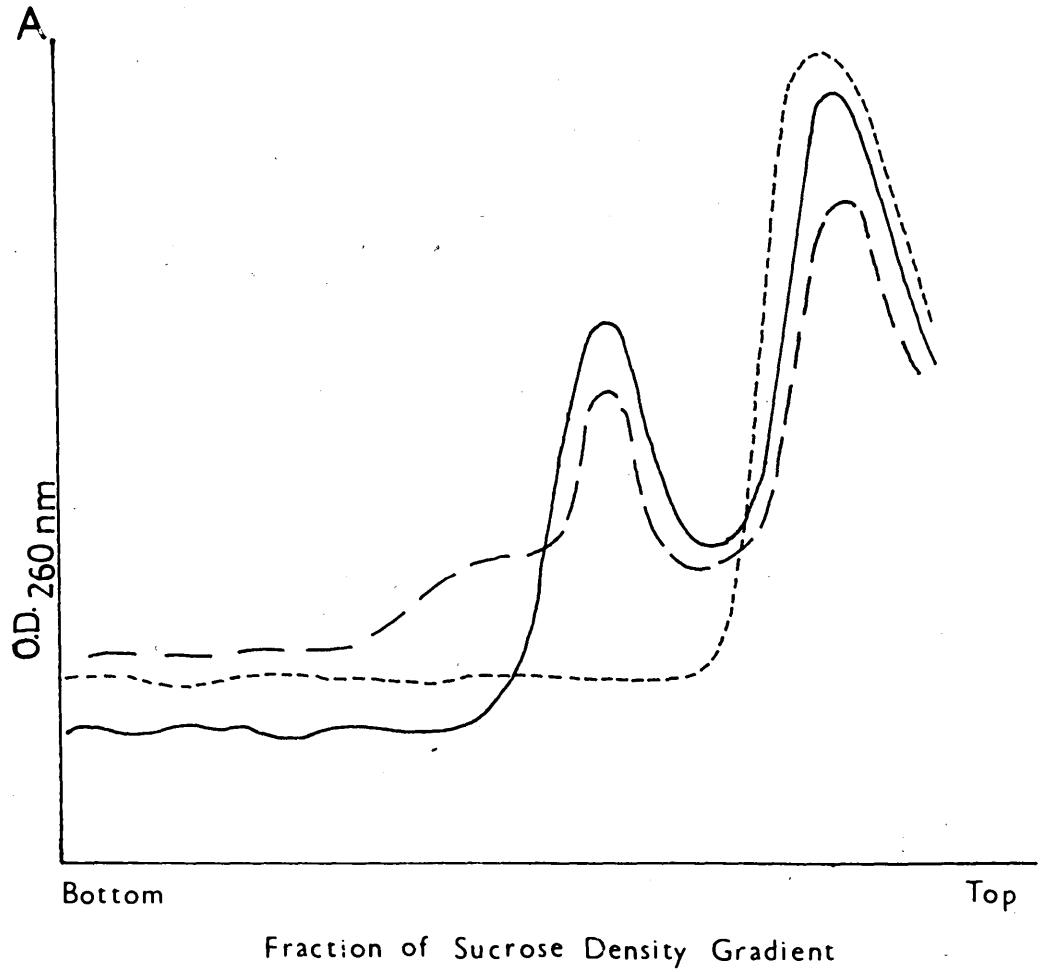
i) sucrose density gradient loaded with STM8 extract of purified nuclei (————)

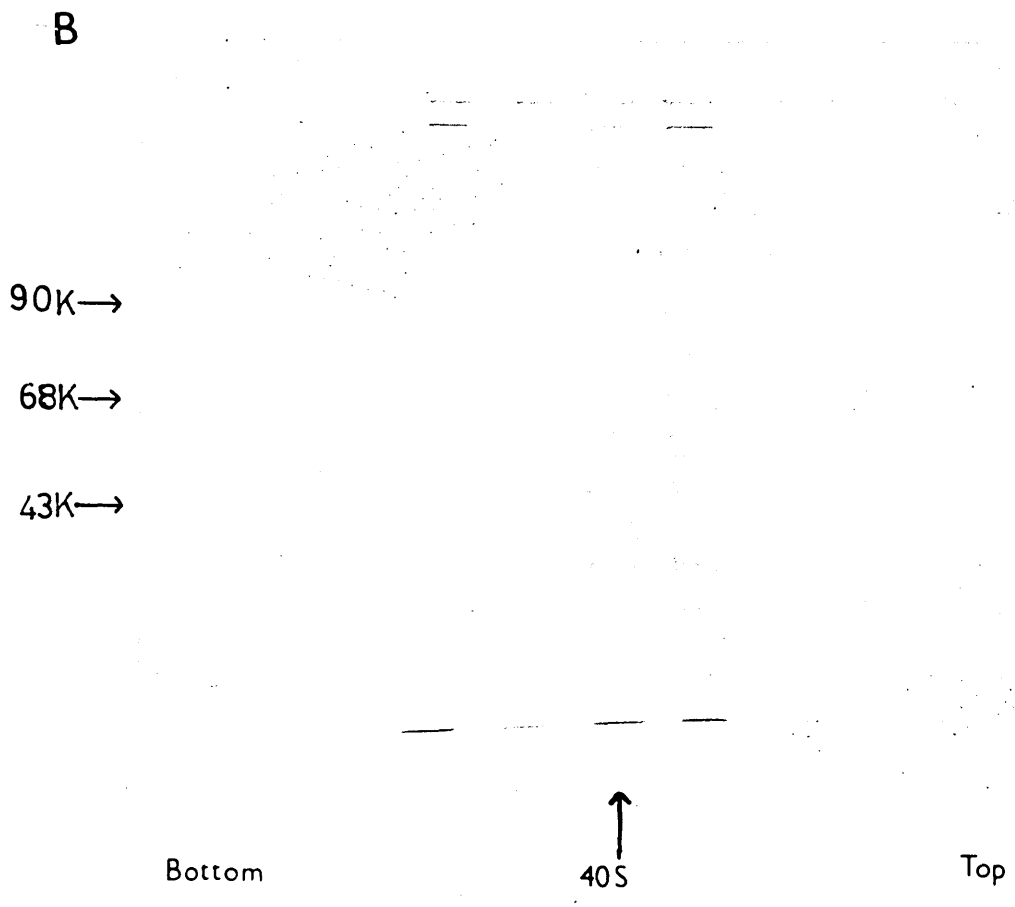
ii) sucrose density gradient containing 1M NaCl, loaded with STM8 extract treated with 1M NaCl (--------)

iii) Sucrose density gradient loaded with STM8 extract which had been treated with 1M NaCl followed by dialysis against STM8 buffer. (— — —)

B: STM8 extract, treated with 1M NaCl, was then dialysed against 100mM NaCl, 10mM Tris HCl pH 8.0, 1mM $MgCl_2$ and loaded on to 15-13% sucrose density gradient. The gradient was spun at 78000x g_{av} for 17 hours in a Beckman SW27 rotor then collected in 5ml fractions as described in Methods (2). The fractions were dialysed against H_2O, precipitated with 2-3 volumes of ethanol and the proteins analysed on SDS-polyacrylamide gels as described in methods (4.3).

The stained gel (B) has been arranged below the O.D. scan (Aiii) so that the relative positions correspond.


A
O.D. 260 nm
Bottom
Top
Fraction of Sucrose Density Gradient


B
90K⟶
68K⟶
43K⟶
Bottom
40S
Top

To look at reconstitution, hnRNP particles were treated with 1M NaCl and then dialysed against STM8 (100mM NaCl, 10mM Tris pH 8.0, 1mM $MgCl_2$) before being loaded on to a 15-30% sucrose density gradient. When the gradients were scanned as described in Methods (2. ·) a 40S hnRNP peak was seen (Figure 26B). However, a higher molecular weight entity was also present and presumably represented an aggregate of particles or atypical aggregates of particle proteins. To test whether the formation of this higher molecular weight complex was caused by the NaCl treatment or by dialysis, intact hnRNP particles were dialysed against STM8 and then loaded on to a 15-30% sucrose density gradient. When the gradients were scanned the 40S peak was seen but there was no higher molecular weight aggregate. Therefore, it appears that treatment with NaCl followed by dilution may result in some rearrangement of the particles or the formation of a higher molecular weight aggregate.

Similar ideas have been expounded by Stevenin et al.,(1979), after examining the effect of ribonuclease on hnRNP particles.

These results showed that fractionation of the particulate kinase would be achieved only when conditions could be found which satisfied the following requirements:

(a) The particle must be dissociated or at least the kinase activity must be dissociated from the particles.

(b) Dissociation must not destroy kinase activity.

(c)/

(c) Either the particle constituents must be fractionated in the dissociating medium or changing the medium must not result in reassociation.

For these reasons it was decided to try gel filtration in the presence of 1M NaCl.

3.3 Fractionation on Sephadex G100

HnRNP particles were brought to 1M NaCl and stirred gently at 0°C for 15 minutes. They were then loaded on to a Sephadex G100 column equilibrated with column buffer (10mM Tris pH7.5, 1M NaCl, 6mM β mercaptoethanol, 1mM EDTA) and the column was run as described in methods (3.1). The fractions were collected and analysed for kinase activity and protein content (Figure 27).

Two peaks of kinase activity were seen ('A' and 'B'), associated with fractions that contained different sets of polypeptides. Peak 'B' contained core proteins while 'A' did not. Calibration of the Sephadex column showed that 'B' had a molecular weight of approximately 33000 while the molecular weight of 'A' was approximately 112000. Clearly 'A' and 'B' could be different activities, different forms of one enzyme, or 'A' could represent kinase still bound to undissociated particles while 'B' could be dissociated kinase. The latter possibility seemed unlikely as treatment with 1M NaCl had been shown to dissociate hnRNP particles and when the Sephadex G100 column was run in the present of 1.5M NaCl the kinase profile was unaltered (data not shown).

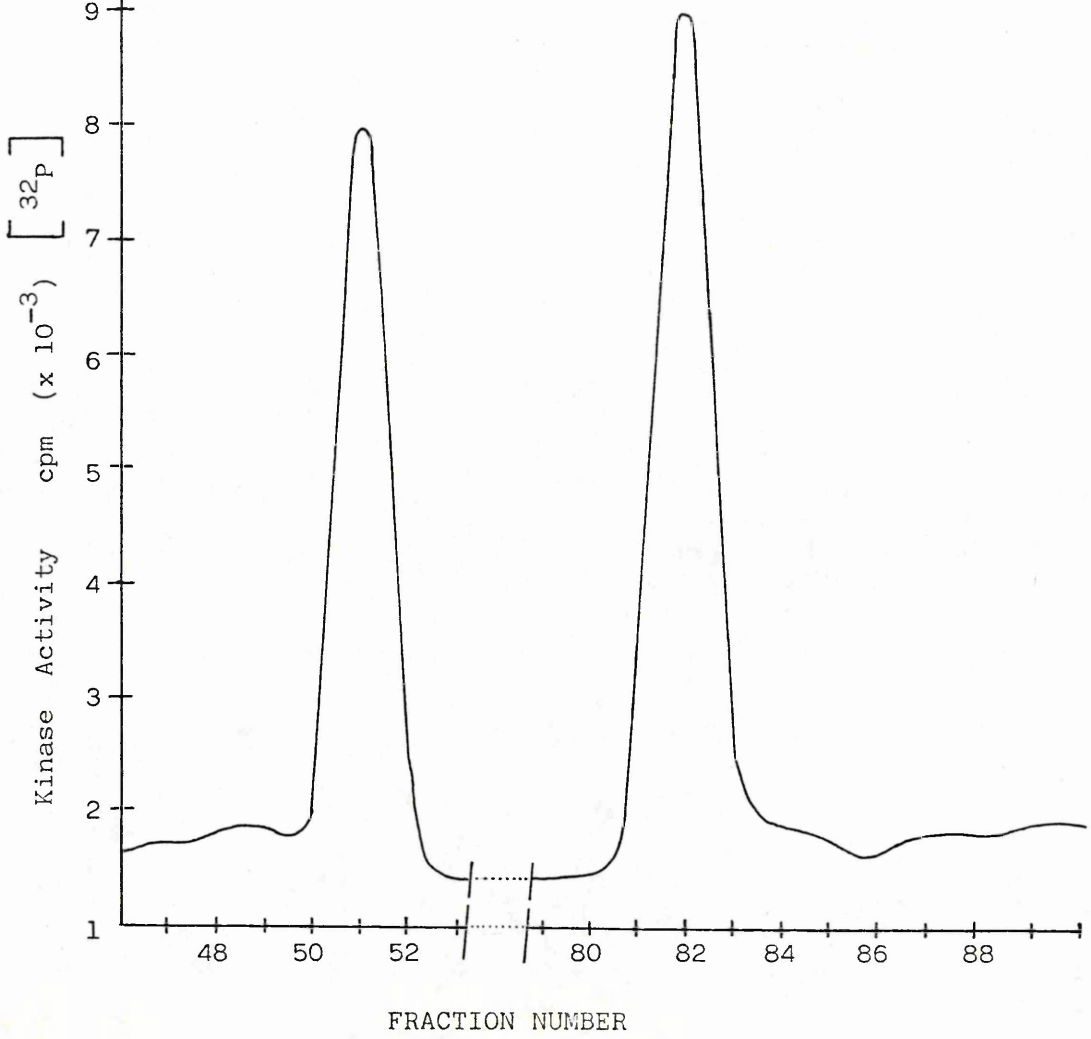
4./

FIGURE 27: Fractionation of hnRNP particle proteins on Sephadex G100

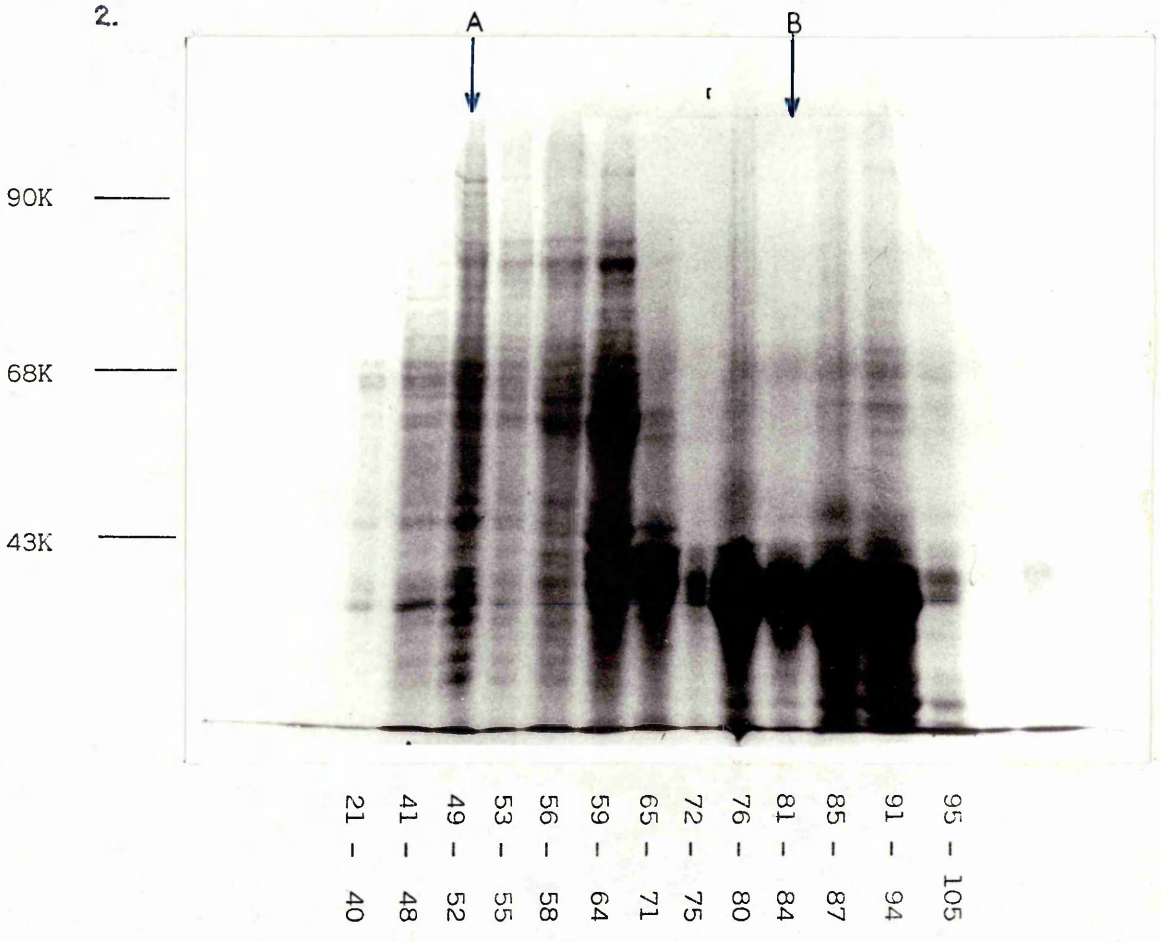
HnRNP particles isolated from sucrose density gradients as described in Methods (2) were dissociated in 1M NaCl and stirred at 0°C for 15 minutes. Then 7-8mg hnRNP in 5mls* of equilibration buffer were loaded on to a Sephadex G100 column equilibrated with 10mM Tris pH 7.5, 1M NaCl, 6mM mercaptoethanol, 1mM EDTA and the column was run as described in Methods (3.1). Some of each 2ml fraction was microdialysed and assayed for kinase activity as described in Methods (4.5). The rest was precipitated with trichloroacetic acid and analysed on SDS polyacrylamide gels as described in Methods (4.3).

The kinase profile (1) is arranged above the stained pattern (2) so that the relative positions correspond.

* This concentration of hnRNP was used when running the Sephadex column as at higher concentration of the hnRNP the particle proteins tended to precipitate.


Kinase Activity cpm (x 10^{-3}) [^{32}P]
1
2
3
4
5
6
7
8
9
48
50
52
80
82
84
86
88
FRACTION NUMBER

2.


A
B
90K
68K
43K
21 - 40
41 - 48
49 - 52
53 - 55
56 - 58
59 - 64
65 - 71
72 - 75
76 - 80
81 - 84
85 - 87
91 - 94
95 - 105
Fraction Number (2ml fractions)

4. Characterisation of 'A' and 'B' Activities

4.1 Optimisation of Assay Conditions

To determine whether the 'A' and 'B' activities were similar or different, they were characterised with respect to their optimum conditions for enzyme activity.

Both 'A' and 'B' activities showed a pH optimum at pH 6.5, the same as that found for intact hnRNP particles. Figure 28 illustrates the pH optimum when casein was employed as the endogeneous substrate but the phosphorylation of histone had the same pH optimum.

The time course for the in vitro incorporation of phosphate was similar for 'A', 'B' and intact hnRNP although when calculated as cpm per µg of total protein the linear reaction was longest for peak 'A'. This was more likely to result from differences in the relative enzyme concentrations than from enzymatic differences (Figure 29).

The stability of 'A', 'B', and intact hnRNP kinase activity was investigated by incubating them at 30, 50 or 60°C for various times then analysing for kinase activity at 30°C in the usual assay (Figure 30). All 3 activities appear to be stable at 30°C while incubation for even 2 minutes at 60°C destroys 70% of the activity in 'A' and 90% of the activity in 'B' and hnRNP. Kinase 'B' activity appeared to be the most labile with only 27% of the activity remaining after 20 minutes incubation at 50°C, while hnRNP was 50% inactivated after 5 minutes and the kinase activity in 'A'/

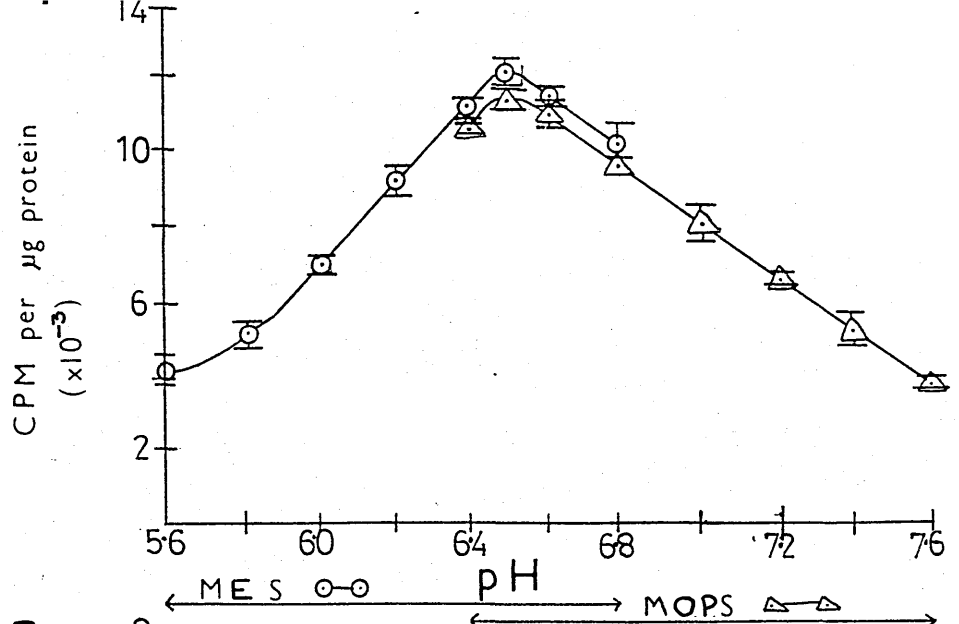
FIGURE 28: Effect of pH on Kinase Activity

HnRNP particles isolated from 15-30% w/v sucrose density gradients were dialysed against buffers, at various pH values, containing 10mM $MgCl_2$. MES was used for the range pH 5.6 - 6.8 while MOPS was used for pH 6.4 - 7.6. The casein substrate was also in the appropriate buffer solution. Kinase activity at the various pH values was then determined as described in Methods (4.5).

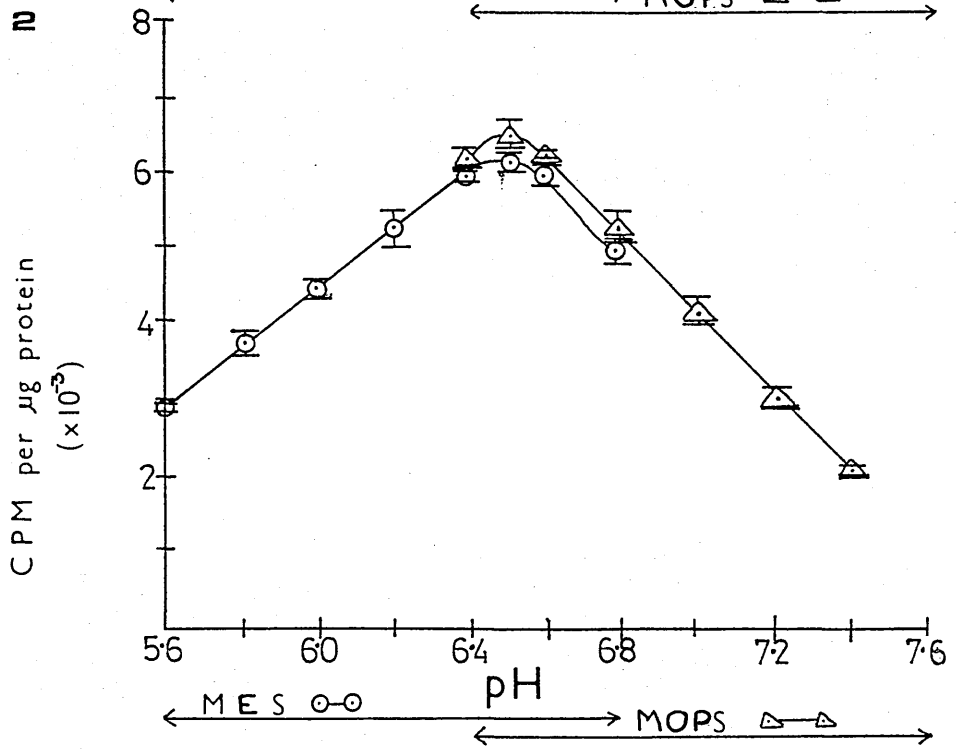
The kinase-containing peaks 'A' and 'B', isolated from a Sephadex G100 column as described in Methods (3.1), were prepared for assay in the same way as the hnRNP particles.

1. 'B'
2. 'A'
3. HnRNP

(The error bars represent standard deviations derived from duplicate analyses).


14
10
6
2
CPM per µg protein
(×10⁻³)
5·6
6·0
6·4
6·8
7·2
7·6
MES
pH
MOPS

2


8
6
4
2
CPM per µg protein
(×10⁻³)
5·6
6·0
6·4
6·8
7·2
7·6
MES
pH
MOPS

3

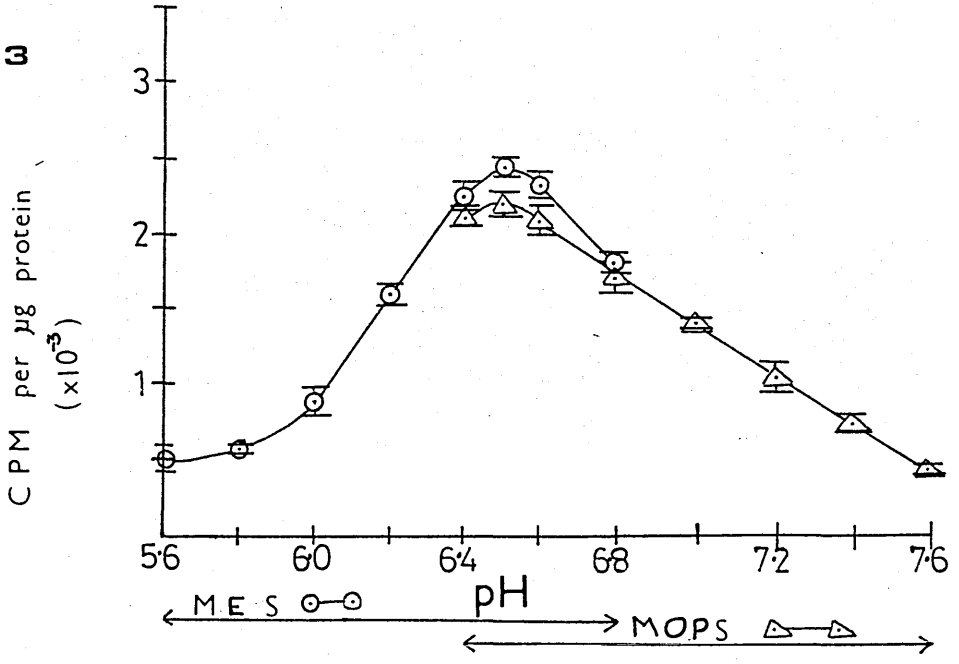
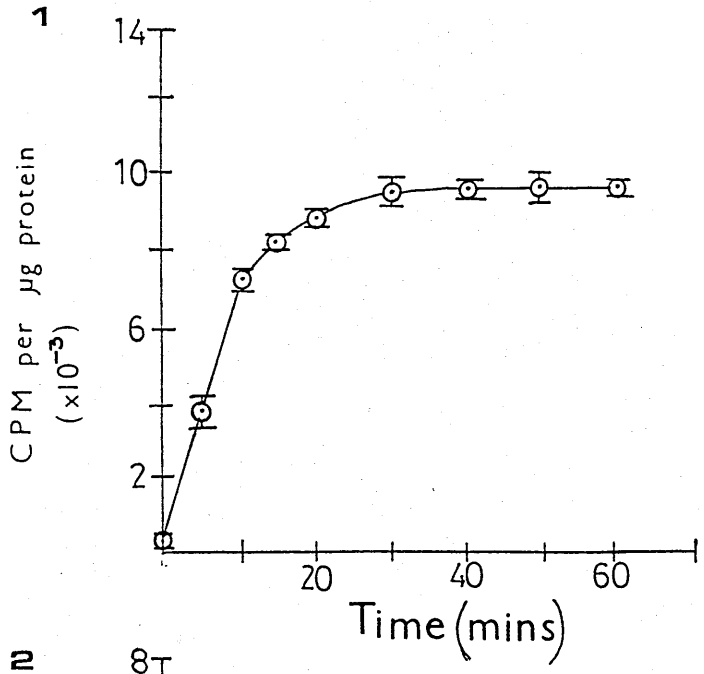

3
2
1
CPM per µg protein
(×10⁻³)
5·6
6·0
6·4
6·8
7·2
7·6
MES
pH
MOPS

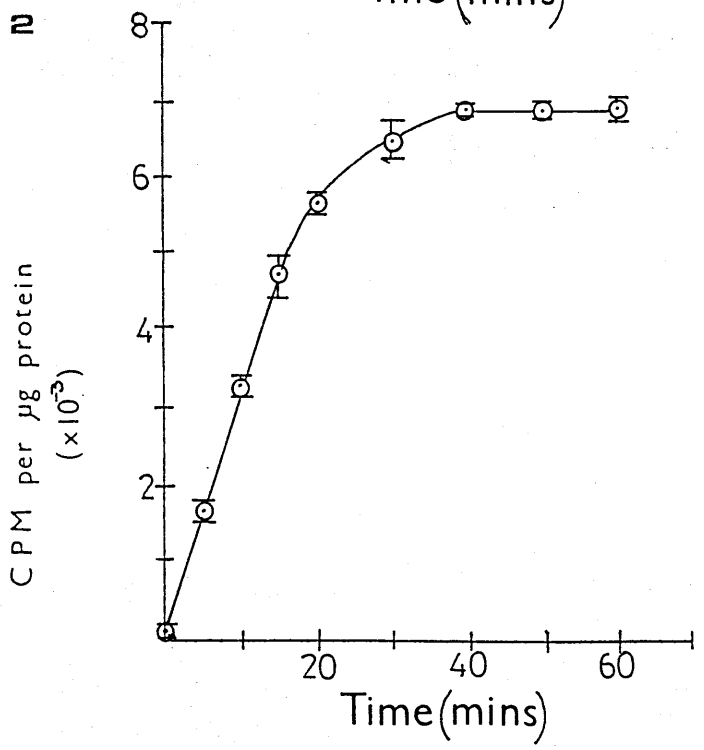
FIGURE 29: Effect of Time of Incubation on Kinase Activity

'A' and 'B' peaks isolated from a Sephadex G100 column were dialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5. They were then assayed for kinase activity as described in Methods (4.5) adding casein as an exogenous substrate and incubating at 30°C for various time intervals. The results were compared with those of hnRNP particles, isolated from 15-30% w/v sucrose density gradients, as described in Methods (2), which were dialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5 and assayed for kinase activity as for 'A' and 'B' above.

1. 'B'
2. 'A'
3. HnRNP

(The error bars represent standard deviations derived from duplicate analyses).


1
14
10
6
2
CPM per µg protein
(x10⁻³)
20
40
60
Time (mins)


2
8
6
4
2
CPM per µg protein
(x10⁻³)
20
40
60
Time (mins)

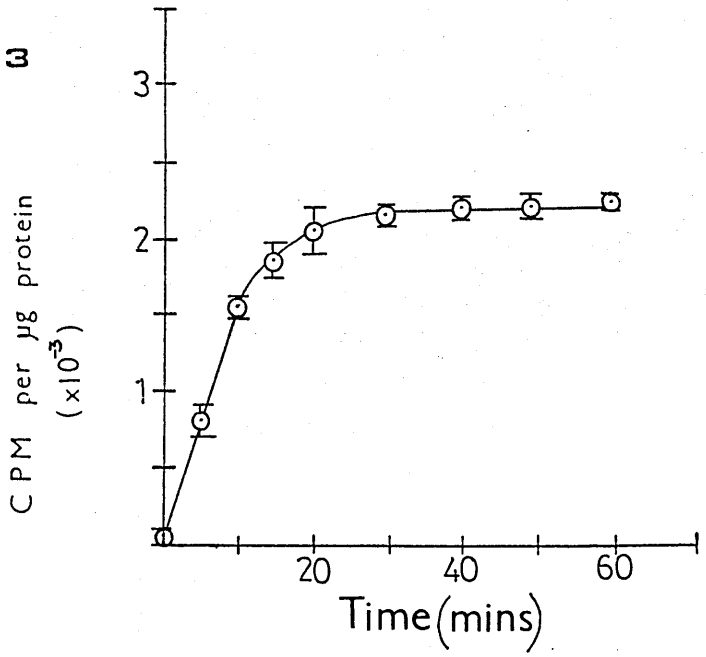
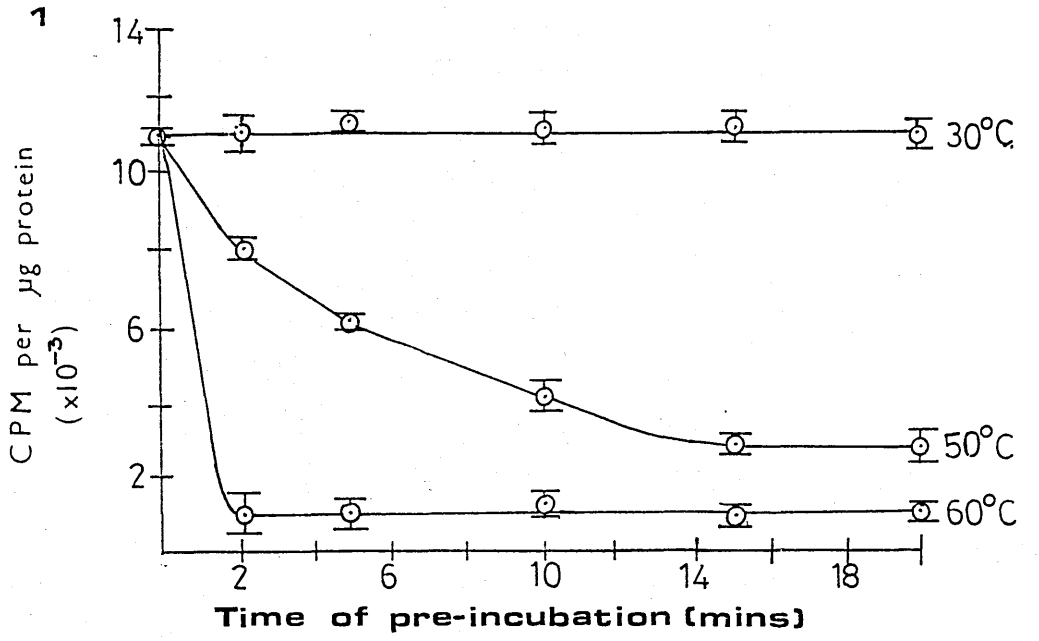

3
3
2
1
CPM per µg protein
(x10⁻³)
20
40
60
Time (mins)

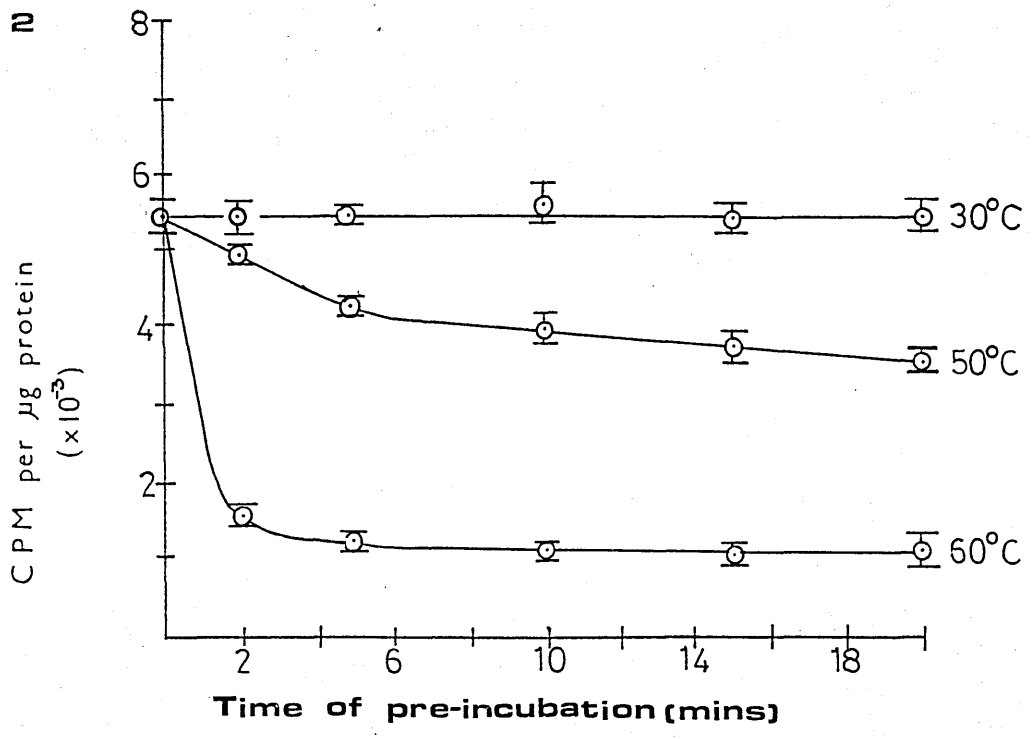
FIGURE 30: Effect of Temperature on Kinase Activity

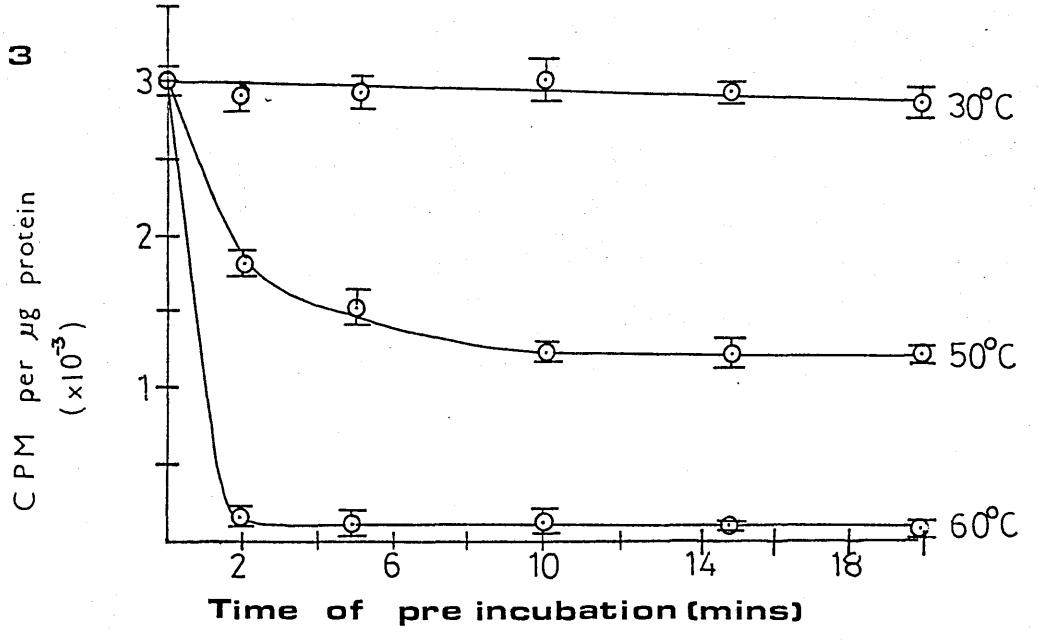
'A' and 'B' peaks isolated from a Sephadex G100 column were dialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5. They were then incubated at 30°C, 50°C or 60°C for various time intervals, cooled then assayed for kinase activity using added casein as described in Methods (4.5) by incubating at 30°C for 10 minutes. The results were compared with those of hnRNP particles which were isolated from 15-30% w/v sucrose density gradients as described in Methods (2), and treated in the same way as 'A' and 'B'.

1. 'B'
2. 'A'
3. HnRNP

(The error bars represent standard deviations derived from duplicate analyses).


1
14
10
6
2
CPM per µg protein
(×10⁻³)
30°C
50°C
60°C
2
6
10
14
18
Time of pre-incubation (mins)


2
8
6
4
2
CPM per µg protein
(×10⁻³)
30°C
50°C
60°C
2
6
10
14
18
Time of pre-incubation (mins)


3
3
2
1
CPM per µg protein
(×10⁻³)
30°C
50°C
60°C
2
6
10
14
18
Time of pre incubation (mins)

in 'A' gradually became inactivated with time but still retained 60% of the activity after 20 minutes.

The kinase activities in peaks 'A' and 'B' as well as that in intact hnRNP, were markedly dependent on the concentration of Mg^{2+} (Figure 31). The activity of 'A', and the undissociated particle was maximal at 20mM Mg^{2+} while that of 'B' showed further small increases up to 40mM. Furthermore Mn^{2+} could partially replace Mg^{2+} in phosphorylation by 'A', and intact particles, but was totally ineffective for peak 'B' kinase activity.

Neither 'A', 'B' or intact hnRNP kinase showed any activity when assayed in the presence of (γ - ^{32}P) GTP rather than (γ -^{32}P) ATP.

One major difference between 'A' and 'B'was in their substrate specificity (Table 5). Whereas 'B', like hnRNP, could use added casein or histone as substrates, 'A' appeared to be specific for hnRNP particle proteins and to be unable to use added casein or histone. That is, no increase over endogenous phosphorylation was observed when these substrates were added to the peak of enzyme activity.

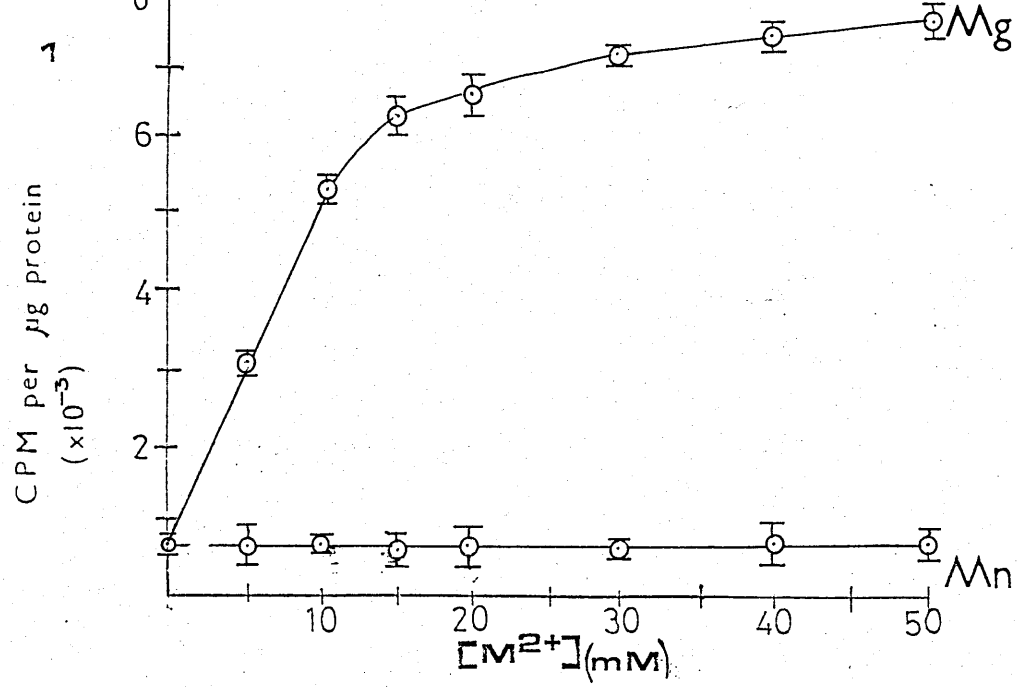
These results show that no additional phosphorylation was seen when casein or histone was added to 'A' but did not show that casein or histone were not phosphorylated. To demonstrate this, casein and histone were incubated with either 'A' or 'B' in the presence of (γ -^{32}P) ATP. The proteins were then analysed on SDS polyacrylamide gels and the phosphorylated proteins detected/

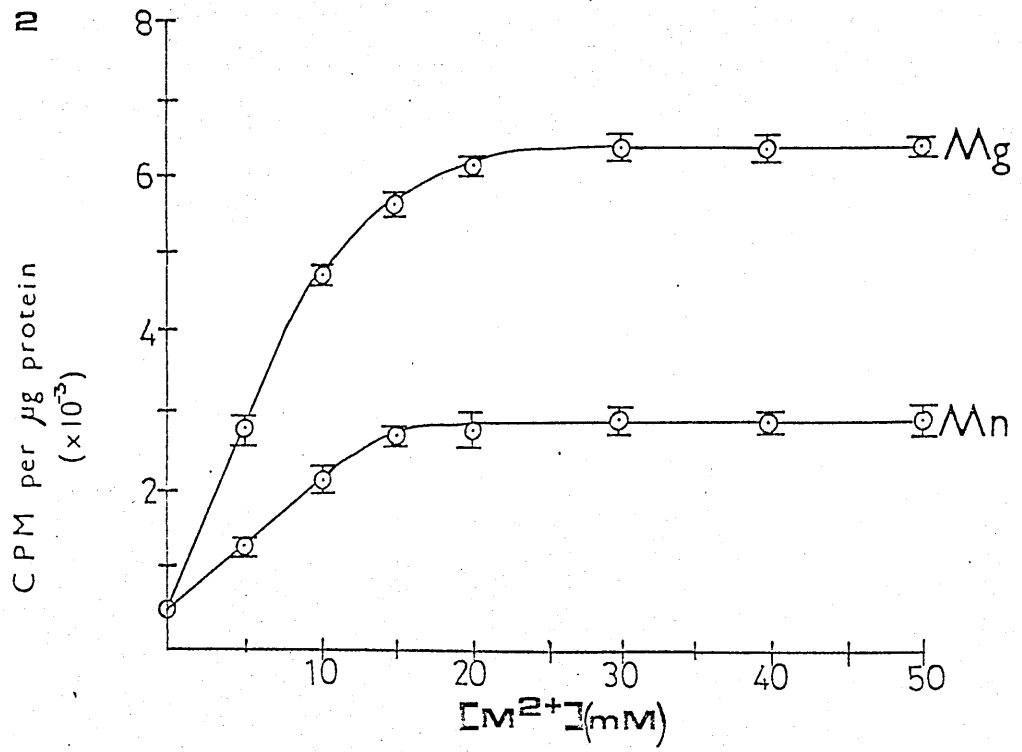
FIGURE 31: Effect of Mg^{2+} and Mn^{2+} on Kinase Activity

'A' and 'B' peaks isolated from a Sephadex G100 column were dialysed against 50mM MOPS pH 6.5. They were then assayed for kinase activity using added casein as described in Methods (4.5) after bringing them to various Mg^{2+} or Mn^{2+} concentrations by adding 250mM $MgCl_2$ or $MnCl_2$. The results were compared with those of total hnRNP particles, isolated from 15-30% w/v sucrose density gradients, which were treated as 'A' and 'B' above.

1. 'B'
2. 'A'
3. HnRNP

(The error bars represent standard deviations derived from duplicate analyses).


1
6
4
2
CPM per μg protein
(×10⁻³)
Mg
Mn
10
20
30
40
50
[M²⁺](mM)


2
8
6
4
2
CPM per μg protein
(×10⁻³)
Mg
Mn
10
20
30
40
50
[M²⁺](mM)

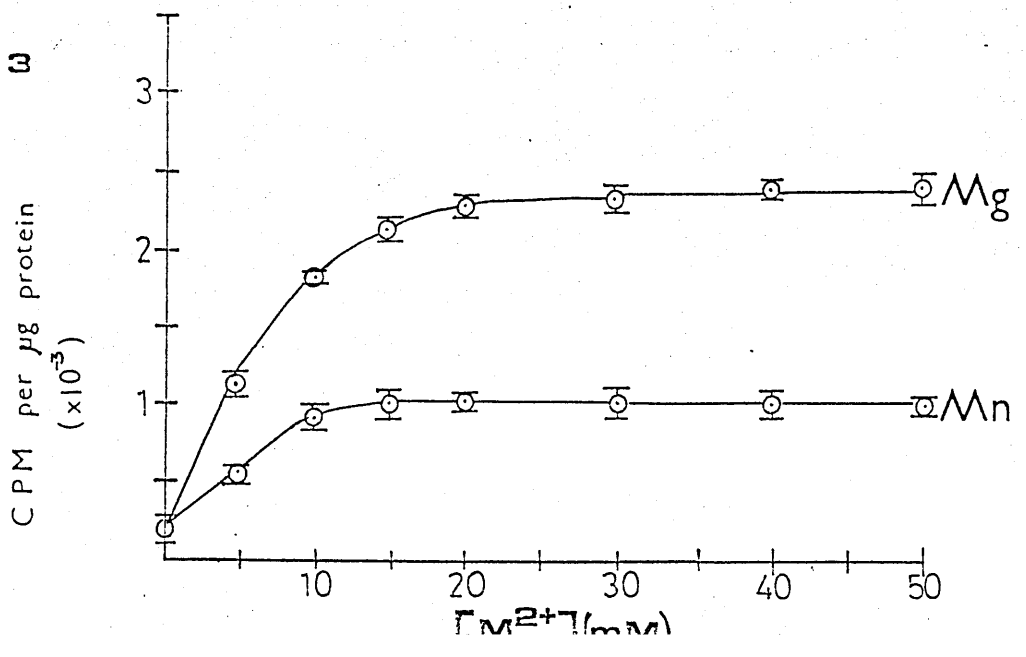

3
3
2
1
CPM per μg protein
(×10⁻³)
Mg
Mn
10
20
30
40
50
[M²⁺](mM)

TABLE 5 : Substrate Specificity of Kinase Activity

'A' and 'B' isolated from a Sephadex G100 column as described in Methods (3.1), and hnRNP particles isolated from sucrose density gradients as described in Methods (2) were dialysed against 50mM MOPS, 10mM $MgCl_2$, pH 6.5. They were then assayed for kinase activity as described in Methods (4.5) with or without the addition of 100 µg casein or 180 µg histone to the assay.

	C.P.M. Added Substrate			
	None	Casein	Histone	Casein + Histone
HnRNP	10000	23000	16000	35000
A	10000	9000	9500	10000
B	5000	43000	9000	50000

TABLE 5

detected by autoradiography (Figure 32). The results showed that histone, and particularly casein were phosphorylated in the presence of 'B' but that 'A' incorporated little or no phosphate into histone and small amounts into casein. Similar results can be seen in Figure 33 in an experiment which was initially designed to investigate the reason for the plateau after 30 minutes of enzyme activity, (Figure 29). At the 30 minute time point the residual assay mixture was divided into several equal portions and various additions made before removing aliquots to test for kinase activity at 40, 50, 60 and 70 minutes. The results showed that the levelling off of kinase activity at 30 minutes was due to substrate exhaustion as the addition of extra (γ -^{32}P) ATP did not increase phosphate incorporation whereas addition of further substrate did. Addition of extra enzyme also caused increased phosphate incorporation but this result was hard to interpret as the enzyme was impure and impossible to add without providing more substrate. The addition of casein to 'A' at 30 minutes showed only a slight increase in phosphorylation, again demonstrating that 'A' did not phosphorylate casein to a great extent. Conversely, the 30 minute addition of casein to 'B' was more effective than adding more endogeneous substrate.

Heparin has been shown to inhibit casein kinase II and so was used here to see its effect on 'A' and 'B' kinase activities. Neither heparin, cAMP, putrescine, spermidine, isobutylmethyl xanthine (IBMX) added alone had any marked effect on hnRNP,

'A'/

FIGURE 32: Phosphorylation of Casein and Histone

'A' and 'B' isolated from a Sephadex G100 column as described in Methods (3.1) were dialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5. 400µl of each of 'A' and 'B' was incubated at 30°C for 10 minutes with 100 µg of casein, 180 µg histone and 0.1 nmoles (γ -^{32}P) ATP at 100mCi/mmol. The proteins were then precipitated with ethanol, resuspended in SDS sample buffer and analysed on SDS polyacrylamide gels as described in Methods (4.3). The gels were then stained, destained, dried and autoradiographed to reveal whether casein or histone had been phosphorylated.

This figure shows the autoradiograph obtained, with the positions of the casein and histone on the gel indicated.

1. 'A' kinase : 20 µg protein loaded on the gel
2. 'A' kinase : 40 µg protein loaded on the gel
3. 'B' kinase : 20 µg protein loaded on the gel
4. 'B' kinase : 40 µg protein loaded on the gel

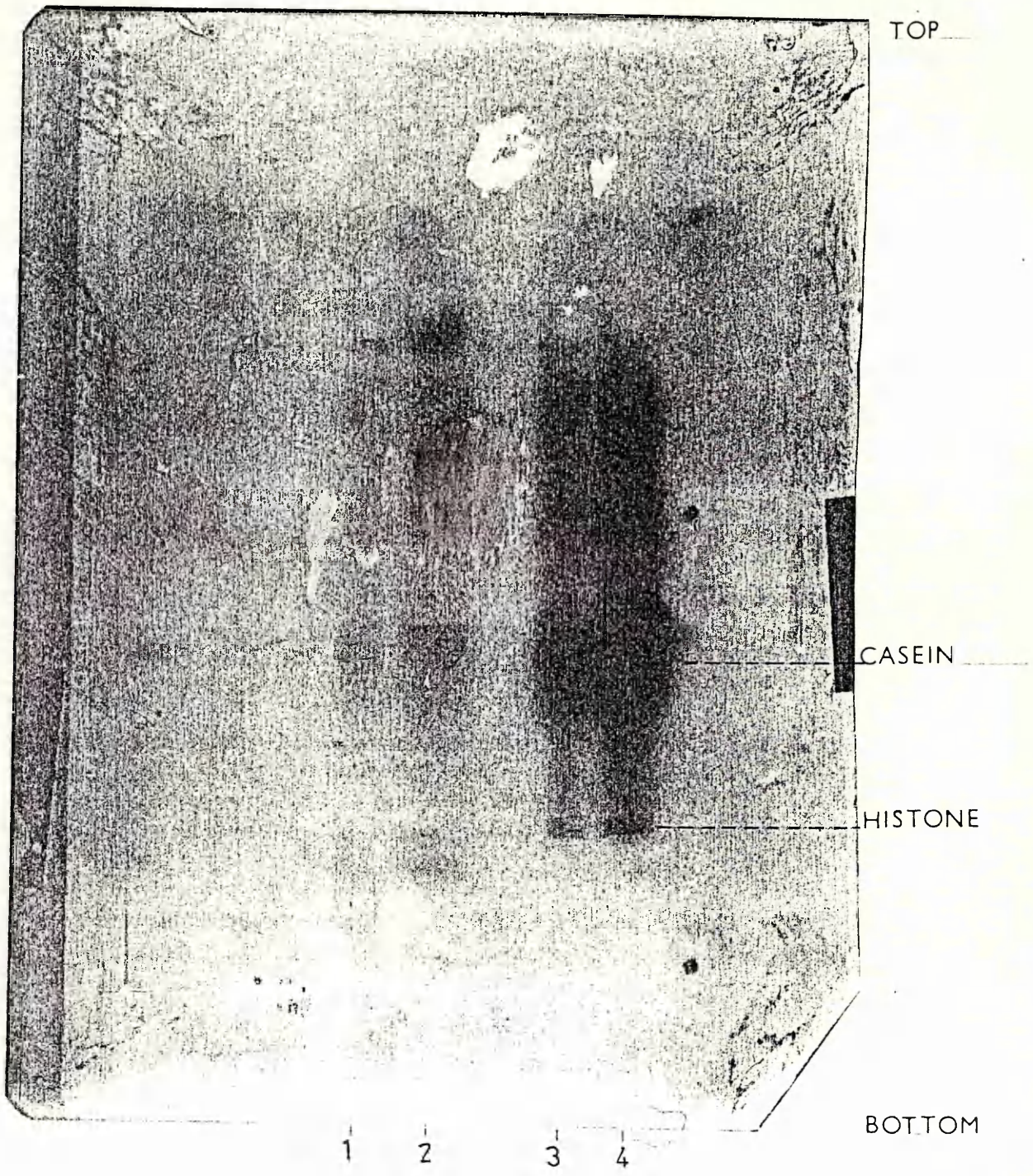

TOP
CASEIN
HISTONE
BOTTOM
1
2
3
4

FIGURE 33: Substrate Specificity of Kinase Activities 'A' and 'B'

'A' and 'B' peaks isolated from a Sephadex G100 column as described in Methods (3.1) were dialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5 and assayed for kinase activity.

The assay mixture was analysed at 0, 10, 20 and 30 minutes by removing aliquots and measuring kinase activity as described in Methods (4.5). The residual assay mixture at 30 minutes was divided into several equal portions and additional substances added as indicated below. Further aliquots were then removed for assay at 40, 50, 60 and 70 minutes.

⊙—⊙—⊙ endogenous kinase activity (i.e. no casein in original assay mixture)

△—△—△ kinase activity with casein present in the original assay mixture

- ⊙ - ⊙ - ⊙ - addition of extra casein (250µg/ml of assay) to assay at 30 or 60 minutes

··⊙···⊙···⊙·· addition of endogenous substrate (300µg/ml of assay) at 30 minutes

(Kinase-free substrate was the pooled fractions C, D, E from the Sephadex G100 column as indicated on p123)

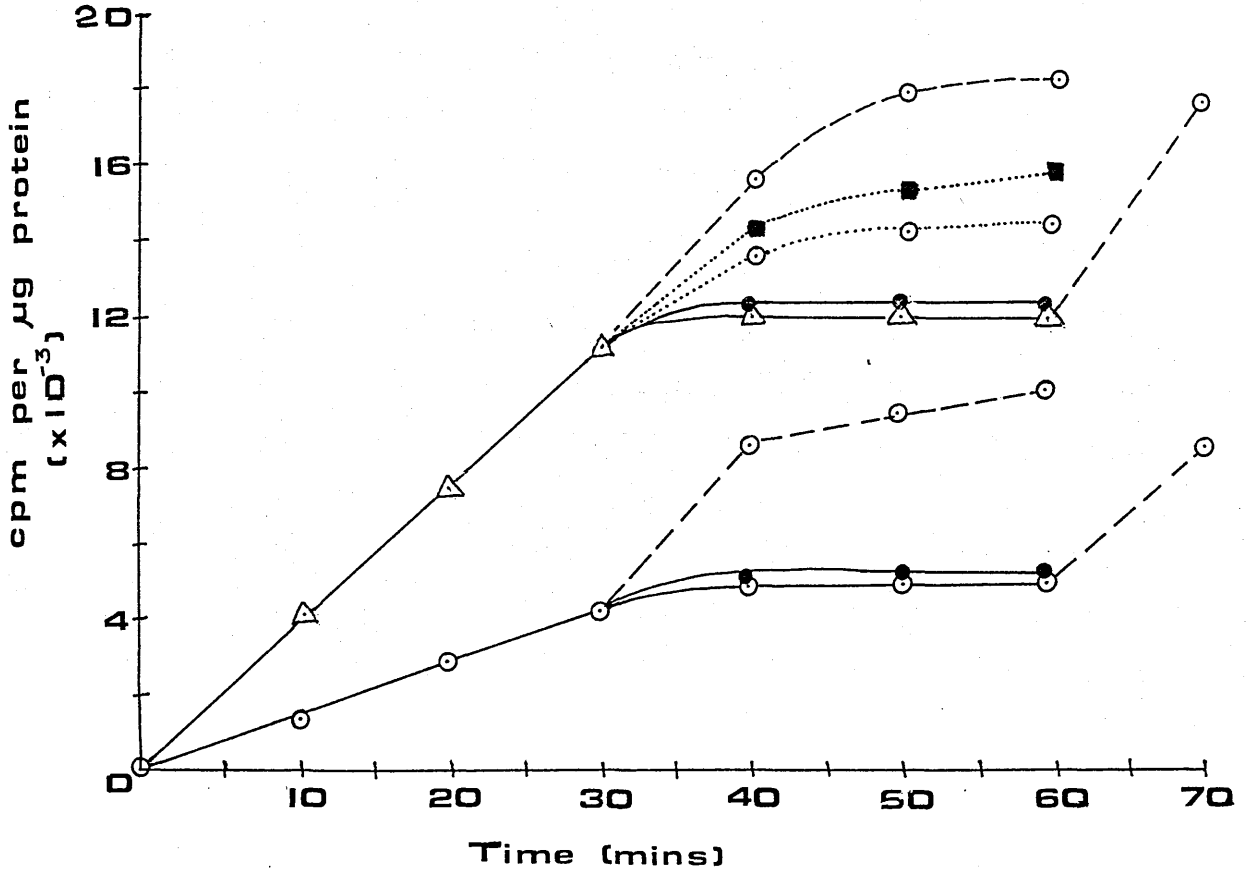
●—●—● addition of extra (γ - ^{32}P) ATP to assay at 30 minutes

■·····■·····■ addition of extra endogenous kinase activity to assay at 30 minutes (i.e. peaks 'A' or 'B' from Sephadex G100 column)

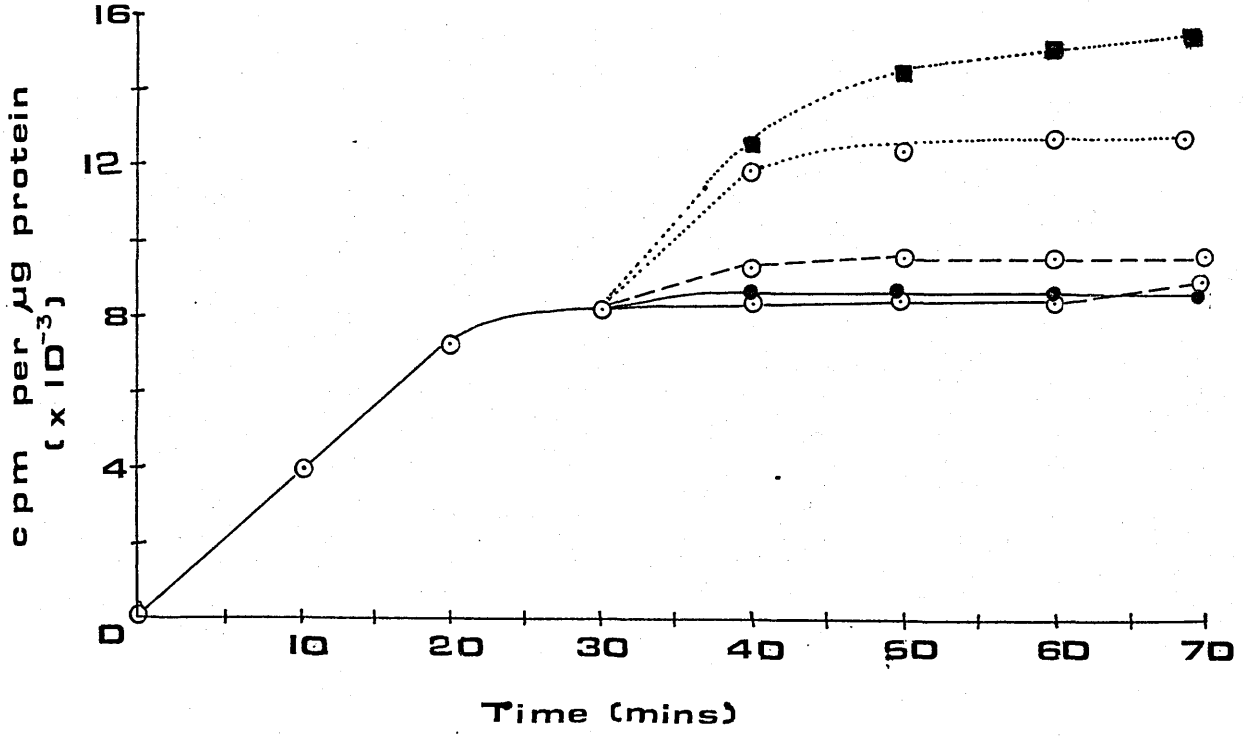
1. 'B'

2. 'A'

1.


20
16
12
8
4
0
cpm per µg protein
(x 10⁻³)
10
20
30
40
50
60
70
Time (mins)

2.


16
12
8
4
0
cpm per µg protein
(x 10⁻³)
10
20
30
40
50
60
70
Time (mins)

'A' or 'B' kinase activities (Table 6). The lack of effect of cAMP and polyamines differed from the results of Wilks & Knowler (1981a), who detected a kinase activity in rat liver hnRNP particles which was stimulated by cAMP and polyamines. However, fluoride, an inhibitor of phosphatases, stimulated particulate kinase activity five fold and peak 'B' kinase activity 1.6 fold. The addition of cAMP slightly enhanced the effect of the fluoride. No effect of fluoride was seen on peak 'A' kinase activity and IBMX, also a phosphodiesterase inhibitor, had no significant effect on any of the activities. EDTA and 1M NaCl inhibited peak 'A', peak 'B' and particulate kinase activity.

4.2 Attempts to further purify 'A' and 'B' kinase activities

As both 'A' and 'B' are clearly heterogeneous, further attempts were made to try to improve the purity of both activities.

4.2.1 Phosphocellulose: 3cm x 1cm phosphocellulose (Whatman No. 11) columns were set up and equilibrated with 10mM Tris pH 7.9, 100mM NaCl, 1.5mM $MgCl_2$, 6mM mercaptoethanol, 10% w/v glycerol. Peaks 'A' and 'B', isolated from Sephadex G100 column, were dialysed against the above equilibration buffer and then loaded on to the column. When 'A' was fractionated in this way the kinase activity was detected in the 0.5M NaCl/5mM ATP wash along with other polypeptides. Some purification was achieved in that many of the loaded polypeptides were not bound to the column. However the enzyme-containing peak was still very impure and/

TABLE 6: Effect of various additives on kinase activity

HnRNP particles were isolated from sucrose density gradients as described in Methods (2) and the peaks 'A' and 'B' were isolated from the Sephadex G100 column as described in Methods (3.1). They were dialysed against 50mM MOPS, 10mM $MgCl_2$, pH 6.5, before being analysed for kinase activity as described in Methods (4.5). The kinase activity was measured in the presence of various substances as indicated in the table.

The results here are the average of 2 separate determinations.

IBMX = isobutylmethylxanthine

ADDITION	A		B		HnRNP	
	cpm x 10^{-3} per assay	% of control	cpm x 10^{-3} per assay	% of control	cpm x 10^{-3} per assay	% of control
cAMP (1μM)	129	= 104%	132	= 100%	93	= 103%
Dibutyryl cAMP (10μM)	126	= 102%	129	= 98%	92	= 102%
cAMP dependent protein kinase inhibitor (10μg per assay)	122	= 98%	128	= 97%	96	= 107%
Putrescine (5mM)	120	= 97%	132	= 100%	90	= 100%
Spermidine (5mM)	124	= 100%	115	= 93%	92	= 102%
Fluoride (25mM	128	= 103%	210	= 159%	460	= 500%
Fluoride + cAMP	134	= 108%	248	= 188%	532	= 590%
Putrescine + cAMP	132	= 106%	128	= 97%	90	= 100%
IBMX (1mM)	122	= 98%	129	= 98%	85	= 94%
Heparin (5μg per ml)	124	= 100%	130	= 98%	92	= 102%
EDTA (10mM)	20	= 16%	25	= 19%	17	= 19%
NaCl (1M)	43	= 35%	57	= 43%	28	= 31%
Control	124	= 100%	132	= 100%	90	= 100%

TABLE 6

and there was a 60% loss of kinase activity (Figure 34). When the experiment was repeated with peak 'B', kinase activity was found in the run-through eluate and there was very little binding of kinase or contaminating protein to the phosphocellulose (Figure 34). Thus, phosphocellulose did not appear to offer a potential means of further purifying the enzymes.

4.2.2 Ammonium Sulphate Precipitation: 'A' and 'B' isolated from the Sephadex G100 column were brought to various concentrations of ammonium sulphate by adding increasing volumes of a saturated solution. The precipitated proteins were then analysed on SDS polyacrylamide gels. The treatment appeared to destroy all kinase activity in 'A' as no activity could subsequently be detected. Kinase activity was detected in the 40% $(NH_4)_2SO_4$ fraction from peak 'B' (Figure 35). However, the majority of 'B' polypeptides were also found in this fraction and no effective purification of kinase was achieved. Futhermore there was a 55% loss of kinase activity.

Time did not permit further attempts to purify the kinase activities.

4.3 Endogenous Phosphorylation of HnRNP Particle Proteins by Kinases 'A' and 'B'

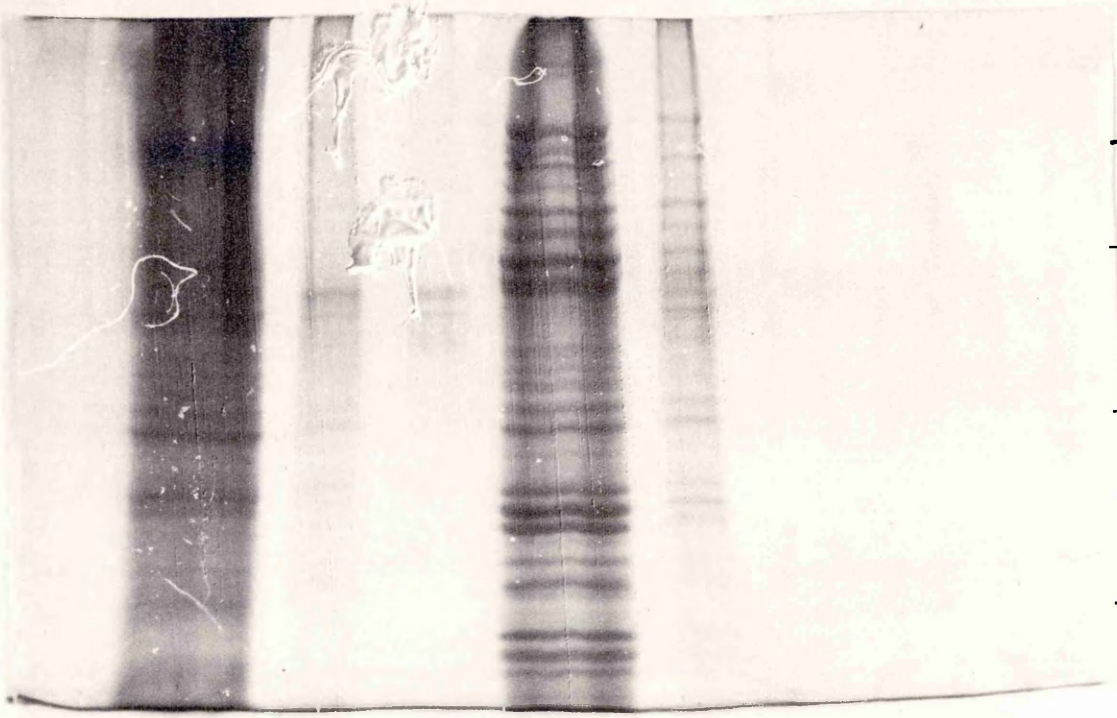
In order to analyse the endogenous phosphorylation of hnRNP proteins/

FIGURE 34: Phosphocellulose columns with 'A' and 'B'

'A' and 'B' peaks isolated from a Sephadex G100 column as described in Methods (3.1) were diluted to 0.1M NaCl with phosphocellulose column buffer (10mM Tris HCl pH 7.9, 100mM NaCl, 1.5mM $MgCl_2$, 6mM mercaptoethanol, 10% w/v glycerol. Amounts containing kinase activity of 3000 cpm/µg protein of peak 'A' kinase activity or 9000cpm/µg protein of peak 'B' kinase activity were added to 3cm × 1cm phosphocellulose columns. The columns were washed with 4ml aliquots of buffer containing increasing concentrations of NaCl. The collected fractions were microdialysed against 50mM MOPS, 10mM $MgCl_2$, pH 6.5, and assayed for kinase activity as described in Methods (4.5) or precipitated and analysed on SDS polyacrylamide gels as described in Methods (4.3).

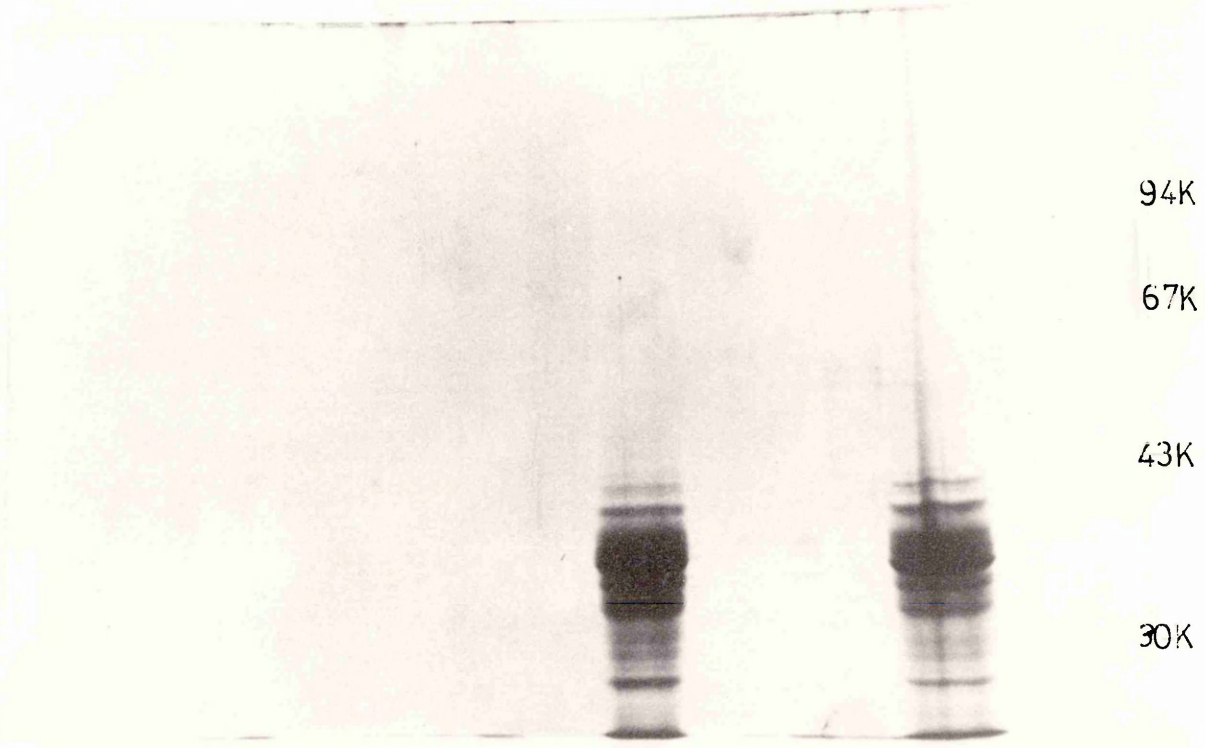
Gel Track	Kinase Activity (cpm/µg protein)	
	'A'	'B'
1. run through eluate	-	8000
2. 0.3M NaCl wash	-	-
3. 0.3M NaCl wash	-	-
4. 0.5m NaCl/5mM ATP wash	1200	-
5. 0.5M NaCl/5mM ATP wash	-	-

A



1 2 3 4 5

B

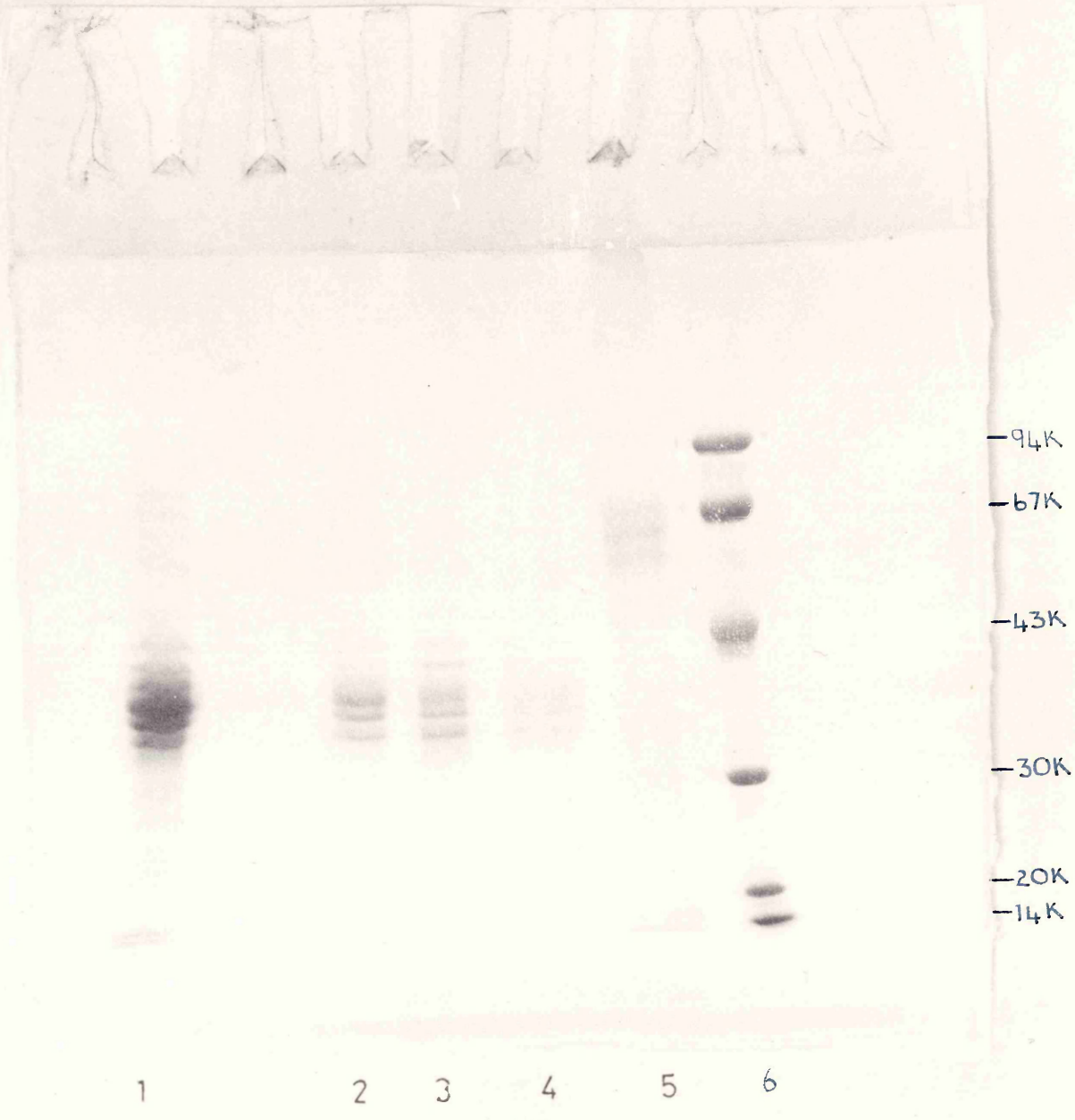


1 2 3 4 5 B

FIGURE 35: Treatment of 'B' with Ammonium Sulphate

Peak 'B' isolated from Sephadex G100 column as described in Methods (3.1) was brought to various concentrations of ammonium sulphate. The precipitated proteins at each stage were analysed on SDS polyacrylamide gels as described in Methods (4.3). Some of each fraction was dialysed against 50mM MOPS, 10mM $MgCl_2$ pH 6.5 and analysed for kinase activity as described in Methods (4.3).

Gel Track	Kinase activity (cpm/μg protein)
1. 'B'	9700
2. 30% $(NH_4)_2SO_4$	-
3. 40% $(NH_4)_2SO_4$	4350
4. 50% $(NH_4)_2SO_4$	-
5. supernatant	-
6. markers	


94K
67K
43K
30K
20K
14K
1
2
3
4
5
6

proteins by the 2 kinase activities, the eluate from Sephadex G100 columns (Methods 3.1) was fractionated as indicated below:-

When 'A' and 'B' were added to the pooled 'C', 'D' and 'E' fractions, dialysed and incubated with (γ -^{32}P)ATP the results, seen in Figure 36, were very similar to those seen with intact hnRNP particles (Figure 23), thus showing that the Sephadex column did not produce anomalies and indicating that differences seen between the proteins phosphorylated by peaks 'A' and 'B' are not caused by the fractionation.

'C', 'D'and 'E' were then added separately to either 'A' or 'B' incubated with (γ -^{32}P)ATP and the phosphorylated proteins analysed on 2-dimensional gels to see if there was any difference in the proteins phosphorylated by 'A' and 'B' kinase activities. (Figures 37, 38, 39, 40).

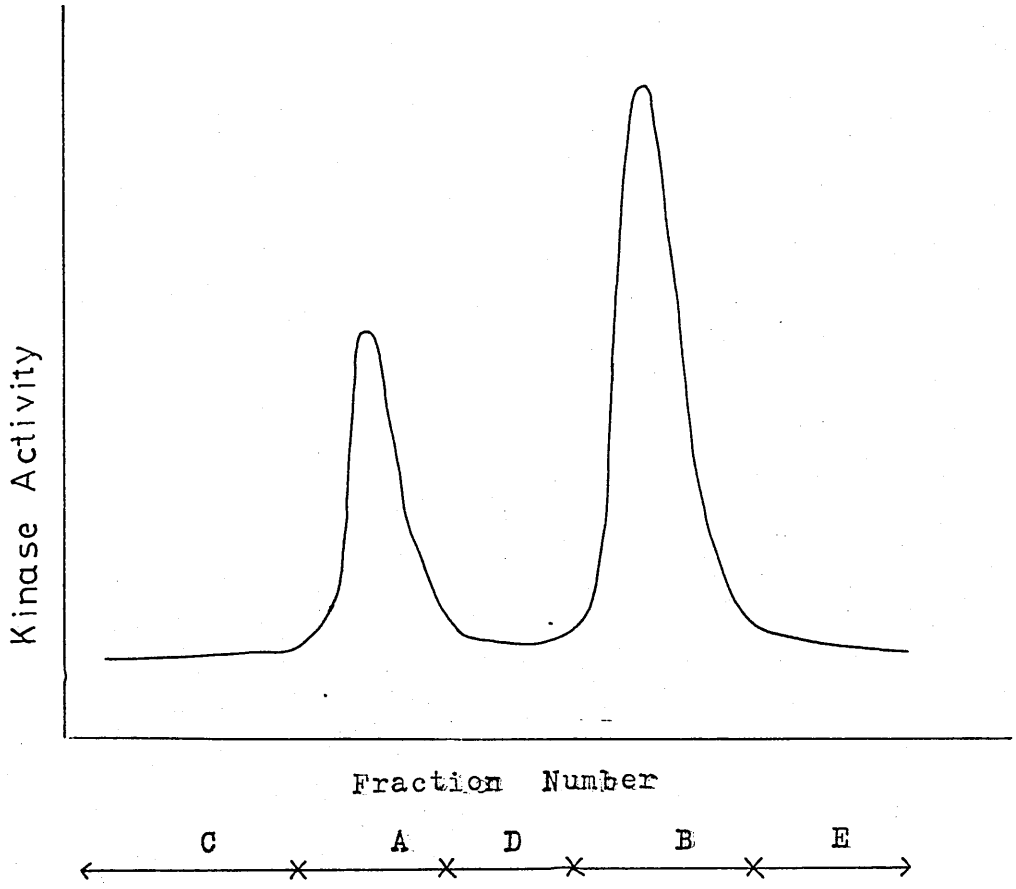
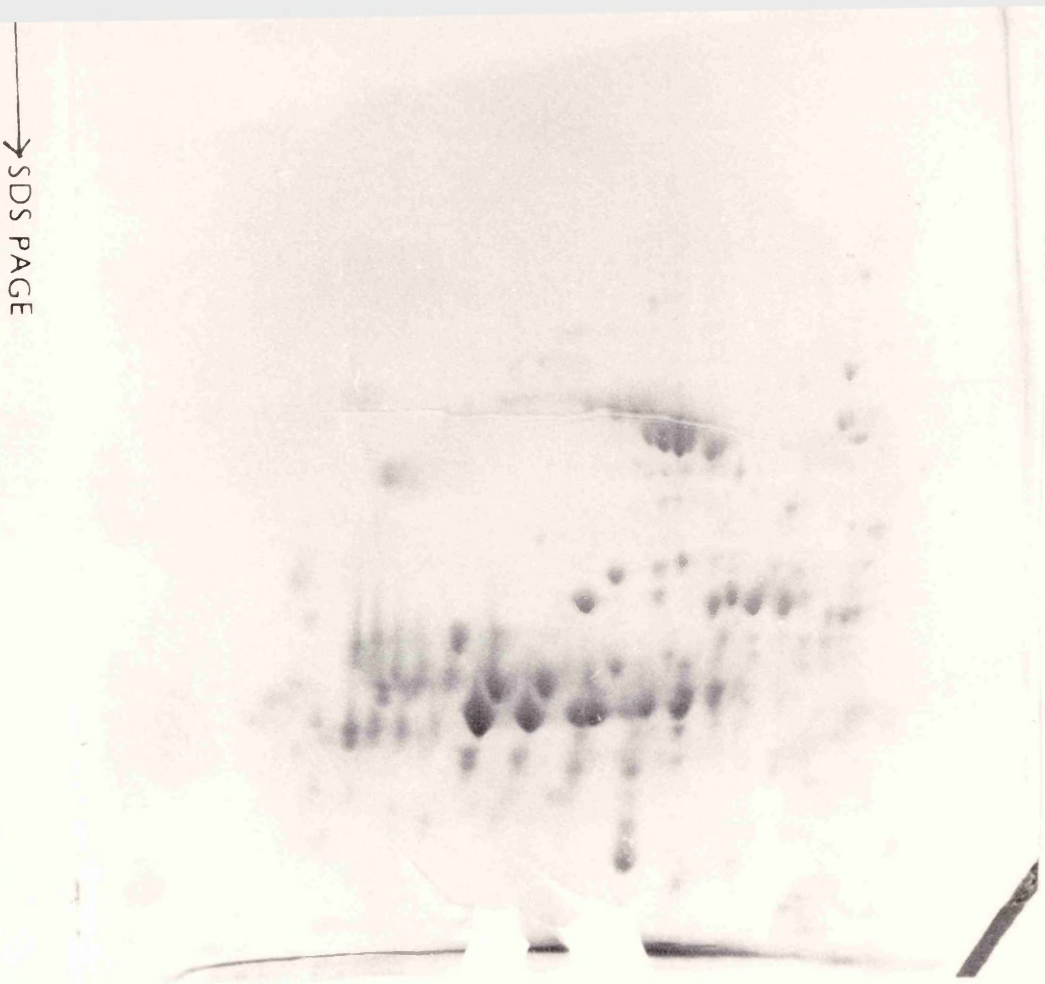


FIGURE 36: Phosphorylation of total protein isolated from Sephadex G100 column

The peaks of kinase activity 'A' and 'B' and the pooled fractions C, D and E (section 43) isolated from the Sephadex G100 column as described in Methods (3.1) were added together in the presence of (γ-^{32}P) ATP at 10m Ci/μmol using 50μ Ci/500μg protein, incubated at 30°C for 10 minutes. The ethanol precipitated proteins were then resuspended in lysis buffer (9.5M Urea, 5% v/v mercaptoethanol, 2% w/v NP-40, 2% v/v ampholines pH 3.5-10) before being loaded on NEPHGE gels. These gels along with the second dimension SDS polyacrylamide gels were run as described in Methods (4.2). The gels were stained, destained then dried and autoradiographed.

1. A + B + C, D and E - stained gel
2. A + B + C, D and E - autoradiograph

SDS PAGE



2

NEPHGE

SDS PAGE

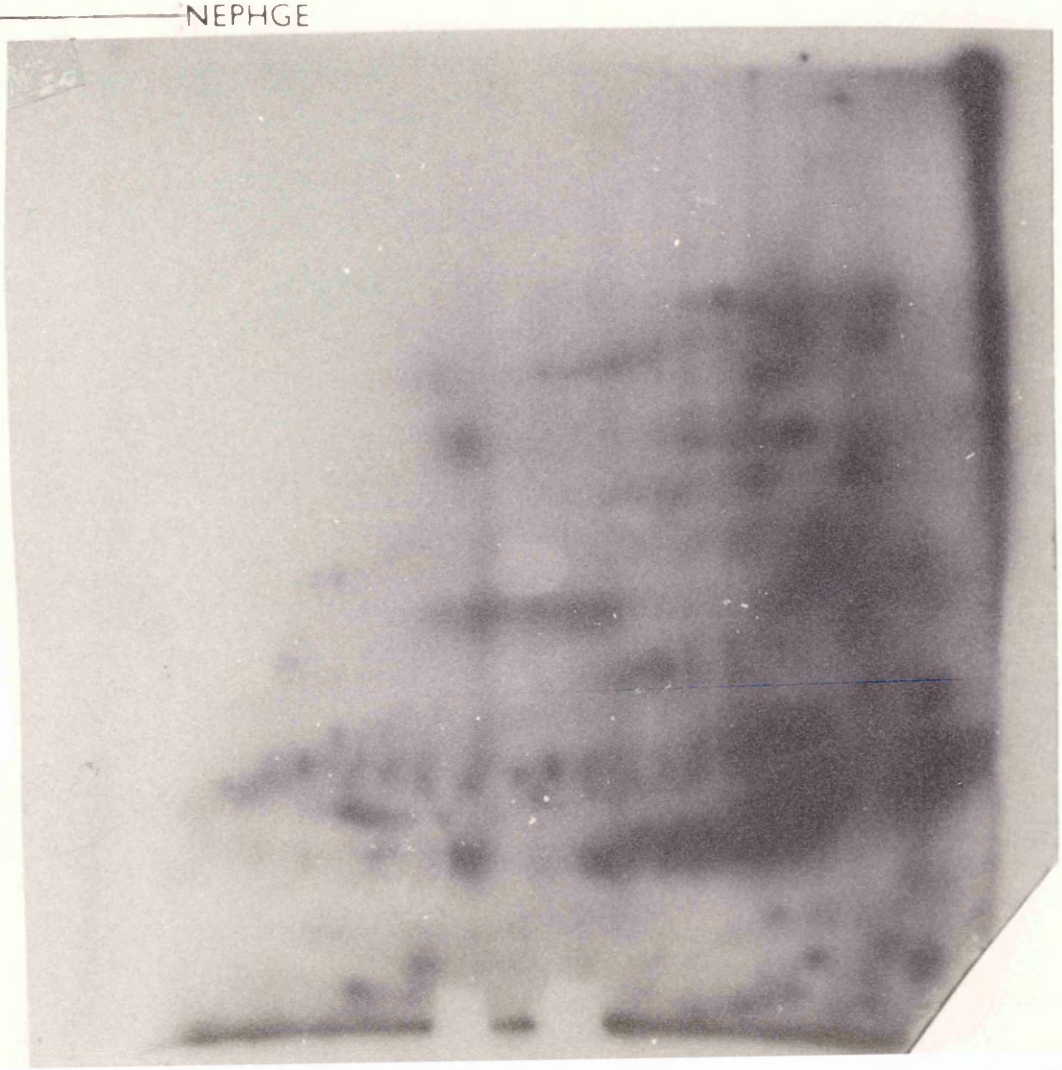


FIGURE 37: Phosphorylation of Proteins in Fractions 'A' and 'B'

Peaks 'A' and 'B' were isolated from a sephadex G100 column as described in Methods (3.1). They were then dialysed against 50mM MOPS, 10mM $MgCl_2$, pH 6.5, before being incubated at 30°C for 10 minutes in the presence of $[\gamma\text{-}^{32}P]$ ATP at 10mCi/μmol using 10μCi/100μg protein. The proteins were then precipitated with 2-3 volumes of ethanol, before being resuspended in lysis buffer and loaded onto NEPHGE gels. The first and second dimensional gels were loaded and run as described in Methods (4.2). The proteins in the gels were then stained and incorporated (^{32}P) detected by autoradiography.

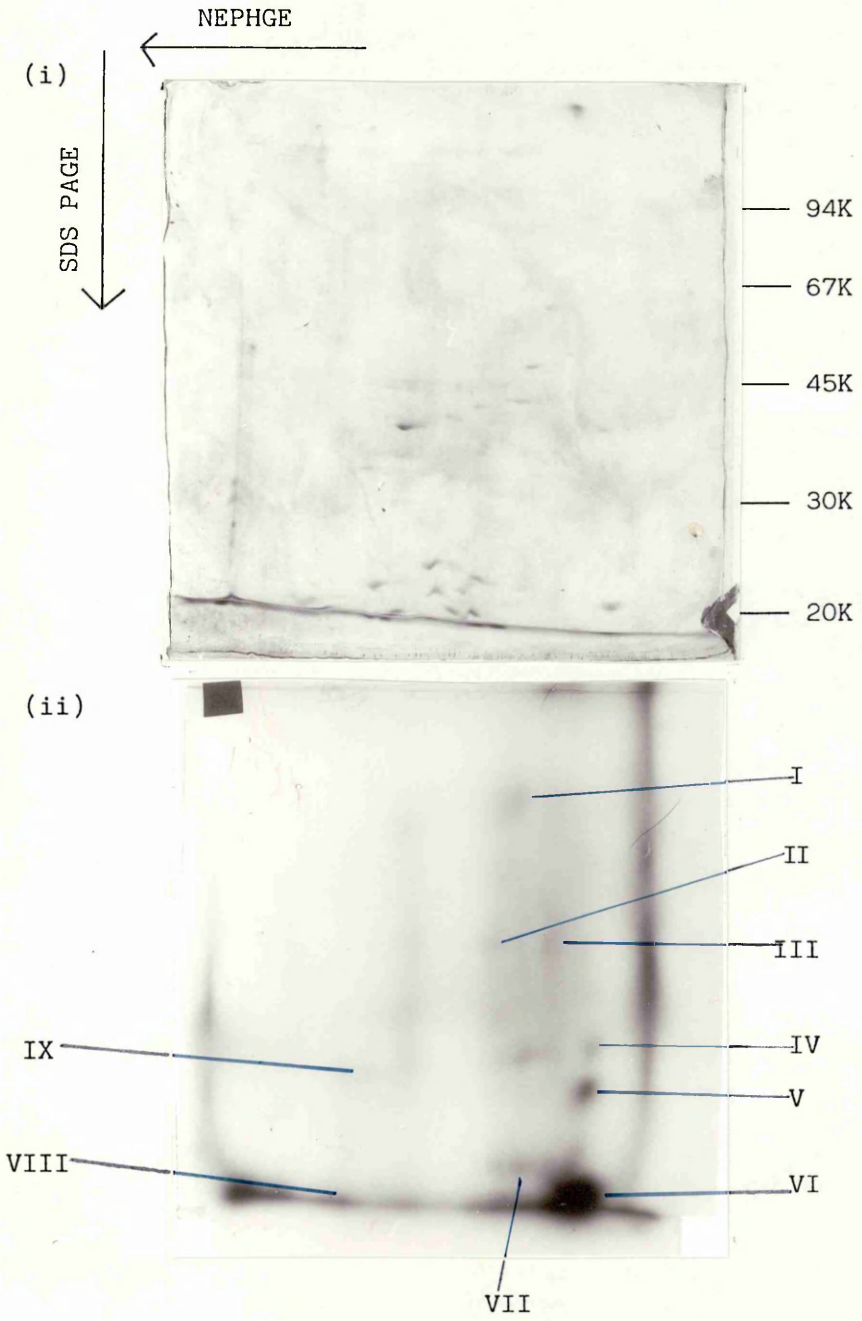
The results here show the stained gels and the corresponding autoradiographs.

(i) stained polypeptides of 150μg of peak 'A' proteins

(ii) stained polypeptides of 150μg of peak 'B' proteins

(iii) autoradiograph of the stained gel (i)

(iv) autoradiograph of the stained gel (ii)


NEPHGE
(i)
SDS PAGE
94K
67K
45K
30K
20K
(ii)
I
II
III
IV
V
VI
VII
VIII
IX

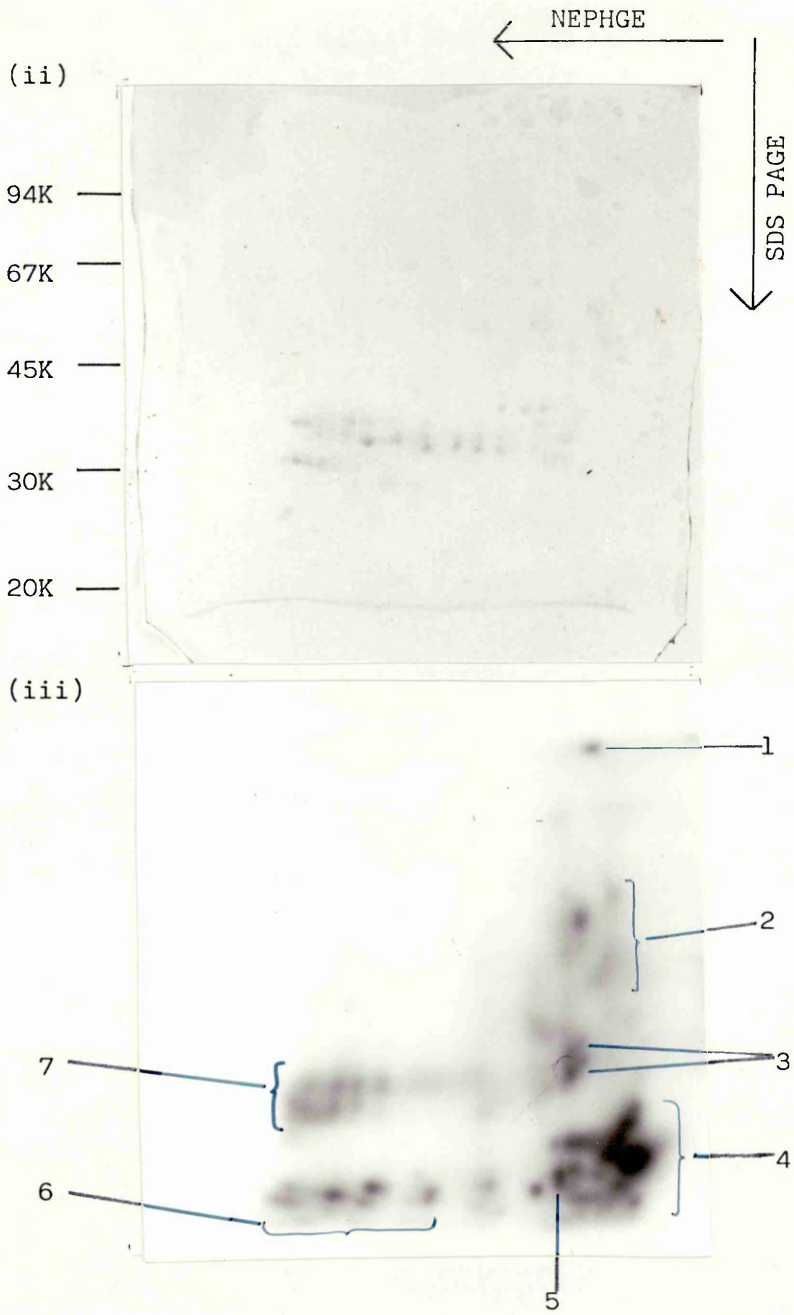

NEPHGE
(ii)
SDS PAGE
94K
67K
45K
30K
20K
(iii)
1
2
3
4
5
6
7

FIGURE 38: Phosphorylation of Proteins in Fraction C by 'A' and 'B'

Peaks 'A' and 'B' and fraction C were isolated from a Sephadex G100 column as described in Methods (3.1). They were then dialysed against 50mM MOPS, 10mM $MgCl_2$, pH 6.5, before being incubated at 30°C for 10 minutes in the presence of $(\gamma\text{-}^{32}P)$ATP at 10mCi/μmol, using 10μCi/100μg protein. The proteins were then precipitated with 2-3 volumes of ethanol, before being resuspended in lysis buffer and loaded onto NEPHGE gels. The first and second dimensional gels were loaded and run as described in Methods (4.2). The proteins in the gels were then stained and incorporated (^{32}P) detected by autoradiography.

The results here show the stained gels and the corresponding autoradiographs.

(i) stained polypeptides of 100 μg of peak A and 250 μg of fraction C proteins

(ii) stained polypeptides of 150 μg of peak B and 150 μg of fraction C proteins

(iii) autoradiograph of the stained gel (i)

(iv) autoradiograph of the stained gel (ii)

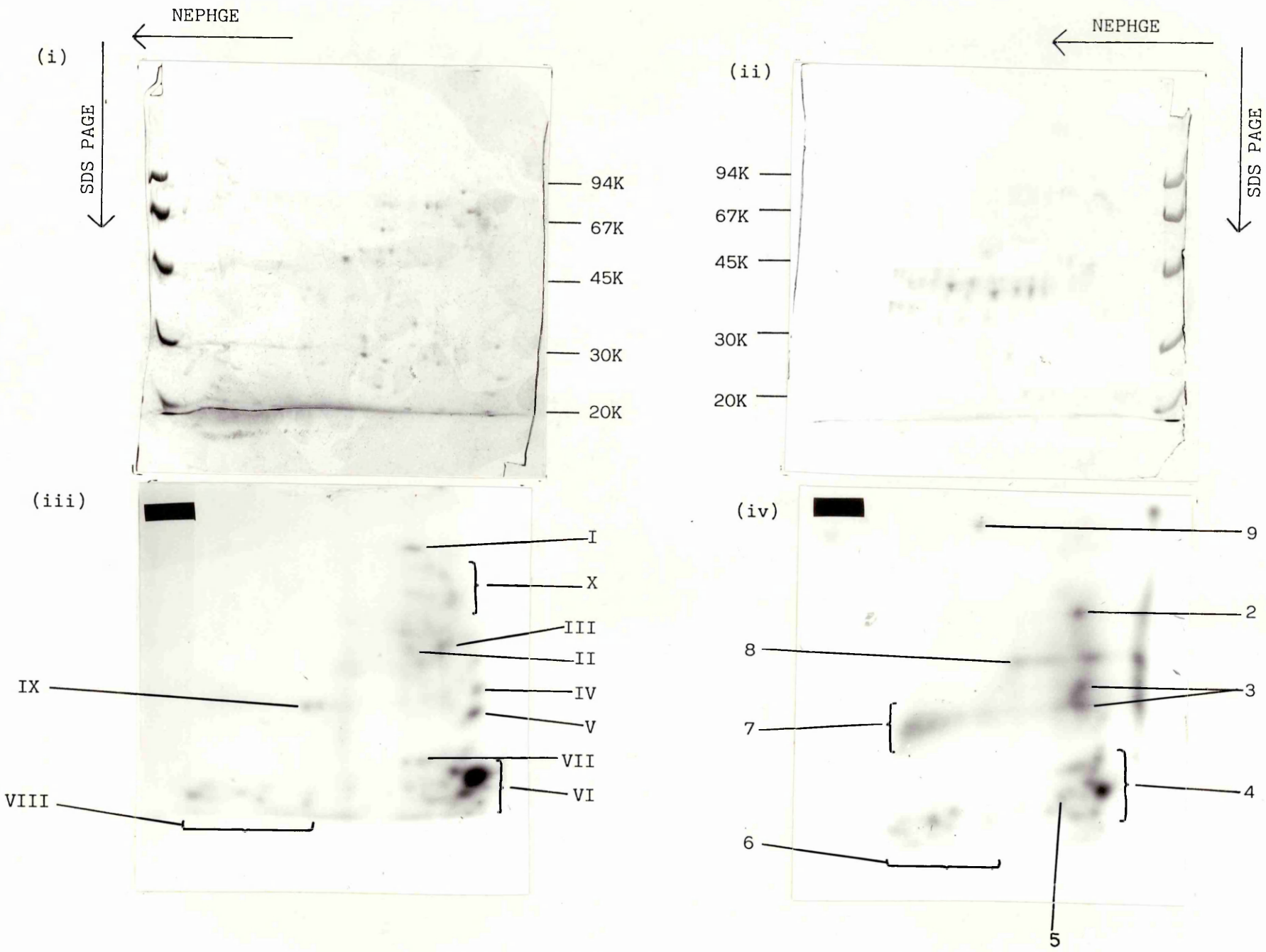

(i)
NEPHGE
SDS PAGE
94K
67K
45K
30K
20K
(ii)
NEPHGE
SDS PAGE
94K
67K
45K
30K
20K
(iii)
I
X
III
II
IX
IV
V
VII
VI
VIII
(iv)
9
2
8
3
7
4
6
5

FIGURE 39: Phosphorylation of Proteins in Fraction D by 'A' and 'B'

Peaks 'A' and 'B' and fraction D were isolated from a Sephadex G100 column as described in Methods (3.1). They were then dialysed against 50mM MOPS, 10mM $MgCl_2$, pH 6.5, before being incubated at 30°C for 10 minutes in the presence of $(\gamma\text{-}^{32}P)$ATP at 10mCi/μmol, using 100μCi/100μg protein. The proteins were then precipitated with 2-3 volumes of ethanol, before being resuspended in lysis buffer and loaded onto NEPHGE gels. The first and second dimensional gels were loaded and run as described in Methods (4.2). The proteins in the gels were then stained and incorporated (^{32}P) detected by autoradiography.

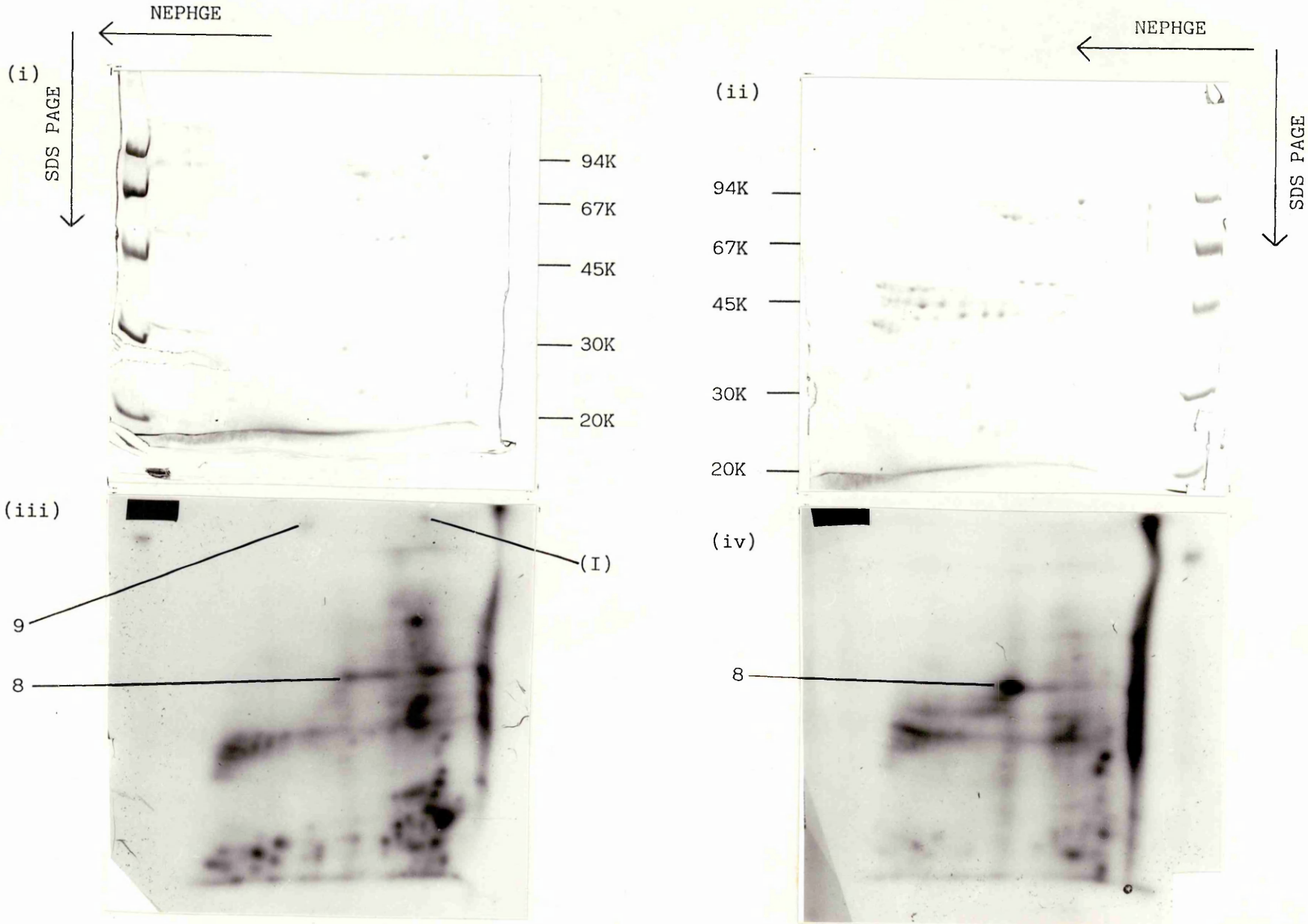
The results here show the stained gels and the corresponding autoradiographs.

(i) stained polypeptides of 100 μg of peak A and 200 μg of fraction D proteins

(ii) stained polypeptides of 100 μg of peak B and 200 μg of fraction D proteins

(iii) autoradiograph of the stained gel (i)

(iv) autoradiograph of the stained gel (ii)


NEPHGE
(i)
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94K
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SDS PAGE
(iv)
8

FIGURE 40: Phosphorylation of Proteins in Fraction E by 'A' and 'B'

Peaks 'A' and 'B' and Fraction E were isolated from a Sephadex G100 column as described in Methods (3.1). They were then dialysed against 50mM MOPS, 10mM $MgCl_2$, pH 6.5, before being incubated at 30°C for 10 minutes in the presence of $[\gamma - {}^{32}P]$ ATP at 10mCi/μmol, using 10μCi/100μg protein. the proteins were then precipitated with 2-3 volumes of ethanol, before being resuspended in lysis buffer and loaded onto NEPHGE gels. The first and second dimensional gels were loaded and run as described in Methods (4.2). The proteins in the gels were then stained and incorporated (^{32}P) detected by autoradiography.

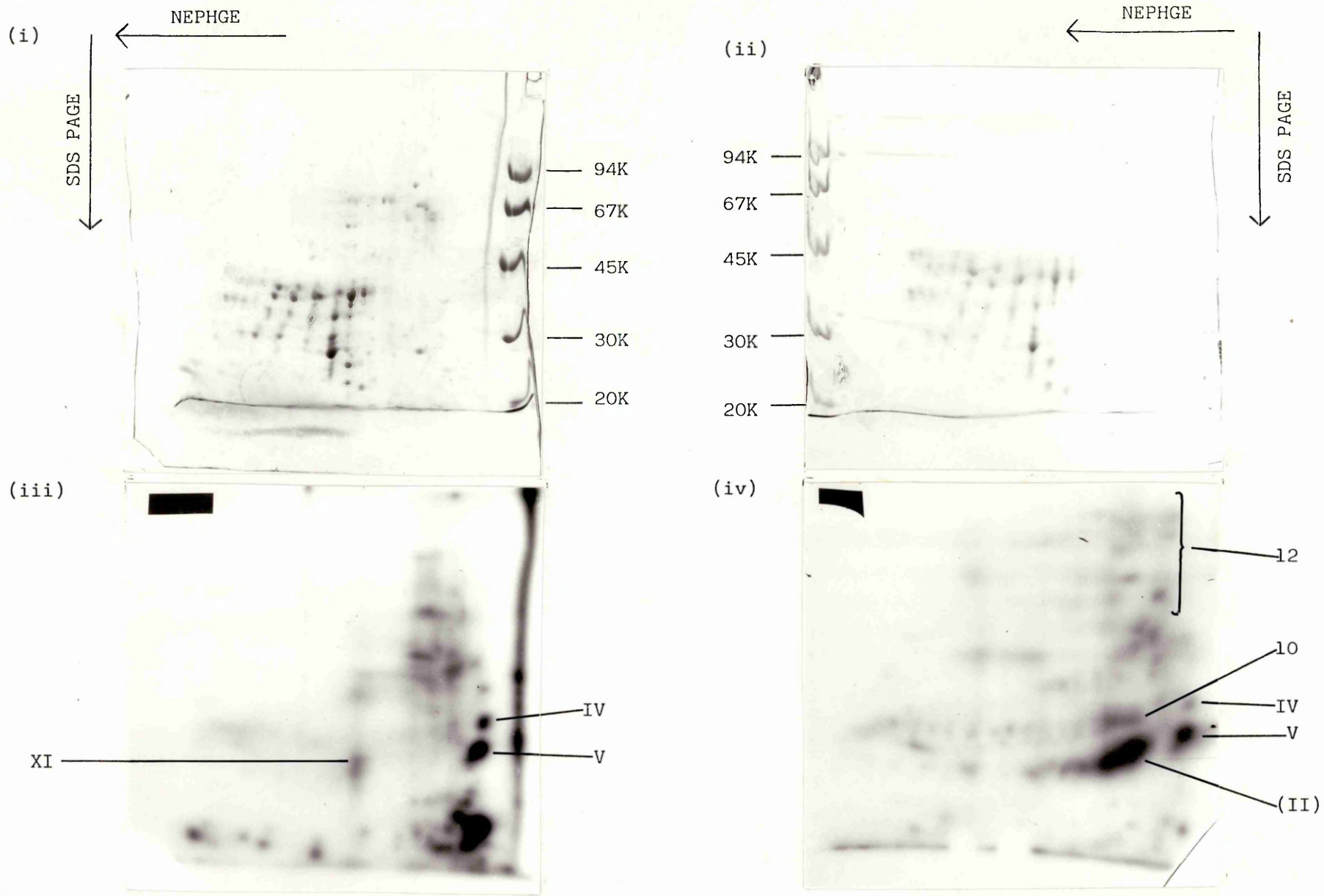
The results here show the stained gels and the corresponding autoradiographs.

(i) stained polypeptides of 100 μg of peak A and 250 μg of fraction E proteins

(ii) stained polypeptides of 100 μg of peak B and 200 μg of fraction E.proteins

(iii) autoradiograph of the stained gel (i)

(iv) autoradiograph of the stained gel (ii)


(i)
NEPHGE
SDS PAGE
94K
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(ii)
NEPHGE
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(iii)
XI
IV
V
(iv)
12
10
IV
V
(II)

DISCUSSION

DISCUSSION

1. Authenticity of hnRNP Particles

The possibility that non-specific adsorption might contribute to the observed protein profile of isolated hnRNP is particularly difficult to exclude as there is no functional assay available to distinguish or define hnRNP proteins. The defining of hnRNP proteins is made more complex as some of the proteins may be true components of more than one subnuclear complex. The solution of this problem needs detailed consideration of the individual proteins, their functions and their locations within the cell.

The authenticity and integrity of hnRNP particles, have, over the years been demonstrated in many ways including:-

1. Electronmicrographs: From a very early stage in their synthesis the transcripts of eukaryotic genes are seen to be associated with protein (Sommerville, 1973; McKnight & Miller, 1976) in the form of 25nm particles on the nascent RNP fibrils.

2. Antibody studies: Antibodies prepared against several hnRNP proteins specifically label RNP associated with DNA loops undergoing transcription (Martin & Okamura, 1981) and it has been shown that hnRNP particles can be formed during *in vitro* transcription (Economides & Pederson, 1982).

3. Protein profiles: The protein profiles of particles isolated by various methods i.e. by extraction method of Samarina *et al.*, (1966) and the lysis method of Pederson (1974a), are very similar.

4./

4. Failure to generate artefactual complexes: It is not possible at present to totally exclude the possibility that small amounts of soluble proteins adsorb on to hnRNP during their isolation. However, particles isolated in the presence of radioactively labelled proteins from other cellular pools have failed to demonstrate significant contamination (Wilks & Knowler, 1981a). HnRNP's have been shown not to be artefacts by the failure to generate hnRNP-like complex by addition of deproteinised hnRNA to cellular extracts (Pederson, 1974a, 1981).

5. Constituent RNA of hnRNP particles can be shown to compete with hnRNA in hybridisation to DNA (Mantieva et al, 1969) and to hybridise to saturation with radioactively labelled cDNA copies of total mRNA (Kinniburgh & Martin, 1976).

6. Crosslinking experiments have been used to look at the integrity of hnRNP particles and have shown that the protein profile of particles crosslinked in situ in HeLa cells with U.V. light was indistinguishable from that of non-crosslinked particles (Mayrand et al., 1981). The same proteins are cross-linked to host RNA and viral RNA sequences in virus infected cells (Van Eekelen et al, 1981b).

In the work presented here rat liver hnRNP was shown to have the same protein composition as that described by other workers and no contamination with histones or ribosomal proteins could be detected (Figure 13). These two groups of proteins serve as markers for chromatin and ribosomes, the two most likely contaminants.

2./

2. Authenticity of protein kinase as an hnRNP protein

The collected evidence, listed above shows that hnRNP particles are not artefacts but correspond to the nascent hnRNP observed in situ. It remains possible, however, that small quantities of proteins adhere to the particles during their purification and it is very difficult to rule this out especially when one is claiming that an enzyme detectable only by its catalytic activity is associated with hnRNP particles. At least 10 enzyme activities have been detected in hnRNP particles and a host of minor proteins are detectable on stained 2D gels, yet the molecular weight of the particle has been estimated at 1,000,000 (Krichevskaya & Georgiev, 1969) of which 60-90% is core protein. Le Stourgeon and co-workers have calculated that the core proteins occur in the ratio of 3 each of A_1 and A_2, 1 each of B_1 and B_2 and 3 of C_1 and C_2. At an average molecular weight of 40000 this gives a molecular weight of 560000 and takes no account of the more acidic D group of core proteins (Wilks & Knowler, 1981). In short, the non-core proteins are likely to have a combined molecular weight of not more than 400000 and possibly as little as 100000, so it is difficult to conceive that they include molecules of ten enzymes and other non-core proteins in each particle.

It is not necessary, however, to envisage that an enzyme such as a protein kinase is a rigid component of every particle. The occurrence of an enzyme activity, even weakly or transiently, and in equilibrium with other cellular components might still be of physiological/

physiological importance. It is to be expected that a kinase activity such as this is not strongly bound to the particles but should be free to dissociate and move from one gene transcript to another. It is difficult to see how it could perform its catalytic function if it was a rigid and integral part of the particle structure.

Few workers regularly purify hnRNP particles more extensively than their recovery as a peak from sucrose density gradients. Particles can certainly be subjected to further purification procedures but it is not certain whether the products are more pure or depleted in their components. Bajszar et al., (1978) have demonstrated the loss of endogenous mRNA capping enzymes during repeated purification of particles. Similarly, in this work when the hnRNP peak from sucrose density gradients was concentrated and subjected to gel filtration (Figure 19) or rapidly dialysed and relayered on a second sucrose density gradient, there were partial losses of endogenous kinase activity and these steps were not therefore used routinely. For the reasons outlined above, it is difficult to know whether the lost entities were contaminants or loosely bound particle proteins.

Similar arguments are relevant to the kinase activity which remains at the top of the sucrose density gradient after the isolation of hnRNP's (Figure 18). This activity could be free nucleoplasmic enzyme extracted from nuclei with the particles. This/

This in itself could be different from or in equilibrium with the particulate activity. Alternatively the enzyme at the top of the gradient could result from dissociation from particles caused by the shear forces of the centrifugation. It would have been desirable to compare and contrast this non-particulate enzyme activity with those characterised on the particles. Such a comparison may have resolved some of the above possibilities but time did not permit it. The fact that kinase activity was still found associated with hnRNP particles after gel filtration on Bio Gel columns, (Figure 19) and after respinning on a second sucrose density gradient, and that a band of kinase activity corresponded to the stained band of hnRNP on non-denaturing gels (Figure 17) suggests that the kinase is genuinely associated with hnRNP particles.

3. Difficulty of Fractionating Kinases and other hnRNP Proteins

The fractionation of hnRNP particle proteins to yield reasonable quantities of purified proteins is not easy as the proteins have strong affinities for each other. Even after extensive RNase digestion of the RNA component, hnRNP proteins still occur in particulate form and then aggregate into even higher molecular weight complexes (Stevenin et al, 1979). Results presented in this thesis show that treatment with 1M NaCl followed by dilution may result in some rearrangement of the particles or the formation of a higher molecular weight aggregate, (Figure 26).

HnRNP/

HnRNP particles can be completely dissociated by high salt concentrations (Figure 26) but Fuchs & Jacob (1979) argue that some particles, (representing a separate population) appear to be resistant to dissociation up to 0.4M NaCl and that complete dissociation required greater than 0.7M NaCl.

Reports suggesting the existence of biochemically distinct monoparticle populations on the basis of salt and nuclease sensitivity of RNP have been disputed and may represent rearrangements of monoparticles as a result of RNase treatment and resedimentation on sucrose density gradients (Stevenin et al., 1977; Stevenin et al., 1979). Nevertheless reports exist that strongly suggest a heterogeneity of structures (Gattoni et al., 1977). Gattoni et al., (1977) have shown 2 distinct populations of hnRNP monoparticles with distinct and characteristic RNA and protein compositions which do not appear to represent rearrangements of RNP components.

The work presented in this thesis employed 1M NaCl to look at dissociation of hnRNP particles. In our hands 1M NaCl caused complete dissociation of hnRNP particles but this dissociation was partially reversible and removal of salt led to reaggregation of a substantial percentage of the proteins, (Figure 26). This could account for the results obtained with ion-exchange columns (Results 4.1). Even when the dilution was made at the moment of application to the column or when the salt was dialysed away with the ion-exchange resin present in the dialysis tubing it seemed likely that aggregation was occuring.

Dialysis/

Dialysis appeared to result in reconstitution of the protein into hnRNP particles as the nature and relative abundancies of the particles resembled those of the hnRNP. However care must be exercised with this interpretation as a considerable amount of work was done with reconstituted chromatin in the early 1970's only to be followed by strong indications that the reconstituted material was not the same as the original.

Fractionation of the particle kinases can only be achieved when conditions can be found which satisfy the following requirements:-

1. Particle must be dissociated or at least the kinase activity dissociated.
2. Dissociation must not destroy kinase activity.
3. Either the particle constituents must be fractionated in the dissociating medium or changing the medium must not result in reassociation, i.e. 1M NaCl fractionation only met the criteria when it was not subsequently diluted.

The only method which met these criteria was the fractionation of hnRNP protein in the presence of 1M NaCl. Under these conditions protein kinase activity was recovered as 2 peaks which are here referred to as 'A' and 'B', (Figure 27).

The main question was whether the 'A' and 'B' activities isolated from the Sephadex G100 column, represented different kinases or different forms of the same enzyme. Thus peak 'A' could represent kinase still bound to undissociated particles and peak 'B' could be the dissociated kinase. Alternatively, 'A' could be a multisubunit kinase/

kinase perhaps in a form attached to a regulatory subunit with 'B' representing the free monomeric form, i.e. similar to cAMP-dependent protein kinase.

Various arguments tend to rule out all of these possibilities. 1M NaCl has been shown to completely dissociate hnRNP particles (Figure 27) but even increasing the salt concentration to 1.5M NaCl did not alter the kinase profile from the Sephadex column. This suggests that 'A' may represent a different activity. When visualised by coomassie blue staining, the peaks 'A' and 'B' contained a different set of polypeptides (Figure 27) though the enzymes may not have been detectable in this way.

Although 'A' and 'B' had similar pH and time profiles to that of total hnRNP kinase activity, their dependence on divalent ions was slightly different. 'A' and hnRNP kinase activity were maximally stimulated at 15-20mM Mg^{2+} whereas 'B' continued to show further slight increases up to 40mM Mg^{2+}. Furthermore the activity of 'A' was stimulated by Mn^{2+} whereas that of 'B' was not.

Kinase 'A' activity appeared to be more stable than 'B' at 50°C and 60°C. However this difference could conceivably result from differential stabilisation by other proteins in the fraction.

Another major difference was the fact that 'A' kinase activity could only use hnRNP particle proteins. It has been shown that the lack of histone and casein phosphorylation by 'A' was not due to excess acceptor proteins in 'A' (Figure 33 and 34), and that the casein and histone were actually phosphorylated by 'B'

Protein/

Protein kinases have been detected in nuclear extracts by several workers (Takeda et al., 1971; Kimayama et al., 1971; Desjardins et al., 1972). Kish & Kleinsmith (1974) have separated the nuclear protein kinase activity into 12 distinct peaks. Each activity exhibited different specificities for casein, histone and non-histone proteins as substrates as well as showing a variety of cofactor requirements. However, they all had a pH optimum at pH 7.5.

The activities investigated in this work showed similarities with the enzyme detected in rat liver hnRNP by Wilks and Knowler (1981a) which also had a pH optimum at pH 6.5. The present enzymes differed however in that they were cAMP independent and were not stimulated by polyamines. In the latter characteristic, they also differed from the activity described by Ohtsuki et al., (1980) in mouse spleen. Enzyme 'B' and total hnRNP kinase activity did show some stimulation (Table 7) with cAMP in the presence of fluoride (phosphatase inhibitor) but the phosphodiesterase inhibitor isobutylmethyl xanthine did not appear to have any effect on either 'A', 'B' or hnRNP kinase activities. No activity strongly responsive to cAMP could be detected in the fractionated particle proteins. It must be remembered, however, that the particles have been subjected to moderately denaturing conditions which may irreversibly inactivate the activity detected in intact particles by Wilks & Knowler (1981a).

The lack of effect of cAMP, phosphodiesterase inhibitor and the/

the cAMP-dependent protein kinase inhibitor provide some evidence that the kinase in peaks 'A' and 'B' are not related to each other in the way that the active and inactive forms of cAMP-dependent kinase are.

Although 'B' and hnRNP kinase activities could use casein as an exogeneous substrate, unlike casein kinase II (Hathaway & Traugh, 1984) they were not inhibited by heparin.

An enzyme activity which is similar to hnRNP particle kinase activity in rat liver is the cAMP dependent nucleoplasmic protein kinase isolated by Neuman et al.,(1978) which has a pH optimum at pH 6.5 but differs in that this kinase is histone specific.

The hnRNP kinase activity detected by Blanchard et al., (1977) in HeLa cells had a maximum activity at pH 8.3 in the presence of 10mM $MgCl_2$ and was also cAMP independent.

The 'A' and 'B' activities detected in this work could not use GTP in place of ATP and were thus similar to the activity detected by Blanchard et al., (1977) in HeLa cells. An activity detected in HeLa cells by Holcomb & Friedman (1984) could use GTP or ATP. It was stimulated by polyamines and inhibited by heparin and was thus similar to casein kinase II. All these differences in the kinase activity characteristics could be explained if multiple nuclear protein kinases were capable of weak and transient association with hnRNP. The different methods used by different workers in this field could favour the detection of different enzymes.

Periasamy/

Periasamy et al., (1979) have reported the partial purification of a kinase activity from HeLa cell hnRNP particles, by using DEAE and phosphocellulose chromatography. The activity was similar to that detected by Blanchard et al., (1977). It had a molecular weight of 48000 as determined by gel filtration and appeared as 2 bands of molecular weight 25-28000 on SDS gels. Periasamy et al.,(1979) suggest that this activity shows similarities to nuclear kinase I from rat liver cells (Thornburg et al., 1977).

Holcomb & Friedman (1984) have purified 2 casein kinases from micrococcal nuclease disrupted hnRNP from HeLa cells using DEAE and phosphocellulose. The first kinase showed greatest activity towards casein but no activity against the major particle proteins. It constituted 70% of the total particle kinase activity. The second kinase activity eluted from the phosphocellulose overlapped the C-core protein peak and Holcomb & Firedman suggested that this activity was the C-protein kinase. The work described in this thesis indicates that aggregation by hnRNP proteins can give rise to problems where ion-exchange resins are used to fractionate hnRNP proteins.

4. Phosphorylation of HnRNP Particle Proteins

4.1 Endogenous Phosphorylation of Proteins within the Peaks A and B

The protein kinase-containing peaks 'A' and 'B' were seen to contain different sets of proteins in Figure 27 and this is again seen in two dimensional fractionations (Figure 37 (i) and (ii)).

Peak/

Peak A contained relatively little protein and gave rise to very faint two dimensional stained profiles. The failure of further attempts at purification of the kinase means that it is not known whether any of the stainable spots represent the enzyme.

The autoradiographs of Figure 37 (iii) and (iv) reveal totally different sets of polypeptides that are phosphorylated by the endogenous enzymes. Peak A contains relatively few clearly defined phosphorylated products (indicated by Roman numerals) while Peak B contained multiple groups of phosphorylated polypeptides (indicated by Arabic numberals) which included phosphorylated core proteins (1). Repeat analysis of peak A, however, (data not shown because of poorly stained protein profile) revealed that the strongly phosphorylated low molecular weight spots VI and VIII in A and (4) and (6) in B were probably identical. In this region of the gels only the spots VII in A and (5) in B appeared different.

It should be emphasised that the differences probably reflect the phosphorylatable components of peaks A and B rather than differences in the specificity of the enzymes.

4.2 Phosphorylation of Proteins in Fractions A, B and C by the kinases of Peaks A and B

The stainable polypeptide profiles of A and C are still very different from those of B + C (Figure 38). The latter is dominated by the core proteins while the former is dominated by other hnRNP proteins.

Many/

Many of the phosphorylated polypeptides are unchanged from Figure 37 and it can now be seen that the spots VI and VIII form an identical pattern to (4) and (6). The spots VII and (5) however remain different. Some new, or much more noticeable spots, have appeared on both of the autoradiographs of Figure 38. Thus, on Figure 38 (iii) a faint spot labelled IX on Figure 37 is now much stronger and co-migrates with the most basic elements of the core proteins. Of perhaps more interest are a group of labelled polypeptides X in Figure 38 (iii) which were never seen with peak A alone and appear to be fraction C specific proteins. Their interest stems from the finding that some of them at least appear not to be phosphorylated by the kinase activity in peak B. Other changes occur in Figure 38 (iv) where the proteins in fraction C are combined with peak B. Most notable of these are the disappearance of the spot (1) and the appearance of the streak (8) and the spot (9). However, all three of these subsequently reappear in Figure 39 (iii), the autoradiograph of A + D.

All of these experiments were performed twice and most of them three times. Nevertheless the appearance of these three spots in experiments A + D and C + B, and their absence in other combinations would appear artifactual.

4.3 Phosphorylation of Proteins in Fractions A, B, and D by the Kinases in Peaks A and B

Mention has been made above of the spots (1), (8) and (9). Apart/

Apart from these, the addition of fraction D to the Peaks A and B resulted in no new clearly identified spots. Rather the addition of the D proteins to each peak made both the stained profiles and the autoradiographs of the phosphorylated proteins much more alike, i.e. there is little difference between A + D and B + D. Comparison of repeat experiments only served to indicate that those spots which did appear quantitatively, if not qualitatively different in Figures 39 (iii) and (iv), were not reproducibly so.

4.4 Phosphorylation of Proteins in Fractions A, B and E by the kinases in Peaks A and B

The stained polypeptides of Figure 40 (i) and (ii) are strikingly different from those with other combinations of gel filtration column fractions. The core protein profile now contains many extra spots which appear to be lower molecular weight derivatives of each of the charged separated polypeptides. They are faintly discernible in all gels containing peak B (Figures 37 (ii), 38 (ii), 39 (ii)) but are now very obvious and must form a major part of Fraction E. It must be likely that they are breakdown products. On the autoradiographs (Figures 41 (iii) and (iv)) core protein phosphorylation is now much less obvious. Futhermore some new spots have appeared on both fractionations. These are the spots labelled XI on Figure 40 (iii) and the spots labelled (10), (11) & (12) on Figure 40 (iv) (12 is a complex of at least 3 components). It would appear that these are E specific polypeptides that are differentially phosphorylated by the 2 kinase activities.

4.5/

4.5 Analysis of the Data on Endogenous Phosphorylation of HnRNP Particle Proteins

Considerable care must be taken with the interpretation of the data on the endogenous phosphorylation of fractionated hnRNP proteins. To begin with the fractionation is arbitrary and incomplete, with many proteins probably present in more than one fraction. Secondly, when the effects of the enzymes on the phosphorylation of the proteins in C, D and E are examined, one is looking for changes against the background phosphorylation of the proteins in A or B. Thirdly, repeat experiments can show greater variation than comparisons between different fractions. This is particularly true of the low molecular weight components which migrate very close to the marker dye, i.e. components (4),(5), (6),VI, VII and VIII. In most gels spot 4 appears the same as VI, and spot (6) the same as VIII. There are differences however in the areas (5) and VII which may be associated with proteins in peaks A and B.

Notwithstanding, all of the above, there do appear to be some differences in the proteins phosphorylated by the enzymes in peaks A and B. These are polypeptides X in Fraction C (Figure 38 (iii)) and the polypeptides XI, (10) (11) and, (12) in Fraction E (Figure 40).

CONCLUSION

In conclusion 2 different protein kinase activities have been detected in hnRNP particles. It has not proved possible to purify or quantitate either protein but they would appear to be minor components of the hnRNP particles. The enzymes may be transiently associated with the particles and may be partitioned between the particle and other nuclear fractions. On the basis of substrate specificity, with both exogenous and endogenous substrates, as well as their apparent molecular weight and, stability in high temperature, and activation by fluoride, it would appear that the two enzymes are genuinely different. However, it cannot be totally excluded that the apparent differences derive from the other proteins with which the impure enzymes are associated. The effect of fluoride in particular could result from phosphatase presence.

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