CORE PROTEINS OF RAT LIVER HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN (hnRNP) PARTICLES

Zeba I. Seraj

Thesis presented for the degree of Doctor of Philosophy, Faculty of Science, at The University of Glasgow, February 1986.

Acknowledgements

I would like to express my sincere thanks to:

Professor R. M. Smellie, Professor M. Houslay and Professor C. Fewson for making the departmental facilities available.

Dr J. T. Knowler, for his patient guidance and helpful suggestions throughout the course of my work.

Dr D. P. Leader, Dr J. R. Coggins, Dr. J. G. Beeley, Dr. H. Nimmo and Dr. D. Mousdale for their valuable suggestions and help.

My friends and colleagues in the department for being generally helpful, keeping my morale up and bearing with me.

My parents who have been a constant source of inspiration to me and my husband who has been a great support.

Abbreviations

BRL	Buffalo Rat Liver
hnRNA	heterogeneous nuclear RNA
hnRNP	heterogeneous nuclear ribonucleoprotein
Iodogen	1, 3, 4, 6-tetrachloro-3α, 6α-diphenyl glycouril
NEPHGE	Non-equilibrated pH gradient gel electrophoresis
MW	Molecular weight
PEG	Polyethylene glycol
snRNA	Small nuclear RNA
snRNP	Small nuclear ribonucleoprotein
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

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SUMMARY

To study the nature of the four core proteins A, B, C, and D, of rat liver heterogeneous nuclear ribonucleoprotein (hnRNP) particles, the proteins were isolated and fractionated by preparative sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. The structure of the four purified proteins were compared by partial peptide mapping on SDS-polyacrylamide gels, using the Cleveland Method. The enzymes used for the partial peptide mapping were α-Chymotrypsin and S. aureus V8 Protease. Products of the proteolytic treatment were detected on the SDS-polyacrylamide gels either by silver-staining or by autoradiography. In the latter case core proteins iodinated in their tyrosine residues were used and therefore only the tyrosine-containing fragments were observed. Experiments were further conducted to determine whether the four proteins represent glycosylated variants of one protein. As an aid for the structural study of the proteins, polyclonal antibodies were raised to total core proteins and separately to core protein A and B. Immunoblots of the core proteins with the antibody preparation were used to establish the relationship between the four core proteins.

Several approaches were made to determine the cause of the observed charge heterogeneity of each of the core proteins when they are fractionated on a pH-gradient. 1) Artefactual formation of the charge isomers was considered. 2) The charge isomers of core proteins A and B were subjectd to partial peptide mapping by the Cleveland Method. One dimensional SDS-polyacrylamide gel electrophoresis and two-dimensional gel

electrophoresis were used to map the peptides produced after V8 protease treatment. 3) The core proteins were subjected to both *in vitro* and *in vivo* phosphorylation. For the *in vitro* studies, qualitative and quantitative phosphorylation patterns were analysed. The *in vivo* phosphorylation was performed with core proteins of a Buffalo Rat Liver Cell Line, and 4) Preliminary attempts were made to determine whether the core proteins are poly ADP-ribosylated.

Results from the comparison of the partial peptide maps of isolated core proteins A, B, C and D indicated that the four proteins are a family of closely related species. A majority of the peptide products from each protein were identical, and there were relatively few unique bands. The immunological experiments confirmed the close relatedness of the proteins. Since the core proteins were found not to be glycosylated variants of each other, and certain unique peptides were observed, it was concluded that they probably are products of separate genes.

The partial peptide maps of the charge isomers of both core protein A and B were shown to be nearly identical. In the A series, charge isomers A_1 to A_6 are probably post-translational modifications of one protein. The B series of charge isomers B_1 to B_5 were concluded to be post-translational variants of one or possibly two proteins. It was not possible to identify the nature of the post-synthetic modification responsible for the charge isomers.

I. INTRODUCTION

Eukaryotic mRNA is derived from precursors collectively known as heterogeneous nuclear RNA (hnRNA). hnRNA is always found complexed with protein in the cell nucleus and evidence presented later in this section suggests that the processing of hnRNA to mRNA takes place while it is complexed with nuclear proteins in ribonucleoprotein particles known as hnRNP.

The aim of the present study was to investigate the nature of the core proteins which form over 80% of the total particle protein. A description of what is known of hnRNA processing and the association of hnRNA with proteins is necessary before explaining the background to the core proteins and the need to study them to elucidate their role, if any, in hnRNA processing.

1.0 TRANSCRIPTION OF EUKARYOTIC GENES

1.1 Eukaryotic Genes

The determination of the structure of the first eukaryotic gene revealed that unlike the genes of procaryotes whose coding sequences are continuous, the coding sequences (exons) of most eukaryotic genes are present in blocks separated by intervening or non-coding sequences (introns). Apart from the suggestion made by Gilbert (1978) and Blake (1983) that exon/intron structure could be a mechanism for increasing the rate of evolution, such a structure could provide a level for regulation of gene expression. For example, control could be exercise at steps of mRNA biogenesis (Nevins, 1982). The primary transcript from these split genes, which form at least a

part of heterogeneous nuclear RNA (hnRNA), contain internal regions that are excised during their maturation, the final messenger being a spliced product (mRNA).

In many cases, the total length of the introns exceeds the total length of the exons. Thus the chicken ovalbumin gene is 7564 nucleotides in length but codes for a mature mRNA of only 1872 nucleotides. The difference of 5692 nucleotides represents noncoding sequences arranged in seven introns (Woo et al., 1978; Woo et al., 1981). By cloning the avian vitellogenin gene, Wilks et al. (1981) have shown that the gene is interrupted with at least 25 introns having a mean length of 940 base pairs. The mean exon length was discovered to be only 250 base pairs in comparison.

1.2 hnRNA and mRNA

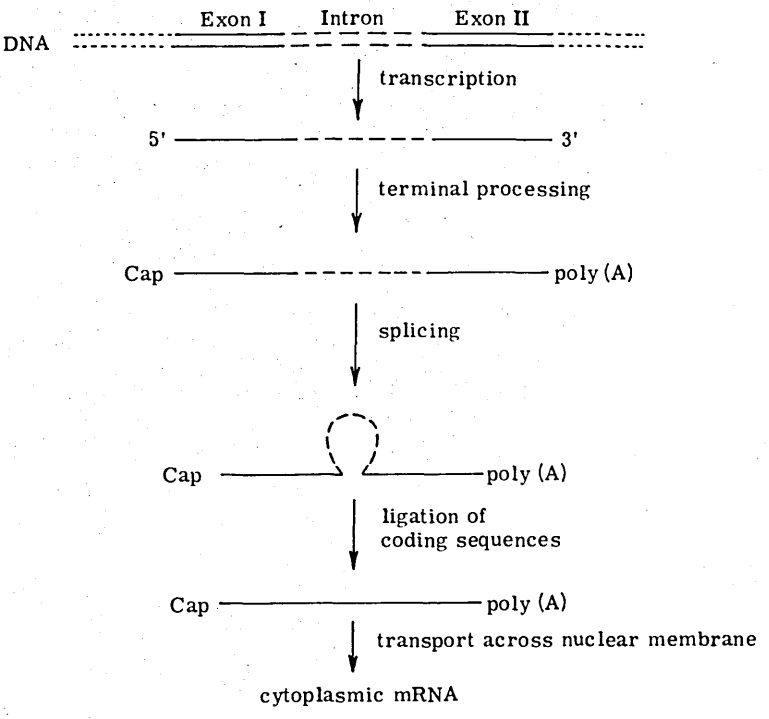
hnRNA is transcribed and localised in the nucleus and exhibits a diversity of size. hnRNA molecules may have lengths of up to 50,000 nucleotides (Greenberg and Perry, 1971). Most hnRNA appears to lie in a size range of from 10 to 100 times the length of mRNA which for animal cells has an average of about 2000 bases. Early kinetic evidence for the relationship between hnRNA and mRNA was ambiguous. However, the precursor-product relationship between hnRNA and mRNA was indicated with the finding that both possess 3' polyadenosine tails and modified 5' termini known as caps and that these tails and caps when labelled could be chased into polysomal mRNA (Darnell et al., 1971; Perry and Kelley, 1976).

It was also shown that all mRNA sequences are detected in hnRNA populations (Hanes and Perry, 1977; Aziz and Knowler, 1980). At the same time it was demonstrated that the intervening sequences or introns in DNA are absent from mature mRNA (Tilghman et al., 1978a; Breathnach et al., 1977; Jeffreys and Flavell, 1977). Subsequently Tilghman et al., (1978b) used the techniques of northern blotting and R loop mapping to show that a 15S precursor to β-globin mRNA contained a transcript of at least the larger of the two introns in the β-globin gene. Maturation of the 15S precursor to 10S mRNA was accompanied by excision of this intron transcript, a process which has come to be known as splicing. Tsai et al. (1980) followed the processing of ovomucoid mRNA using a kinetic approach combined with northern mapping and R loop mapping. Their results showed that all seven introns of the ovomucoid gene were transcribed into a large nuclear pre-mRNA and that these introns were removed in a preferred but not obligatory order. Similar processing has been followed in the transcript of a number of other genes, including a 7.8 Kb ovalbumin pre-mRNA (Tsai, et al., 1980), 10.6 Kb precursor to immunoglobulin light chain mRNA (Herbert and Wall, 1979) and a large precursor to amphibian vitellogenin mRNA (Ryffel et al., 1980) figure 1.1 shows a scheme for the processing of gene transcripts based on the studies quoted above (Knowler, 1983).

2.0 hnRNP PARTICLES

2.1 Proteins are Associated with hnRNA at Early Stage of Transcription

Evidence from spread chromatin preparations (McKnight and Miller, 1976) as well as from autoradiographic studies (Gall and



Diagramatic representation of the processing of the transcript of a gene containing a single intron.

Figure 1.1

Callan, 1962) indicates that protein/RNA associations are established immediately and continuously in the growing transcripts of eukaryotic genes, at virtually all of the transcriptional sites. It is therefore apparent that from a very early stage in its transcription hnRNA exists as ribonucleoprotein, extractable as hnRNP particles as detailed in section 2.2. Spread prepartions of chromatin prepared by the Miller technique (Miller and Beatty, 1969), show two classes of nucleoprotein structure. The chromatin fibre is seen as beaded filaments in which DNA complexed with histones, is formed into linear arrays of nucleosomes which are 12 nm in diameter and connected by a thin deoxyribonucleoprotein (DNP) fibre (Kornberg, 1977). In addtion to this, in transcriptionally active regions of chromatin, ribonucleoprotein (RNP) fibres are seen branching off the DNP backbone and at the point of attachment, there is a small dense granule. These lateral ribonucleoproteins are nascent gene transcripts and the dense granule at their origin is RNA polymerase.

Nascent transcripts of non-nucleolar genes have been extensively studied in developmental stages of Diptera (McKnight and Miller, 1979; Beyer et al., 1981b; Beyer, 1983), in giant polytene chromosomes of amphibian oocytes (Malcolm and Sommerville, 1974; Scheer and Sommerville, 1982) and in cells of mammalian origin (Hamkalo and Miller, 1973; Puvion-Dutilleul et al., 1978; Derenzini et al., 1981). In a small number of cases, it has been possible to identify the nascent transcripts with specific gene products (McKnight et al., 1976; Lamb and Daneholt, 1979; Beyer et al., 1981a). The variation in observed structure

of nascent transcripts across the different species described above has been reviewed by Sommerville (1981). These variations include the density of RNP fibres on the DNP fibre, the degree to which the DNP fibre of actively transcribed regions is free of nucleosomes and the density of particles on the RNP fibre. In general, nascent RNP chains have the following characteristics in common. They consist of 4nm RNP fibrils which is complexed with or aggregated into 24-25 nm RNP particles at non-random intervals along its length.

2.2 Isolation of hnRNP Particles

Methods employed for isolation of hnRNP particles have been reviewed by Heinrich et al. (1978) and van Venrooij and Janssen (1978). There are two basic methods for their isolation. The first of these two methods involves extraction of the particles from purified intact nuclei (Samarina et al., 1968). This method of isolation of hnRNP is applicable to most tissues, except tissue culture cells. The purified nuclei are first extracted with an isotonic buffer at pH 7.0 which helps in eliminating contamination by perinuclear ribosomes and pre-ribosomal particles. The nuclear residue is then repeatedly re-extracted with the same buffer at pH 8.0. The extracts contain hnRNP which can be further purified by centrifugation on sucrose density gradients.

In the second method of hnRNP preparation, particles are collected after disruption of nuclei achieved by various methods including 1) osmotic shock (Raj et al., 1975), 2) sonication in either hypotonic or isotonic buffers (Pederson 1974a; Stevenin and

Jacob, 1974), 3) detergent (Stevenin et al., 1970) or 4) mechanical shearing (Faiferman and Pogo, 1975; Faiferman et al., 1970). The nuclear extract obtained after lysis is usually purified further on sucrose density gradients followed by isolation of the particle - containing fractions. Nuclear lysis methods give higher yields of hnRNP than extraction at pH 8.0 but are more likely to result in contamination with histones and non-histone chromatin proteins (Gallinaro et al., 1975; Thomas et al., 1981b).

For isolation of hnRNP particles from tissue culture cells, nuclear lysis employing sonic disruption is usually the method of choice. Such cells do not respond well to extraction at elevated pH because yields are low (Pederson, 1974a) and can be only improved by the use of more extreme conditions such as elevated temperature (Pederson, 1974a) and higher pH (Quinlan et al., 1974).

Particles isolated by nuclear lysis and extraction methods are very similar (Pederson, 1974a). Protein composition of particles isolated by both methods have been shown to be near-identical on both one and two dimensional gels (Wilks, 1980; Brunel and Lelay, 1979).

Two basic types of hnRNP particles have been isolated. In cases where endogenous ribonuclease (RNase) is low, for example in HeLa cells or brain cells (Pederson, 1974a) and where RNase inhibitor is used, polymeric particles are obtained. These polymeric particles have long RNA transcripts (approaching 200S, Pederson 1974b) with proteins associated in bead-like structures at intervals. When high endogenous RNase levels are present (for

example, rat liver), hnRNP particles are usually isolated as "monomer" subunits of about 40S which consist of a small piece of RNA (5-15S) coupled with a complement of hnRNP particle proteins (Samarina et al., 1968). Samarina and Coworkers (1968) have observed that treatment of large polyparticles with small amounts of RNase quantitatively converts them to 40S particles. The protein profile of polymeric particles has been shown to be almost indistinguishable from that of monomers (Wilks, 1980; Suria and Liew, 1979). Evidence that the isolated monomeric hnRNP particles are identical to the 25 nm particles seen on nascent transcripts is presented in section 2.2.1.

Several groups have studied the possible contamination of hnRNP particles by the adsorption of proteins from other cellular fractions. hnRNP proteins prepared by the methods described above have been found to be free of ribosomal proteins (Maundrell and Scherrer, 1979; Wilks, 1980). Particle proteins have also been shown to be distinct from chromatin proteins and histones (Beyer et al. 1977) and when particles were prepared in the presence of radioactively labelled proteins from other cellular pools, no significant contamination was detected (Wilks, 1980; Jacob et al., 1981). Cross-linking and reconstitution studies have also demonstrated that isolated hnRNP particles are generally free of contamination from other proteins (see sections 3.4 and 5). Recently, Choi and Dreyfuss (1984a) have described a method of isolating hnRNP particles by immunoprecipitating them from nucleoplasm with monoclonal antibodies. The antibodies were raised against HeLa proteins which had been cross-linked to hnRNA in vivo. This method is drastically different from the previous

isolation procedures in that it is specific and rapid, avoiding lengthy sedimentation and extraction. The particle isolated by these workers are similar to those described above. Choi and Dreyfuss (1984a) have used their antibodies to isolate hnRNP particles from hamster, chicken and human nuclei.

Nuclear Matrix and hnRNP

When hnRNP particles are isolated by nuclear lysis methods which do not employ shearing forces (e.g. nitrogen cavitation bomb or high salt buffers), hnRNP is not observed free but is bound to the nuclear matrix (Faiferman and Pogo, 1975; Pogo, 1981). Pogo and coworkers suggest that hnRNP should not be considered in isolation but as a matrix structure and that particles are artefacts of proteolysis and fragmentation of the matrix. van Eekelen and van Venrooij (1981) have shown that hnRNP can be cross-linked with the matrix in situ. Nothwithstanding these observations, the study of hnRNP and its role may be best achieved with preparation separated from the nuclear matrix which, most evidence suggests contains a different set of proteins (Grebanier and Pogo, 1979; Maundrell et al., 1981). More recently, Gallinaro et al. (1983) have reported that nuclear matrix and hnRNP share a common structural constituent as certain residual fibrils resistant to salt. Both these fibrils are associated with the same premessenger RNA and maturation products. Their results led the latter workers to propose that pre mRNA may be associated with the same basic constitutive unit in nucleoplasmic matrix as in hnRNP.

2.2.1 hnRNP Particles *in situ* and *in vitro*

There is growing evidence that isolated hnRNP is derived from the nascent hnRNP seen in Miller spreads. Isolated particles and those observed *in situ* are the same size (20-30 nm) and isolated polymeric hnRNP appear the same as nascent hnRNP under the electron microscope (Samarina *et al.*, 1968; Tsanev and Djundurov, 1982). The RNA from isolated particles competes with hnRNA in hybridization to DNA (Mantieva *et al.*, 1969) and will hybridize to saturation with cDNA copies of total mRNA (Martin *et al.*, 1974) and of specific mRNA species (Kucherer, *et al.*, 1982). Antibodies to proteins from isolated hnRNP have been shown by *in situ* immunofluorescent techniques to specifically label the RNP associated with DNA loops undergoing transcription (Sommerville *et al.*, 1978; Martin and Okamura, 1981; Fakan *et al.*, 1984). It has been further shown that hnRNP particles can be reconstituted *in vitro* (Kulguskin *et al.*, 1980; Pullman and Martin, 1983; Wilk *et al.*, 1983) and that a specific RNA polymerase II transcript is assembled into RNP *in vitro* (Economidis and Pederson, 1983b). *In vivo* and *in vitro* identity of hnRNP proteins has also been shown from *in situ* cross-linking of the proteins to hnRNA (Mayrand *et al.*, 1981; Economidis and Pederson, 1983a).

3.0 COMPONENTS OF hnRNP

3.1 Particle Structure

hnRNP particles contain considerably more protein than RNA. After fixation of the particles in formaldehyde (Samarina *et al.*, 1968) or glutaraldehyde (Pederson, 1974a; Karn *et al.*, 1977), they have buoyant densities in $CsCl_2$ of 1.39-1.43 (Samarina *et*

al., 1968; Pederson, 1974a; Karn et al., 1977; Northemann et al., 1977; Knowler, 1976). This indicates protein: RNA ratios of 4:1. Polymeric and monomer hnRNP exhibit similar buoyant densities (Samarina and Krichevskaya, 1981). Monomeric hnRNP particles are recovered from sucrose density gradients in a peak of UV absorbing material with a sedimentation coefficient of approximately 40S by most of the above workers.

Electron microscopy of fixed particles and various treatment of the particles with salt or urea extraction and degradation by ribonucleases and proteases (Samarina et al., 1968; Jacob et al., 1981; Le Stourgeon et al., 1981) have led to the following model for hnRNP. It is assumed that hnRNP consists of an array of particulate monomers with identical protein composition arranged along hnRNA or an hnRNP fibre. The RNP associated with the 3' poly (A) tail have a different protein composition (see section 3.4.2). The hnRNA is either placed on the outside of the particles (Samarina et al., 1968; Sommerville, 1981) or within and at least partially protected by the proteins of the particle (van Venrooij and Janssen, 1978; Le Stourgeon et al., 1981). It is thought that some double stranded RNA may be involved in the fibrillar or particulate part of hnRNP structure (Sommerville, 1981; Heinrich and Northemann, 1981). The hnRNA between the particles may also be shrouded in protein (Jacob et al., 1981). It has also been reported that stable low molecular weight RNA (snRNA) may form integral part of the particles (Heinrich and Northemann, 1981). Recent in vitro reconstitution studies have indicated that the RNP particles along hnRNA backbone consist of identical ratios of the major packaging proteins of

hnRNP, the core proteins (see section 3.4.1) (Pullman and Martin, 1983; Wilk et al., 1983). Monoclonal antibodies raised to some of the minor proteins of Drosophila (Saumweber et al., 1980) have shown that the antigens are differentially distributed along polymeric hnRNP complexes (Risau et al., 1983). Very recently Lothstein et al. (1985) have reported on cross-linking studies between the major proteins within monomer 40S particles which are discussed in section 3.4.1.

3.2 The hnRNA Component

Monomeric particles are associated with approximately 600 nucleotides of RNA and there is a good correlation between the size of polyparticles and the molecular weight of their RNA (Samarina et al., 1968). As outlined in section 1.2 and 2.2.1, it has been demonstrated that this rapidly labelled and rapidly degraded component of hnRNP is hnRNA and includes pre-mRNA sequences (see section 4 for details of hnRNA processing to produce mRNA).

3.3 The snRNA Component

Several authors have identified another RNA component of hnRNP particles, the small nuclear RNA (snRNA) (Sekeris and Neissing, 1975; Gallinaro and Jacob, 1979; Northemann et al., 1977; Heinrich and Northemann, 1981). Most evidence however points to snRNA being associated with hnRNP in the form of small ribonucleoprotein particles or snRNP. Antiserum from patients with some autoimmune diseases and monoclonal antibodies have been shown to react uniquely with various subsets of the polypeptides

found in association with the snRNA (Lerner and Steitz, 1979; Lerner et al., 1981). snRNP have been isolated by Hinterberger et al. (1983) as stable RNA protein complexes, that are completely immunoprecipitable by anti Sm or anti UI RNP human autoantibodies (anti Sm and anti UI RNP antibodies are derived from patients with the autoimmune disease, systemic lupus erythematos). These workers have shown that the proteins associated with snRNP are distinctly different from major hnRNP proteins. There is now substantial evidence that snRNA in the form of snRNP is involved in mRNA processing reactions (see section 4.3).

3.4 The Protein Component

hnRNP proteins have been studied from a wide variety of organisms ranging from non vertebrates such as Artemia salina (Thomas et al., 1981a) and Physarum polycephalum (Le Stourgeon et al., 1978) to mammalian cells such as rat liver (Karn et al., 1977) monkey (Martin et al., 1974) and HeLa cells (Beyer et al., 1977). These nucleus specific proteins comprise nearly 85% of monomer particle mass. On one dimensional SDS-polyacrylamide gel electrophoresis, the proteins from most species form about 13 to 18 bands that are dominated by a group of 4-6 major polypeptides of 30,000 to 43,000 MW. These major proteins are the so-called core proteins. Fig. 1.2 shows a typical one dimensional SDS-polyacrylamide gel fractionation of the hnRNP proteins, in this case extracted from purified rat liver nuclei at elevated pH and fractionated on a sucrose gradient. The core proteins are totally different to the non-particulate proteins of the extract which remain on top of the gradient and are not visibly contaminated by

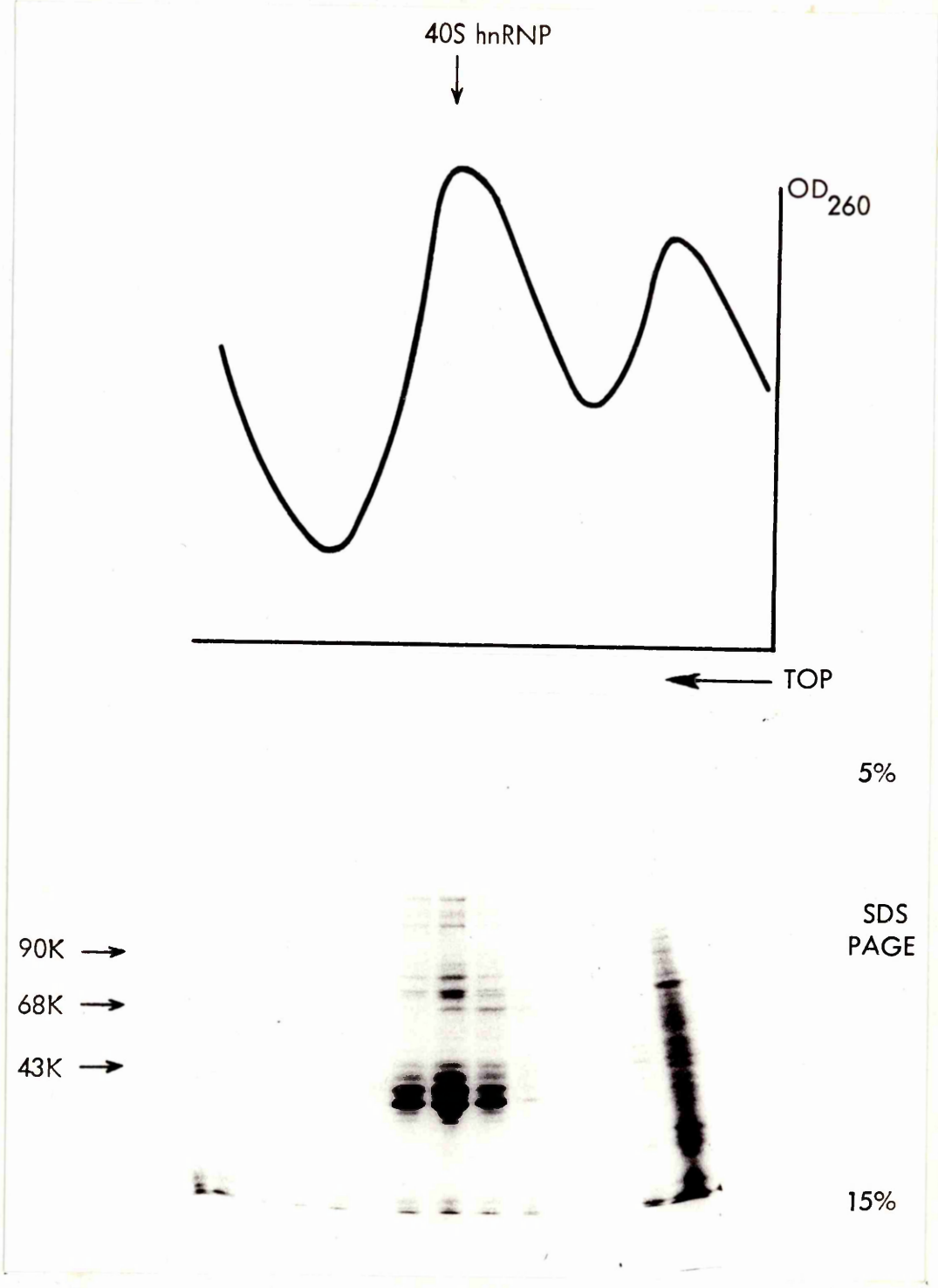
histones or ribosomal proteins. Similar protein profiles to that of figure 1.2 have been presented by other workers despite variation in isolation technique, tissue and species of origin and conditions for SDS-polyacrylamide gel electrophoresis (Beyer et al., 1977; Karn et al., 1977; Northemann and Heinrich, 1979; Stevenin and Jacob, 1979; Walker et al., 1980).

Two dimensional fractionations of O'Farrell (1975) and O'Farrell et al. (1977) which employ isoelectric focusing or non-equilbrated pH gradient electrophoresis in the first dimension and SDS-polyacrylamide gel electrophoresis in the second dimension, have been employed to fractionate hnRNP proteins. Similar fractionations have been produced by many workers again with core proteins dominating the protein pattern (Suria and Liew, 1979; Maundrell and Scherrer, 1979; Brunel and Lelay, 1979; Wilks and Knowler, 1980; Peters and Comings, 1980; Comings and Peters, 1981). On both one dimensional and two dimensional polyacrylamide gel fractionations of hnRNP proteins, minor differences have been detected in the composition of different tissues (Gallinaro-Matringe et al., 1977; Wilk et al., 1985); different species (Beyer et al., 1977; Wilks and Knowler, 1980), different stages of development (Maxwell and Fischer, 1979) and after viral infection (Pagoulatos and Yaniv, 1978; Gattoni et al., 1980).

3.4.1 The Core Proteins

As discussed in section 3.4, core proteins are the major proteins of hnRNP particles from many species. These proteins may comprise more than 80% of the total particle protein (Beyer et al., 1977).

Figure 1.2



Analysis of the protein components of each fraction of a sucrose density gradient on which a pH 8.0 nuclear extract containing hnRNP particles was sedimented at 78,000 g_{av} for 17 hours at 4°C. Each of the 1-ml fractions of the gradient was dialyzed against water, lyophilized, and analyzed on 5–15% polyacrylamide gradient gels. The stainable protein patterns are arranged below the OD_{260} trace of the gradient so that their relative positions correspond. Reproduced from Wilks and Knowler (1980).

a) General Characteristics. Amino acid composition of core proteins have shown that they have a high percentage of glycine, as much as 25 mol %, little detectable cysteine and low methionine. They also contain the unusual amino acid dimethyl arginine (Christensen et al.. 1977; Beyer et al., 1977; Karn et al., 1977; Fuchs et al., 1980). Karn et al. (1977) found that after labelling in vivo with [methyl-^{3}H] methionine, more than 70% of the radioactivity found in 40S particle proteins could be recovered as dimethyl arginine.

Core proteins are basic, of low molecular weight (Karn et al., 1977; Comings and Peters, 1981) and have low turnover rates (Martin et al., 1979; Ivanova et al., 1981).

b) Conservation across species. Core proteins vary in number in different organisms. In Artemia salina and Physarum polycephalum, there is a single major component of hnRNP particles having the properties of core proteins outlined above (Thomas et al., 1981a; Le Stourgeon et al., 1978). In rat liver there are four core proteins (Karn et al., 1977; Wilks and Knowler, 1981a) and in the HeLa cell, there are six (Beyer et al., 1977). Despite such variation in number, core proteins are highly conserved across species. Antibodies raised in chicken against mouse core proteins, cross-react with core proteins of a range of mammalian, avian and amphibian species (Martin and Okamura, 1981). A monoclonal antibody raised against nuclear proteins of Drosophila cross-reacted with a 44,000MW protein of HeLa cell hnRNP (Hugle et al., 1982). Monoclonal antibodies raised against HeLa cell proteins cross-linked with hnRNA have been used to isolate hnRNP complexes from nuclear extracts of chicken and hamster (Choi and

Dreyfuss, 1984a) and monoclonal antibodies against chicken raised in mice cross-reacted with proteins of Xenopus, PtK_2 and HeLa cells (Leser et al., 1984). Cruz-Alvarez et al. (1985) have recently reported isolation of a cDNA clone for Artemia salina core protein HD40 (MW = 40,000). In Southern blot analyses, performed with DNA of different species such as yeast, peas, chicken, human and mouse, cross-hybridizing bands showed up when probed with DNA from one of the positive DNA clones.

c) Cross-linkage studies. Ultraviolet light has been used to cross-link hnRNA and the proteins in contact with it in vivo. Most of these studies have been performed in vivo by irradiation of HeLa cells. The core proteins of HeLa cell migrate on SDS-polyacrylamide gels as three groups of doublet bands which have been named group A (MW, A1:32000 and A_2:34000), group B (MW, B_1 :37000 and B_2:39000) and group C (MW, C_1:42000 and C_2:44000) (Beyer et al., 1977). Cross-linking of core protein C to hnRNA has been shown by van Eekelen et al. (1982) and Choi and Dreyfuss (1984b). Van Eekelen et al. (1982) also showed that the 42000 hnRNP proteins are non-randomly localized on the RNA sequence of adenovirus transcripts. Beyer et al. (1977) have also reported that group C proteins interact directly with hnRNA to form smaller, high salt-resistant complexes. Recently, by using a combination of cross-linking, protease and ribonuclease treatment of HeLa hnRNP, Lothstein et al. (1985) have indicated that proteins A_1, C_1 and C_2 and most of the pre-mRNA sequences occupy a peripheral position in intact monoparticles. Protein-protein cross-linking by these same workers has further indicated that He La hnRNP monoparticles are composed of 3 or 4 repeating units,

each containing 3(A_1), 3(A_2), 1(B_1), 1(B_2), 3 (C_1) and 1 (C_2) molecules.

d) Localization of core proteins by immunoflourescence. It was mentioned in section 2.2.1 that by using immunoflourescence techniques, protein antigens from isolated hnRNP were localised in RNP associated with DNA loop undergoing transcription. Some of these antigens were core proteins (Martin and Okamura 1981). These workers have also shown that heterochromatin and nucleolar regions in the nuclei did not contain core proteins. Furthermore, Martin and Okamura (1981) have shown that core proteins become distributed throughout the cell plasm of cells in mitosis but the proteins are not associated with mitotic chromosomes. Using antibodies to core proteins, Lahiri and Thomas (1985) have isolated complexes between high molecular weight RNA and core proteins ranging from 80S to 200S in mitotic cells. C proteins of HeLa cell have been located in nuclei but were absent in nucleoli (Dreyfuss et al., 1984). Fakan et al. (1984) were the first to report ultrastructural distribution of the core proteins. With the help of monoclonal antibodies, these workers have shown that core proteins associate preferentially with border regions of condensed chromatin and in particular with perichromatin fibrils and perichromatin granules.

e) Some other properties of core proteins. Properties of core proteins which indicate their possible functional significance are discussed in section 5.

f) Charge heterogeneity of core proteins. On two dimensional gels, the core proteins exhibit marked heterogeneity in charge (Wilks and Knowler, 1980; Comings and Peters, 1981).

Each of the bands detected on a one-dimensional SDS-polyacrylamide gel can be resolved on a pH gradient into a string of stainable spots (see results section, figure 4.15). Most of these cluster around a marker with isoelectric point 8.1 but one string spreads between markers with pIs of 6.1 and 6.7 (Peters and Comings, 1980; Comings and Peters, 1981).

The cause of the charge heterogeneity of core proteins is unknown but could be due to their post translational modification. Core proteins have been shown to be phosphorylated (Karn et al., 1977; Wilks and Knowler 1981b; Wilk et al., 1985). Holcomb and Friedman (1984) and Choi and Dreyfuss (1984b) have reported the phosphorylation of only the C proteins of HeLa. Core proteins may be glycosylated (Jacob et al., 1981) and ADP-ribosylated (Kostka and Schweiger, 1982).

3.4.2 Other Proteins and Enzymes

Enzymic activities associated with hnRNP have been reviewed by Jeanteur (1981). These enzymic activities detected in hnRNP may play a key role in splicing, polyadenylation and capping, in addition to any role the core proteins might play.

Sequence specific endonulceases have been detected by Neissing and Sekeris (1970) and Calvet and Pederson (1977). Molnar et al. (1978) have detected a 5' exonulcease activity. RNase activities specific for double stranded RNA (Rnase D) have also been detected in hnRNP (Molnar et al., 1978; Rech et al., 1979). Any role which these enzymes may play in hnRNA splicing is unknown. In the mechanism for pre-mRNA splicing proposed recently (see section 4.3), it may turn out that RNA itself catalyses the splicing of introns (Sharp, 1985).

Protein kinase activities have been found associated with hnRNP. Karn et al. (1977) and Blanchard et al. (1977) reported a cAMP-independent protein kinase and Wilks and Knowler (1981b) reported a cAMP-dependent protein kinase.

There is good evidence that the 3' poly (A) tail of hnRNA forms a ribonucleoprotein particle which differs in size and composition from the remaining hnRNP complex. Poly-adenylate is a part of polymeric hnRNP but when particles are isolated in the absence of RNase inhibitors, or if polymeric hnRNP is subjected to mild RNase digestion, poly (A) is not recovered with 40S monomers but as separate particles which sediment at 15S (Samarina et al. 1973). These particles contain at least 60% of the nuclear polyadenylate in lengths of upto 230 nucleotides (Quinlan et al., 1977; Kish and Pederson, 1975). Setyono and Greenberg (1981) identified a protein of 60000MW cross-linked to poly (A). Recently, Sachs and Kornberg (1985) have reported 55000MW protein bound to poly (A). The latter workers have suggested that these two proteins may be the same and the discrepancy in molecular masses could be due to residual nucleotide cross-linked to the apparent 60000MW protein.

Poly (A) polymerase activities have also been detected in hnRNP by a number of workers including Niessing and Sekeris (1972) and Louis et al. (1978). Bajszar et al. (1978) have shown particle associated enzyme activities capable of catalyzing reactions necessary for cap formation. These are the guanylyl transferase, the N^7 methyl transferase and the 2-O methyl transferase (see section 4.1). Phosphodiesterase activity has also been found in hnRNP (Periasamy et al., 1977).

4.0 MATURATION OF hnRNA

4.1 Capping

The 5' end of most eukaryotic gene transcripts are capped, by the addition through a pyrophosphate bond, of a 7-methyl guanosine. Capping is an early reaction occurring at the 5'-triphosphate ends of nascent hnRNA shortly after initiation (Salditt-Georgieff et al., 1980; Babich et al., 1980). It is catalysed by guanylyl- and methyl transferases, which are nuclear enzymes, but most animal viruses that replicate in the cytoplasm contain similar activities and produce capped mRNAs (reviewed by Shatkin, 1976). The 7-methyl guanosine at the 5' end is called Cap 0. The cap may be further modified by the 2'-O-methylation of the riboses of the first or first two nucleotides of the transcript (Cap 1 and Cap 2 respectively). Cap 2 methylation has only been reported in mRNA and may only occur in the cytoplasm (Shatkin, 1976).

Caps are retained during processing of nuclear transcripts and serve as stabilizing elements on hnRNA (Green et al., 1983) and mRNAs in the cytoplasm (Furuichi et al., 1977). Cap structure has also been shown to be important for correct and efficient splicing of hnRNA (Konarska et al., 1984; Ruskin et al., 1984). Recognition of the capped end of most mRNAs by specific proteins is also important for their translation in the cytoplasm (Banerjee, 1980).

4.2 Polyadenylation

The polyadenosine tract, poly (A), at the 3' terminus of most eukaryotic mRNAs is added during post-transcriptional

processing. Since transcription typically continues beyond the end of the mature mRNA, the final step in generating the 3' terminus is the result of precise endonucleolytic or exonucleolytic cleavage of the precursor hnRNA. This cleavage is thought to create the site at which a poly (A) polymerase adds approximately 200 A residues (Hofer and Darnell, 1981; Nevins and Darnell, 1978). The site of polyadenylation is probably specified by sequences in the precursor RNA. A highly conserved sequence AAUAAA is present 11-30 nucleotides upstream of most poly (A) sites (Proudfoot and Brownlee, 1976). Fitzgerald and Shenk (1981) have shown that polyadenylation was blocked in SV40 deletion mutants lacking this hexanucleotide. Single base pair mutation within this hexanucleotide have been shown to reduce severely (Montell et al., 1983) or abolish normal polyadenylation (Higgs et al., 1983).

Many genes contain internal copies of AATAAA that do not appear to function as processing and polyadenylation signals (Reddy et al., 1978; Woo et al., 1981). This suggests that AATAAA alone may not be a sufficient signal for processing and polyadenylation. Evidence has been presented by some workers indicating that sequences between 20-40 bases downstream of the recognised polyadenylation signal are necessary for efficient processing and polyadenylation, for example, in the case of the adenovirus type 2E2A gene (McDevitt et al., 1984) and thymidine kinase gene of Herpes simplex virus (Cole and Stacy, 1985). The latter workers have shown that this second polyadenylation signal has G+T-rich sequence. When McLauchlan et al. (1985) and Birnstiel et al. (1985) used sequence comparisons to suggest elements downstream of the AAUAAA site involved in 3' end

processing, they came up with the consensus sequence YGUGUUYY (Y denoting pyrimidines) and G-U clusters respectively. At a slight variation to the above data, Sadofsky et al. (1985) reported that a functional element within the sequence AGGUUUUUU beginning 59 nucleotides downstream of the recognised AAUAAA is important for correct processing and polyadenylation of the simian virus 40 (SV40) late mRNA. There are also reports about the use of more than one of the recognised polyadenylation signal AAUAAA for 3' end processing (Mason et al., 1985; Boardman et al., 1985).

Sadofsky et al. (1985) suggest that the available data on a signal for processing and polyadenylation of the 3' end of mRNAs other than the AAUAAA sequence indicates the possiblity of multiple downstream signals (often rich in Us) which may function alternately under different conditions. Such a scenario could help explain situations in which a specific mRNA utilizes variable 3' end sites in a tissue or developmentally specific manner (Capetanaki et al., 1983; Early et al., 1980; Mather et al., 1984).

Some trans-acting factors have also been implicated in 3' end processing of hnRNA. Polyadenylation in vitro at the L3 site of the adeno major late precursor RNA can be prevented by antisera against snRNPs with Sm and U1 specificities as well as with antisera against the nuclear antigen La (Moore and Sharp, 1985). Therefore snRNPs may be involved in 3' processing of some polyadenylated RNAs. From theoretical considerations, Berget (1984) and Sadofsky et al. (1985) have suggested that U4 RNA might be instrumental in these processes.

Poly (A) sequences at the 3' end of eukaryotic mRNA seem to make the latter stable (reviewed by Brawerman, 1981). Some eukaryotic mRNAs lack poly (A) tails, for example some histone mRNAs. Those histone mRNAs which lack poly (A) have instead consensus sequences which produces stem-loop structures at their 3' end (Busslinger et al., 1979; Birchmeier et al., 1983). In histone mRNAs, these terminal sequences stabilize them against 3' to 5' exonuclease attack (Georgiev and Birnstiel, 1985).

4.3 Splicing

The recent development of in vitro cell-free systems which faithfully splice mRNA precursors (Padgett et al., 1983; Hardy et al., 1984; Hernandez and Keller, 1983; Krainer et al., 1984; Di Maria et al., 1985) has helped to unravel the mechanism of pre-mRNA splicing significantly. Among the few clues that existed prior to the advent of the in vitro splicing systems, about the possible mechanism of pre-mRNA splicing is the presence of consensus sequence at the splicing points of different genes catalogued by Mount (1982). This sequence is $^{C}_{A}AG\uparrow GT^{A}_{G}AGT$ for the exon-intron boundaries (or the 5' splice site) and $(^{T}_{C})_{n}N^{C}_{T}AG\uparrow G$ for intron-exon boundaries (or the 3' splice site). The most invariant aspect of the consensus sequence is the GT at the beginning of the intron and the AG at its end. The role of this sequence in splicing is supported by studies of β-thalassemias, a group of heredity anemias in which the production of globin is absent or faulty due to mutations in the splice point sequences (Baird et al., 1981; Busslinger et al., 1981; Treisman et al., 1982). It was also known that the 5' terminus of the most

abundant snRNA U1 exhibits considerable sequence complimentarity with the consensus sequence of both the exon-intron and intron-exon boundaries (Lerner et al., 1980; Rogers and Wall, 1980). The most notable feature of this complementarity is the tetranucleotide ACCU which would hybridize with the invariant GU/AG ends of the intron transcript when these were brought together to form the sequence UGGA.

Recent findings about pre-mRNA splicing using in vitro systems are summarized below: Adenovirus 2 (Ad 2) RNA substrate containing the first and second leaders (Hardy et al., 1984) and the human β-globin mRNA precursor (Krainer et al., 1984) have been shown to be accurately spliced when added to HeLa cell nuclear extracts. The reactions require ATP absolutely and in the case of the Ad 2 RNA substrate containing the first and second leaders, Mg^{2+} was also required. This splicing is inhibited by antisera against small nuclear ribonucleoprotein (snRNP) particles containing U1RNA (Padgett et al., 1983; Di Maria et al., 1985; Moore and Sharp, 1985). Other evidence which suggests that U1 snRNA is essential at least for the cleavage of the 5' splice site is that purfied U1 snRNP will bind to and protect 5' splice sites of an RNA from nuclease digestion (Mount et al., 1983). Furthermore, enzymic removal of the 5' end of U1RNA prevents splicing (Kramer et al., 1984).

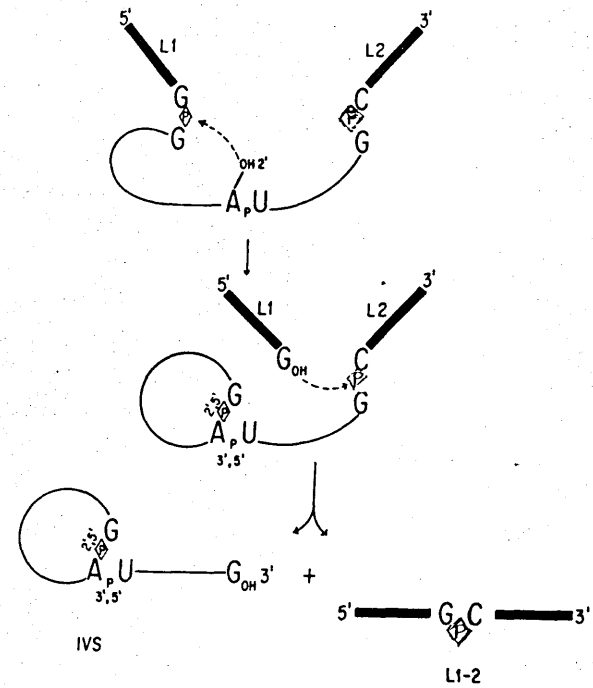
Both the Ad 2 pre-mRNA and human globin pre-mRNA needed a 5' terminal cap for efficient and accurate splicing (Konarska et al., 1984; Moore and Sharp, 1985; Krainer et al., 1984). The products of the in vitro splicing are the spliced exons and the intervening sequence excised as a lariat RNA (Grabowski et al.,

1984; Padgett et al., 1984; Ruskin et al., 1984). These workers have also detected a probable reaction intermediate, consisting of the lariat form of the intervening sequence joined to the 3' exon. Both lariat RNAs have been shown (by the same workers) to contain an unusual nuclease resistant structure, an oligonucleotide containing 2'-5' and 3'-5' phosphodiester bonds joined to a single adenosine residue. Recently Konarska et al. (1985) have characterized this branched intermediate as being $A\begin{matrix}2'p5'G\\3'p5'U\end{matrix}$. These workers have also shown that 1) the 5'-terminal G of the intervening sequence is joined directly to the adenosine residue at the branch site, 2) the phosphate group from the 5' splice site is incorporated into the 2'-5' phosphodiester bond; 3) the branch site is 24 nucleotides upstream of the 3' splice site and 4) the probable intermediate in the reaction, the 5' exon terminates in a 3' hydroxyl group.

On the basis of the recent findings about pre-mRNA splicing described above, Konarska et al. (1985) have proposed a scheme for the splicing of the mRNA precursors as shown in figure 1.3. A multicomponent precursor mRNA-ribonucleoprotein complex is thought to be formed first; it probably contains U1snRNP particle bound to the 5' splice site (Padgett et al., 1983; Mount et al., 1983; Kramer et al., 1984). An involvement of other snRNP such as U2 snRNP (Ohshima et al., 1981; Keller and Noon, 1984) is also possible.

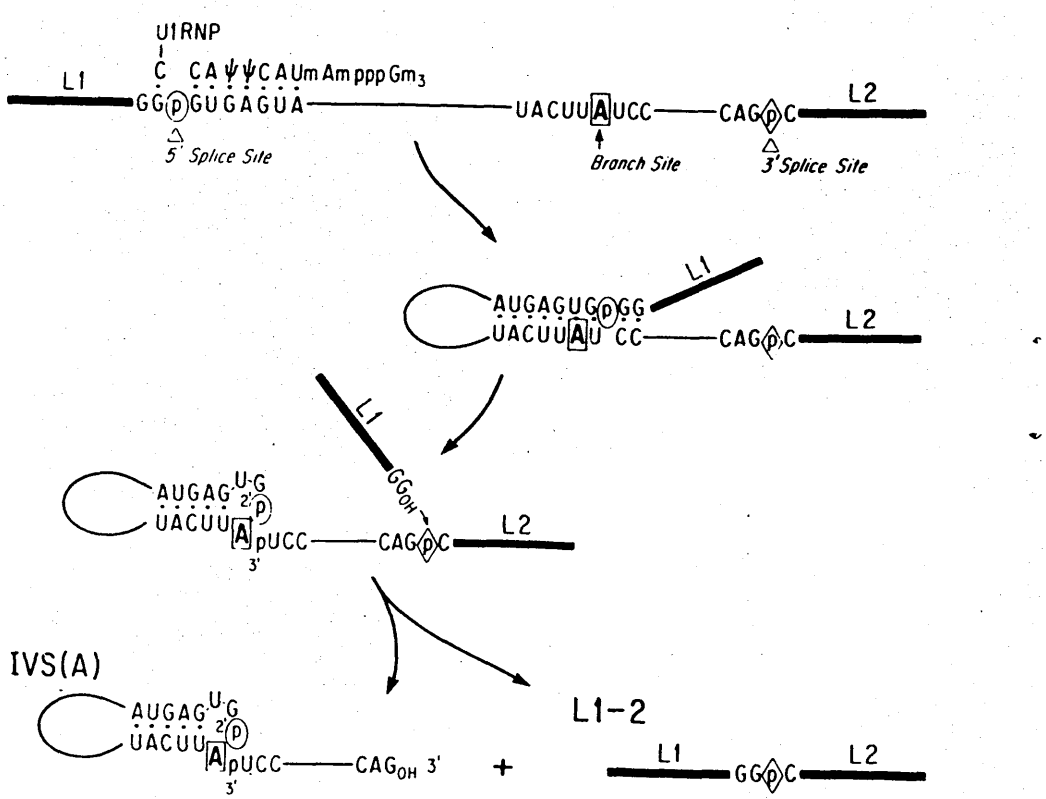
The sequences near the 5' splice site are complementary to both the 5' terminal sequences of U1RNA (Lerner et al., 1980; Rogers and Wall, 1980) and branch site sequences shown in figure 1.3. Base pairing between the 5' terminus of U1RNA and the 5'

Figure 1.3



Splicing of Adenovirus mRNA Precursor

Intermediates and products of splicing process. Precursor RNA consists of 5' exon (L1, thick line), intervening sequences (thin line) and 3' exon (L2, thick line) from the major late transcript from adenovirus 2. The sequences at 5' and 3' splice sites are G–G and G–C, respectively, where the phosphodiester bond between the two nucleotides is cleaved during splicing. The sequence at the branch site is A–U. The first covalent modification of the RNA is the formation of a phosphodiester bond between a 2' position on the ribose moiety of adenosine and the 5' phosphate at the 5' splice site (a). Formation of this branch gives rise to the lariat RNA, intervening sequences-3' exon, and the second RNA, 5' exon. This two part intermediate is reasonably stable in vitro *but (c) is ultimately resolved into the two products, the excised intervening sequences (IVS-A) and the spliced exons (L1–2). The intervening sequences are released in a lariat form and the phosphate at the 3' splice site is retained in the spliced product.*



The splicing process. See above for structure of precursor and splicing intermediates. The complementarity between the sequences at the 5' splice site and branch site is indicated by base pairs. Phosphate moieties from the 5' splice site (p) and 3' splice site ◇p are retained during processing.

splice site is thought to mediate recognition for splicing in the model of mRNA splicing proposed by Konarska et al. (1985). Following selection of the 5' splice site, the U1 RNA could be displaced by the branch site sequences.

The significance of sequence specificity and location of branch site within introns is not clear and recent data (Black et al., 1985) indicate that the choice of the adenosine employed in the branch point may be dictated more by the distance from the 3' splice site than by its associated base sequence. Studies of in vivo deletions within introns of yeast genes have identified a higly conserved sequence UACUAAC which is essential for splicing (Langford and Gallwitz, 1983; Pikielny et al., 1983). This sequence is located between 20 and 60 bases upstream of the 3' splice sites and the adenosine nearest the 3' end is the site of branch formation. Point mutations in this sequence in yeast can inactivate splicing (Langford et al., 1984). A search of many mammalian introns for the analogy of the yeast sequence has only yielded a limited consensus sequence ($CT^{A}_{C}A^{T}_{C}$) (Keller and Noon, 1984). Recently, van Santen and Spritz (1985) have reported from a study of deletions within the second intervening sequence of the human γ-globin gene that at the 3' splice site, a minimum of 16 bases upstream is required for splicing to occur at a significant level.

4.4. Possible Control of Gene Expression During hnRNA Processing

There is growing evidence that control of gene expression can occur in some instances via control of hnRNA processing. In

most cases, it is not yet clear whether control of hnRNA processing is due to selection of particular polyadenylation sites or due to differential splicing. In the late transcription unit of adenovirus, one site of polyadenylation is used efficiently during the early stages of the lytic cycle, while other downstream sites are more efficiently used in later stages (Nevins and Wilson, 1981). However the mRNAs produced during both stages also differ in splicing patterns. An analysis of the expression of immunoglobulin heavy chain has indicated that resting B lymphocytes produce membrane-bound immunoglobulin M by synthesizing mRNA with a downstream polyadenylation site, while stimulated B lymphocytes produce secreted immunoglobulin M by using an upstream poly (A) site (Alt et al., 1980; Early et al., 1980). However in this case also in the production of both these mRNA, alternative mRNA splicing occurs as well.

Regulation of gene expression by polyadenylation has been proved in the case of the calcitonin gene (Rosenfeld et al., 1984). In thyroid tissue the calcitonin gene transcription unit gives rise to an mRNA retaining exons that specify calcitonin, while in brain tissue, RNA from the same unit is spliced to generate a calcitonin-related-polypeptide. While the two mRNAs have different sites of poly (A) addition and different splicing patterns, analysis of nuclear precursors from the two tissues suggests that the sites of polyadenylation is specified before splicing commences. Processing control by polyadenylation may also occur in the case of the human fibrinogen gene product producing either the γ or the γ' chain of fibrinogen (Chung and Davie, 1984).

Regulation of gene expression by alternative splicing probably occurs in the case of the rat fibronectin gene (Tamkun et al., 1984), human argininosuccinate synthetase gene (Freytag et al., 1984) and simian virus 40 (SV40) late transcripts (Somasekhar and Mertz, 1985). In case of the rat fibronectin gene, it has been shown that three fibronectin mRNAs are generated by a pattern of alternative splicing in which one 5' splice site can be paired with any one of 3' splice sites, one at the beginning of and two within a single complex exon. All three mRNAs have identical 3' ends. The human argininosuccinate synthetase gene has also been shown to code for two mRNAs that differ in the 5' untranslated sequences and arise by alternative splicing involving the inclusion of deletion of an entire exon. Somasekhar and Mertz (1985) have shown that mutations within the first exon of the late mRNAs can affect dramatically the utilization of downstream donor (5' splice) and acceptor splice (3' splice) sites. Other examples of differential splicing without the use of alternative polyadenylation sites occur in the production of mouse myelin basic protein (Takahashi et al., 1985) and chicken myosin light chains (Nabeshima et al., 1984).

5.0 Possible Function of hnRNP

In sections 2 and 3 evidence of the association of hnRNA with proteins in the form of hnRNP was presented. hnRNP is therefore a candidate for being the site of hnRNA processing reactions. Whether the proteins of hnRNP are simply 1) a metabolically inactive packaging device for hnRNA and/or 2) are actively involved in the hnRNA processing reactions outlined in section 4 is unknown.

In the splicing mechanism proposed by Konarska et al. (1985) (see section 4.3), a complex multicomponent RNP structure which would hold the various intermediates of the reaction in juxtaposition was implicated. Whether hnRNP is involved is still to be proven, although there is evidence of the involvement of U1 snRNP (see section 4.3). Recently Di Maria et al. (1985) have reported purifying pre-mRNA splicing activity which contained protein bands from MW10000 to 62000. It is not known whether core proteins (the major proteins of hnRNP) are among these proteins.

Recently, the presence of a splicing complex has been reported by two groups of workers using a functional in vitro splicing system. Brody and Abelson (1985) have reported the presence of pre-mRNA in a 40S splicing complex prepared from yeast cells. Similarly Philip Sharp and Coworkers have identified a similar but larger complex from mammalian cells, which sediments at 60S (Lewin, 1985). In both these cases pre-mRNA associates with the splicing complex (which has been named "spliceosome" by Brody and Abelson, 1985) only if splicing is functional. What this splicing complex is and whether hnRNP is a component awaits further work.

Enzymic activities found associated with hnRNP were discussed in section 3.4.2. However since the major proteins found in association with hnRNA are core proteins (see section 3.4.1) some of their properties which may give a clue to their in vivo role are outlined below:

1. Reconstitution studies indicate that core proteins have the intrinsic capability of associating with nascent single-stranded hnRNA regions to form native like hnRNP

complexes with constant protein stoichiometry (Pullman and Martin, 1983; Wilk et al., 1983; Economidis and Pederson, 1983b). Although the stoichiometry of core proteins is maintained during reconstituion, they may be non-uniformly distributed in the reconstituted 30-40S subunits.

2. There are indications that core proteins denature some regions of their RNA template within particle structure (Thomas et al., 1981a; Northemann et al., 1979; Thomas et al., 1983).

3. Core Proteins exhibit charge heterogeneity (Comings and Peters, 1981). There are also reports that core proteins may be phophorylated, glycosylated, ADP-ribosylated. (See section 3.4.1) Such modifications may change the interaction between the protein and the RNA and this may in turn lead to changes in the rate or extent of the maturation events.

4. Changes in the relative proportions of the core proteins have been reported under certain conditions. These include rapidly dividing, slowly dividing and non-dividing cells (Le Stourgeon et al., 1978) and cells under heat shock (Pederson, 1983).

5. There are reports that core proteins interact in a sequence-dependent manner with adenovirus transcripts (van Eekelen et al., 1982).

Further structural studies of individual core proteins are required to understand the function of core proteins and hence of hnRNP particles.

II MATERIALS AND SUPPLIERS

1.1 Radiochemicals

A. Radiochemical Centre, Amersham, Bucks.

[γ-^{32}P] ATP 5500 Ci/mmol

[^{32}P] Inorganic Phosphate 10mCi/ml

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

^{125}I, Carrier free 250mCi/ml

C. [γ-^{32}P] ATP made in the department by the method of Maxam and Gilbert (1980).

70% pure preparation 175Ci/mmol

1.2 Reagents For Electrophoresis

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

Acrylamide, Methylene-bis-acrylamide, and

Sodium Dodecyl Sulphate-Electrophoresis Grade

Urea-AristaR Grade

Bromophenol Blue

N N N'N' Tetramethylenediamine (TEMED)

2-Mercaptoethanol

B. Sigma Chemical Co., London, England.

Brilliant Blue R

C. LKB, South Croydon, Surrey.

Ampholines - 40% w/v Solution, pH ranges 3.5-10.0 and 5.0-7.0

D. Pharmacia Fine Chemicals, Milton Keynes.

Low molecular weight Calibration Kit

E. May and Baker Ltd., Dagenham, England.

Formaldehyde, Glacial Acetic Acid

F. Johnson Matthey Chemicals Ltd., Hertfordshire.

Silver nitrate

1.3 Enzymes and Proteins

A. Sigma

Snake venom phosphodiesterase I Type IV from Crotolus atrox

Ribonuclease A Type 1-A from Bovine Pancreas

Deoxyribonuclease I from Bovine Pancreas

α-Chymotrypsin type II

Soybean Trypsin Inhibitor, Ovomucoid and Histones, 11, 11S, 11AS and 111S

B. Boehringer Mannheim, Lewes, East Sussex.

Alkaline phosphatase Grade I from Calf Intestine

C. Miles Scientific, Stoke Poges, Slough.

S. aureaus V8 protease

D. Gifts from Dr. D. P. Leader of the department:

a. Protein Kinase C, homogeneous preparation isolated from Bovine Brain according to Parker et al. (1984).

b. Catalytic Subunit of cAMP dependent Kinase from Rabbit Muscle prepared according to Beavo et al. (1984).

c. Psuedorabies induced Kinase of Baby Hamster Kidney cells prepared according to Katan et al. (1985).

1.4 Metabolites

A. Sigma

ATP, sodium salt

Adenosine 5'-diphosphoribose, sodium salt

B. BDH

Adenosine 5'-monophosphate

1.5 Chromatographic Materials

A. Pharmacia

Sephadex G-10, G-75 and G-100

Sephacryl S-1000 superfine

DEAE Sephadex

B. BioRad Laboratories, Hertfordshire.

Biogel P-60

C. Sigma

Cyanogen Bromide activated Sepharose 4B

D. Camlab, Cambridge.

Polyethyleneimine impregnated cellulose TLC plates

1.6 Immunological reagents

A. Schleicher and Schull, Dassel, W. Germany.

Nitrocellulose

B. Miles Scientific.

Horseradish peroxidase-bound goat antirabbit IgG

C. Difco Laboratories, Central Avenue, Surrey.

Freunds Adjuvants

D. Sigma

Bovine Serum Albumin (BSA)

O-Phenylenediamine (OPD), Protein A

1.7 Miscellaneous

A. Gibco Europe, Glasgow.

Calf serum and Eagle's medium

B. Pierce Warriner, Chester, Cheshire.

Iodogen (1, 3, 4, 6-tetrachloro - 3α, 6α-diphenyl glycouril

C. Boehringer

Trizma base (Tris).

D. Sigma

Coomassie Brilliant Blue G-250

E. Aldrich Chemicals, Gillingham, Dorset.

Trifluoro methane sulphonic acid

(TFMS) - a gift from Dr J G Beeley of the department.

F. Kodak Ltd., Dallimore Road, Manchester.

Kodak X-omat H film

G. Amicon Ltd., Upper Mill, Stonehouse.

Diaflo Ultrafiltration membrane YM2

Wherever possible all other reagents were AnalaR grade and purchased from BDH Ltd.

III METHODS

1.0 ISOLATION OF hnRNP PARTICLES

Composition of solutions used in section.

Homogenising buffer

Sucrose	2.3M	
$MgCl_2$	10.0mM	

RSB

Tris.HCl	10.0mM	pH7.4
NaCl	10.0mM	
$MgCl_2$	1.5mM	

STM 7

Tris.HCl	10.0mM	pH7.0
NaCl	0.1 M	
$MgCl_2$	1.0mM	

STM 8

As in STM 7 but having a pH of 8.0

Sucrose density gradient solution

15% w/v sucrose in STM 8

30% w/v sucrose in STM 8

1.1 Preparation of Nuclei

1.1.1 Rat Liver Cells

Rat Liver cell nuclei were prepared by a modification of the method of Chauveau et al. (1956). The livers of freshly killed male Wistar rats (250-350g) were finely chopped and homogenised at 4°C in 20ml per liver of homogenising buffer using 2 strokes of a Potter Elvehjem glass/teflon homogeniser. The homogenate was filtered through four layers of cotton muslin and the filtrate layered over 7ml cushions of homogenising buffer in cellulose nitrate SW 27 centrifuge tubes. The homogenates were centrifuged at $40,000g_{av}$ for 1 hour in a SW 27 rotor in a Beckman Ultracentrifuge. The supernatant was discarded and the purified nuclear pellet resuspended in a buffer appropriate for the next stage of the experiment (see method 1.2.1 and 1.2.2).

1.1.2 Rat Liver Cell Line

Buffalo Rat Liver cells (growth conditions described in method 4.2.2) were removed from roller-bottles using a rubber policeman and suspended in Eagles medium (Glasgow modification) (Busby et al. 1964). Cells were collected at $170g_{av}$, in a Sorval HB 4 rotor and resuspended in 1%v/v Triton in RSB, 2 ml per roller-bottle. The cells were allowed to swell for 10 minutes on ice and then gently homogenised by 30 strokes of a hand-held glass/teflon homogeniser. The nuclei were collected at $200g_{av}$ for 5 minutes and washed once in RSB. Lysis of the cells was monitored by phase contrast microscopy and homogenisation repeated if necessary. The nuclei were resuspended in RSB for the next experimental stage (see method 1.2.2).

1.2 Extraction of hnRNP Particles

1.2.1 Extraction at Elevated pH (Samarina et al., 1968)

This method was the one of choice for Rat Liver nuclei. Purified nuclear pellets (method 1.1.1) were resuspended in STM 7 buffer (3ml per liver) and left on ice for 10 minutes. Nuclei were recovered from suspension by a 10 minute centrifugation at $6000g_{av}$ in a Sorval HB 4 rotor.

The "pH 7" extract obtained in this way contains few hnRNP particles and was routinely discarded. Three further 45 to 60 minutes extractions of the residual nuclei were then performed at 0°C with gentle stirring in STM 8. The pH 8 extracts were pooled (1.5ml per liver) and applied to sucrose density gradients as described in method 1.3.1.

1.2.2 Sonication

hnRNP particles were routinely obtained from Buffalo Rat Liver (BRL) nuclei by sonication. Purified nuclei from 4 roller-bottles (method 1.1.2) were resuspended in 2ml of RSB. Sonic disruption was carried at 0°C for 2 x 30 seconds at 1.7A in a MSE ultrasonic power unit. Nuclear lysis was monitored by phase contrast microscopy. The sonicate was layered onto 4ml of 30% w/v sucrose in RSB, and centrifuged at $5500g_{av}$ for 5 minutes in a Sorval HB 4 rotor. This step removed nucleoli and chromatin and the material remaining above the sucrose layer was used for the preparation of hnRNP particles on sucrose gradients as described in method 1.3.1.

For the sonication of nuclei from Rat Liver cells (Pederson, 1974a), purified nuclei (method 1.1.1) were resuspended

in 0.88M sucrose, 25mM NaCl, 5mM $MgCl_2$, 50mM Tris.HCl, pH 7.0 and recovered from suspension by centrifugation at $5000g_{av}$ for 5 minutes in the Sorval HB 4 rotor. The purifed nuclei were washed twice in RSB and resuspended in the same buffer. Sonic disruption and centrifugation through 25ml 30% w/v sucrose was as described above except that the centrifugation was for 15 minutes.

1.3 Purification of hnRNP particles

1.3.1 Sucrose Density Gradients

hnRNP particles from extraction of nuclei at elevated pH (method 1.2.1) or from sonicated nuclei (method 1.2.2) were applied to 36ml, 15 to 30% sucrose gradients at 4.0ml of STM 8 or RSB per gradient. The gradients were centrifuged at $95000g_{av}$ for 17 hours at 4°C in a Beckman SW 27 rotor. The gradients were harvested via a narrow bore tube (introduced from the top of the gradient) using a Sigma motor pump, and the OD_{260nm} was continuously monitored by passage through the flow cell of a Gilford 240 spectrophotometer. Portions of the gradient containing hnRNP particles and in some experiments, other fractions of the gradient were collected for further analysis.

1.3.2 Sephacryl S-1000

A 25ml column (diameter 1cm, length 25cm) was packed at 0.66ml/min with Sephacryl S-1000 superfine in STM 8. Column was run at 10ml/hr with STM 8 buffer. One ml of crude hnRNP particles (4-6 mg/ml) extracted at elevated pH (method 1.2.1) was loaded on the column and 0.8ml fractions were collected. Fractions showing peak at 260 nm were collected and analysed on sodium dodecyl

sulphate (SDS) polyacrylamide gels. hnRNP particles were usually obtained in fractions 25-40 followed by other proteins only in fractions 110 to 140.

2.0 FRATIONATION OF hnRNP PROTEINS

Composition of solution used in section.

Gel buffer

Tris.HCl	10.0mM	pH 7.5
NaCl	1.0M	
2-mercaptoethanol	6.0mM	
EDTA	1.0mM	

2.1 Sephadex G-100 and G-75

hnRNP proteins from sucrose gradients (method 1.3.1) were dialysed against 10mM Tris.HCl, pH 7.5, containing 10mM $MgCl_2$ and were then concentrated to 0.2mg/ml. 10 to 15ml of this solution was made 1M with respect to NaCl and loaded on top of either a G-100 or G-75 sephadex column (100 x 1.8cm) running at 10ml/hr with gel buffer. 1 to 2ml fractions were collected and appropriate fractions were analysed on SDS-polyacrylamide gels (method 3.2).

2.2 Biogel P-60

Fractions from Sephadex G-75 eluate containing core proteins (method 2.1) were pooled, dialysed against 5mM Tris.HCl, pH 7.5 and concentrated by lyophilization. 1ml of a 0.1mg protein/ml solution made 1M with respect to NaCl was loaded on a Biogel P-60 column (25cm x 1cm). The column was run at 4ml/hr in gel buffer. 1½ml fractions were collected and appropriate fractions analysed on SDS-polyacrylamide gels.

2.3 HPLC

Attempts were made to fractionate the 4 rat liver core proteins (mol.wt. 34000 to 43000) on a C18 Biorad Hi-Pore^{tm} reverse phase protein HPLC column. Core proteins from Sephadex G-75 column (method 2.1) were dialysed against 10mM Tris.HCl, pH 7.6, and 1ml of a 0.2mg/ml solution loaded on to the column. Proteins were eluted with a 15-60% gradient of HPLC grade acetonitrile in 0.1% HPLC grade trifluoroacetic acid over a period of 30 minutes (Gurley et al., 1983). The two pumps used were 303 pumps in Gilson HPLC system. Proteins were detected with an M300 UV detector at 214nm with a 0.5 absorbance at full scale deflection. Fractions from 0.1ml to 0.5ml were collected.

2.4 DEAE-Sephadex

Core proteins from Sephadex G-75 column fractions (method 2.1) were pooled and dialysed against 10mM Tris.HCl, pH 7.6. The proteins were then loaded on a DEAE-Sephadex column which had been previously equilibrated with 10mM Tris.HCl, pH 7.6. Proteins were eluted with a 0.1 to 1M NaCl gradient in 10mM Tris.HCl, pH 7.6. Fractions were analysed on SDS-polyacrylamide gels.

3.0 GEL ELECTROPHORETIC SYSTEMS FOR CORE PROTEINS

Stock solutions for sodium dodecyl sulphate (SDS) polyacrylamide gels, Laemmli system.

Acrylamide solution

Acrylamide	30%	w/v
Methylene-bis-acrylamide	0.8%	w/v

Resolving gel buffer

Tris.HCl	1.5M	pH 8.8
SDS	0.4%	w/v

Stacking gel buffer

Tris.HCl	0.5M	pH 6.8
SDS	0.4%	w/v

Ammonium per sulphate

10% w/v solution (made fresh)

N, N, N',N'-Tetramethylenediamine

TEMED

Composition of SDS-polyacrylamide gels

Resolving gel

	Final polyacrylamide concentration			
	ml			
	8.75%	10%	12%	15%
Acrylamide solution	11.7	14.0	18.6	28.0
Resolving gel buffer	10.0	10.0	12.0	15.0
Water	18.0	18.0	16.0	13.0
	Solution degassed			
Ammonium per sulphate	0.2	0.2	0.3	0.4
TEMED	0.01	0.01	0.015	0.02

Stacking gel

	4% polyacrylamide concentration
	ml
Acrylamide solution	1.2
Stacking gel buffer	2.5
Water	6.5
Ammonium per sulphate	0.06
TEMED	0.01

Composition of other solutions used in section

Electrophoresis buffer

Tris	0.025M
Glycine	0.192M
SDS	0.1% w/v

Sample buffer (stored in frozen aliquots)

Tris.HCl	0.0625M	pH 6.8
SDS	2.3% w/v	
2-Mercaptoethanol	5% v/v	
Glycerol	10% v/v	
Bromophenol Blue	0.1% w/v	

Elution buffer

Tris	0.05M
Glycine	0.394M
SDS	0.1% w/v

Stain

Coomassie Blue R250	0.25% w/v
Methanol	50% v/v
Glacial acetic acid	10% v/v

Destain

As for stain without Coomassie Blue

Mixture for Non-equilibrated pH gradient (NEPHGE) gels

Following quantities is enough for preparing 10 gels.

AristaR urea	5.5g
Acrylamide solution	1.33ml
Nonidet P-40	
10% w/v solution	2.0ml

Water	2.0ml
Ampholines	
40% w/v pH 3.5-10	0.5ml
Mixture degassed	
Ammonium per sulphate	0.015ml
TEMED	0.010ml

Lysis buffer (Stored in frozen aliquots)

AristaR urea	9.5M
Nonidet P-40	2% w/v
Ampholines	
1.6% pH 5-7	
0.4% pH 3.5-10	2%
2-mercaptoethanol	5% v/v

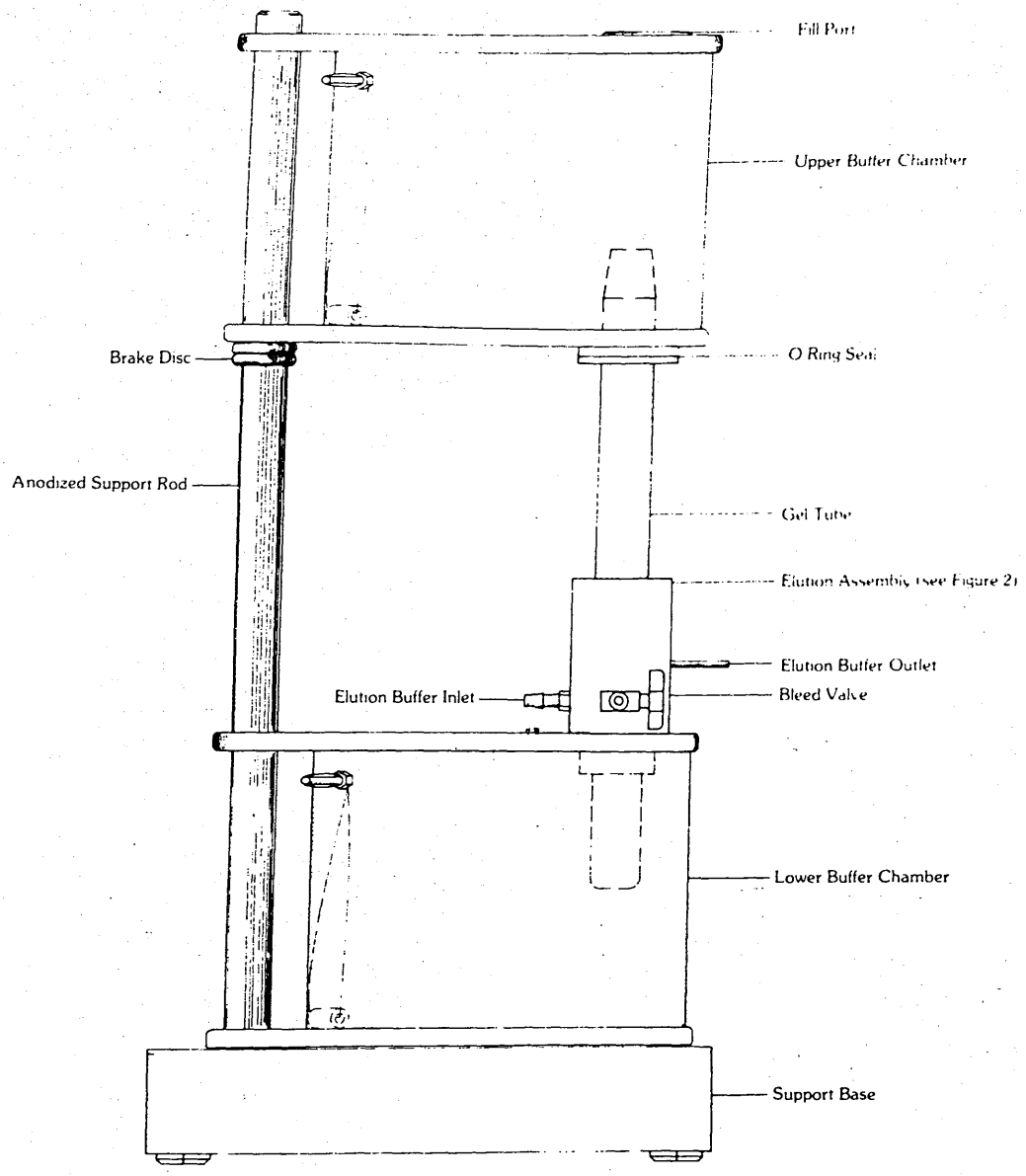
Gel electrophoresis was performed using the discontinuous SDS-polyacrylamide procedure of Laemmli (1970) as described by Le Stourgeon and Beyer (1977) in all the systems outlined below.

3.1 Preparative Gels

3.1.1 Preparative Rod Gel

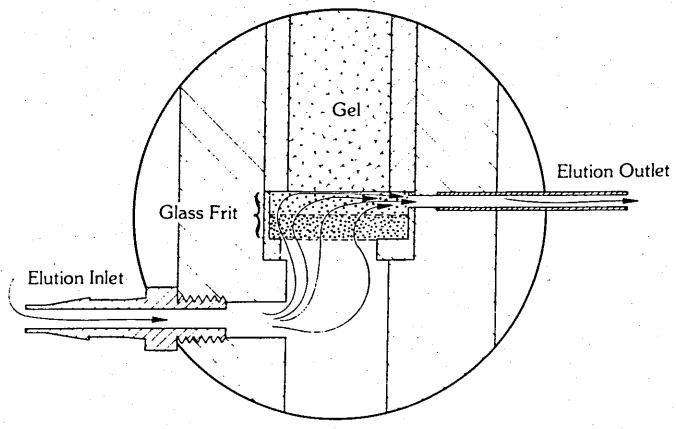
Core proteins were routinely recovered as pooled or fractionated proteins by preparative rod gel electrophoresis in the BRL 1100 PG system. The system allows recovery of the proteins with very low loss of the resolution obtained by the gel electrophoresis. Figures 3.1 and 3.2 show how the system was assembled.

Figure 3.1

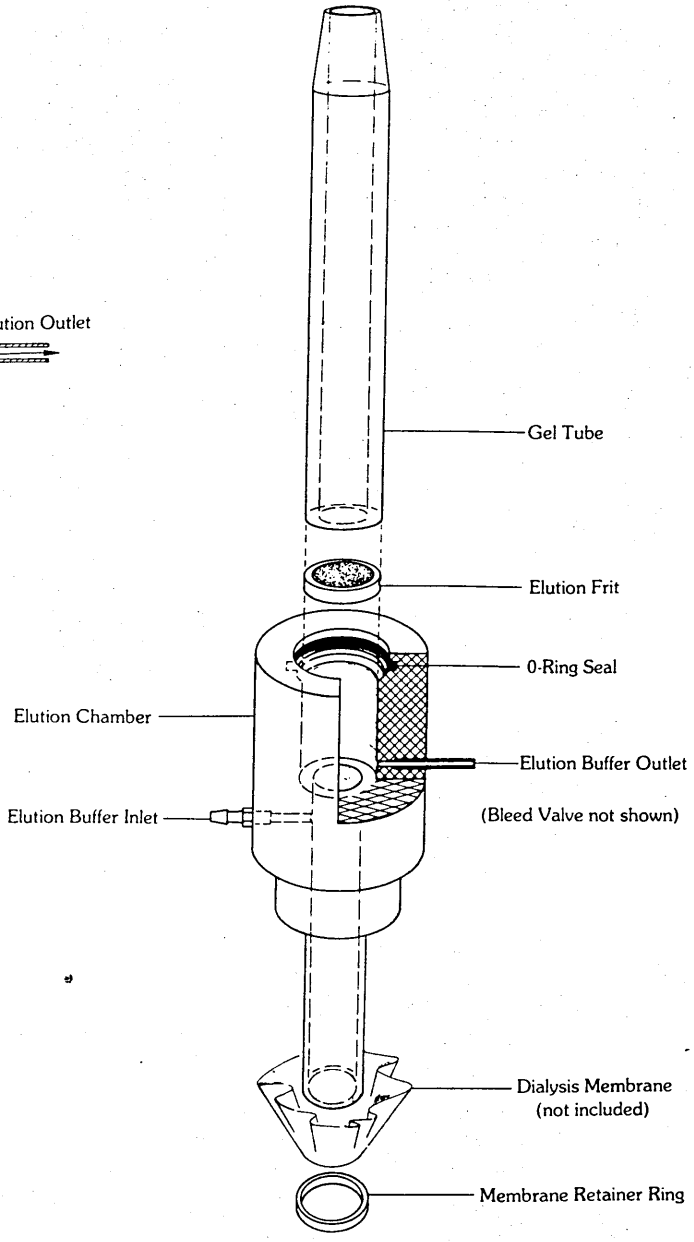


The BRL Preparative Gel Electrophoresis System

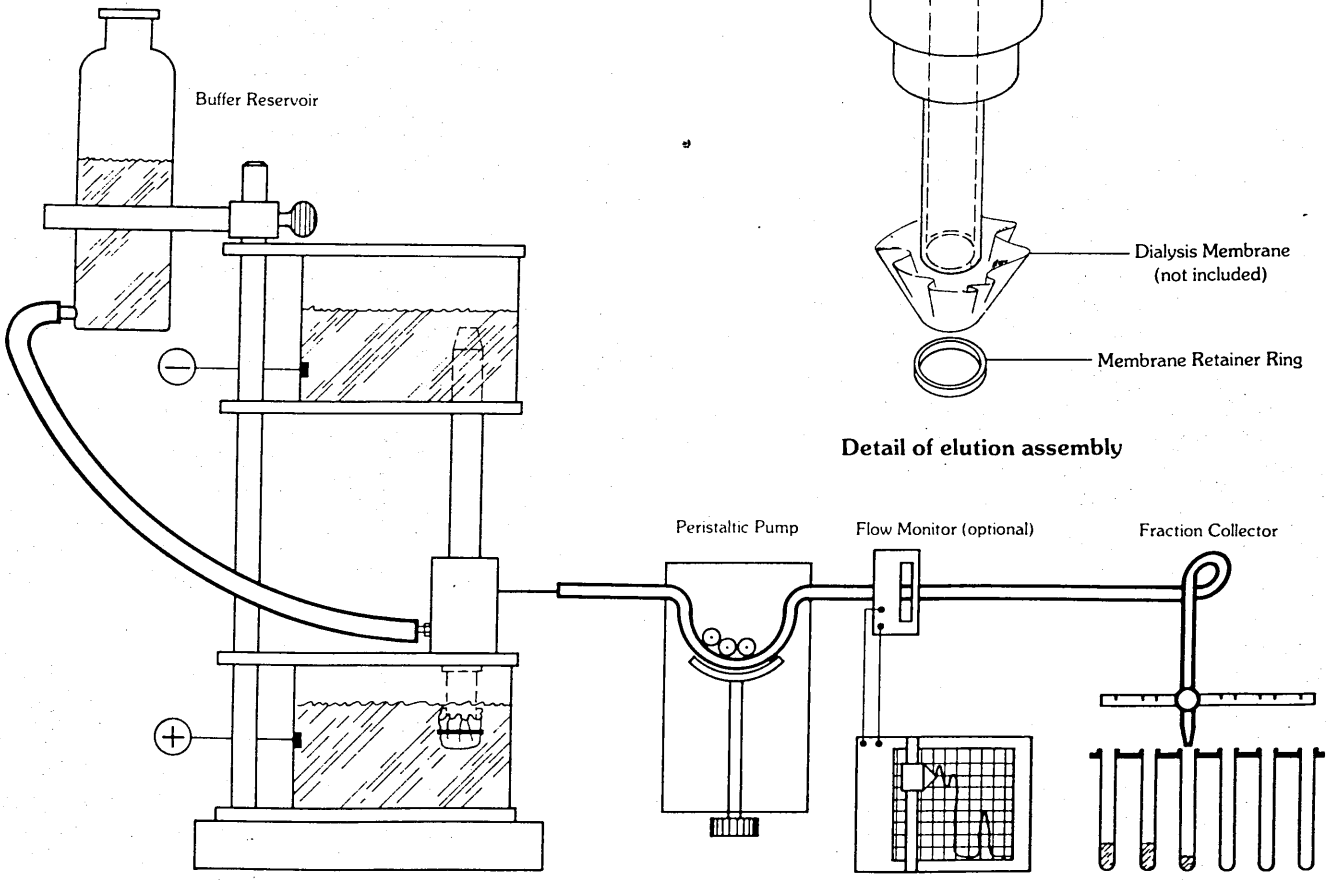
Figure 3.2



Cross-section of elution chamber.



Detail of elution assembly



Complete set up for a preparative run using the 1100PG.

To obtain total core protein from hnRNP particle proteins, 8.75% polyacrylamide resolving gel was used. Gel solution was poured into the 1cm diameter gel tube upto a height of 8cm. Before the gel solution was poured, the bottom of the gel tube was sealed by several layers of parafilm. Gels were overlaid with water and left to set for an hour. Stacking gel was poured on top of the set rod gel to a height of 1cm.

Total hnRNP proteins from sucrose density gradients (method 1.3.1) were dialysed against 10mM Tris.HCl, pH 7.6 and precipitated with polyethylene glycol (see method 6.4). The precipitated hnRNP proteins were taken up in sample buffer upto 3mg/ml. Samples of upto 0.5ml were loaded on top of the rod gel with a pasteur pipette under electrophoresis buffer. Electrophoresis was carried out at a constant voltage of 120 volts. 1ml fractions were collected at a speed of 5-6ml/h. Collection of fractions started when the bromophenol blue dye reached the bottom of the gel. Aliquots of appropriate fractions were precipitated with 10% trichloroacetic acid (TCA) and run on SDS-polyacrylamide gels to determine the location of core proteins.

When core protein fractionation was desired, 15% polyacrylamide resolving gels were used. For the 15% rod gels, the sample loaded consisted of total core proteins obtained from the 8.75% polyacrylamide preparative rod gel described above. Fractions from the 8.75% polyacrylamide gels containing core proteins were pooled, dialysed against 10mM Tris.HCl, pH 7.6, acetone precipitated and dissolved in sample buffer before loading onto 15% polyacrylamide preparative gels.

3.1.2 Preparative Slab Gel

An all glass slab gel apparatus as described by O'Farrell (1975) was used except that the spacers were 3mm thick and the comb had a single well, 8.5cm long for loading sample and one small well, 1.2cm long for loading markers. Also the compressible silicon tubing was replaced by a 3rd thick spacer used to block the bottom.

For electrophoresis a 15% polyacrylamide resolving gel and 4% polyacrylamide stacking gel was used. The apparatus was sealed before the gel solution was poured by addition of 1cm of 15% polyacrylamide gel mixture made 10% with respect to ammonium per sulphate to facilitate rapid polymerisation.

Total hnRNP proteins or total core proteins in up to 0.5ml of sample buffer were electrophoresed at 40mA for 7 hours. To obtain maximum resolution of the core proteins, electrophoresis was continued for up to 2 hours after the dye had reached the bottom of the gel. These thick slab gels took longer to stain - 2 to 3 hours and were destained over night (see method 3.4).

3.2 Analytical Gels

Core proteins were analysed by polyacrylamide gel electrophoresis on the BRL V161 vertical gel electrophoresis system. The slab gels used were 1mm thick. Molecular weights were assigned by comparison to known protein standards in the same slab gel. The percentage of the resolving gel varied from 8.75% to 15% polyacrylamide according to requirements. A 4% polyacrylamide stacking gel was used in which a 20 well-forming teflon comb was embedded. Samples to be run were either acetone

or TCA precipitated (see method 6.4) and dissolved in sample buffer. Gels were run for 4 hours at 30mA.

3.3 2-dimensional Gels

Two dimensional fractionation of total hnRNP proteins and core proteins was performed essentially as described by O'Farrell et al. (1977).

Protein fractionation in the first dimension gel was by charge and was usually performed by non-equilibrated pH gradient electrophoresis (NEPHGE) (O'Farrell et al., 1977) as the basic core proteins are lost during isoelectric focusing (Wilks and Knowler, 1980; O'Farrel, 1975).

The first dimension NEPHGE gels were run in pyrex glass tubing of internal diameter 2.5mm and length 14cm. The tubes were prepared for electrophoresis by washing in Decon, followed by thorough rinsing and air drying. One end of each tube was sealed with several layers of parafilm and tubes were arranged in vertical position for pouring the gel mixture. Gel mixture was added to gel tubes with a pasteur pipette to a height of 11cm. The gels were overlaid with water and left for half an hour to polymerise.

Pre-running is not required for NEPHGE gels. Precipitated or lyophilised samples were dissolved in lysis buffer and loaded anodically. Internal protein markers in lysis buffer were run with samples as described by Peters and Comings (1980). Electrophoresis was performed using disc gel electrophoresis apparatus with 10mM phosphoric acid as the upper reservoir solution and 20mM sodium hydroxide as the lower reservoir

solution. Proteins were electrophoresed for 3½ hours at 500 volts (1750 volt-h) unless otherwise stated.

Second dimension gel was exactly as described for analytical SDS-polyacrylamide gels (method 3.2) except that no comb was put in the stacking gel and a space of 1.5cm was left over the gel solution surface for the first dimension gel to fit in. The first dimension gels were incubated with sample buffer for 30 minutes. The gels were then applied to the polymersied second dimension gels by squeezing them into direct contact with the gel surface (Garrels, 1979). No agarose stacker was used. Gels were run for 4 hours at 30mA (method 3.2). Molecular weight markers in sample buffer were run to one side of the slab gel.

3.4 Coomassie Blue Staining and Autoradiography of Gels

Completed gels were stained and fixed for 1 hour in a shaking water bath at 25°C. Destaining was performed by diffusion using several washes of destain, until background was clear. The gels were then dried onto 3mm filter paper under vacuum, on a BioRad gel drier. Where necessary, autoradiography was performed on the dried gel using an intensifying screen and Kodak X-omat H film.

4.0 ANALYSIS OF CORE PROTEINS

Composition of solutions used in section.

Media without phosphate for Rat Liver Cell Line

BSS*X1	450ml
ES**X10	50ml
$NaHCO_3$ 6.8% w/v	20ml
Calf serum	50ml

***BSSX10**

NaCl	68g
KCl	4g
$MgSO_4.7H_2O$	2g
$CaCl_2.6H_2O$	3.93g
Phenol Red (1% w/v)	15ml
Distilled water to:	1 litre

1ml chloroform per litre added for storage.

****ESX10**

MEM amino acids x100 (Gibco)	20ml
MEM vitamins x100 (Gibco)	20ml
L-Glutamine	0.64g
Glucose	4.0g
pH to 7.1 (KOH)	
Distilled water to:	220ml

Extraction buffer (stored in frozen aliquots)

Triethanolamine.HCl	50mM	pH 8.0
SDS	1% w/v	
EDTA	0.05mM	
DTT	0.1mM	

EDTA_Ethylene diamine tetraacetic acid

DTT_Dithiothreitol

SDS_Sodium dodecyl sulphate

Cleveland sample buffer

Tris.HCl	0.125M	pH 6.8
SDS	0.5% w/v	
glycerol	10% v/v	
Bromophenol Blue	0.0001% w/v	

Silver staining solutions

Method of Steck et al. (1980) and Eschenbruch and Burk (1982).

Solution A

Coomassie Blue R250	0.8g
Absolute alcohol	180ml
Formaldehyde 35%	100ml
Water (deionised)	420ml

Solution B

Coomassie Blue R250	1.2g
Absolute Alcohol	250ml
Water (deionised)	750ml
Formaldehyde 35%	10ml

Developer

Citric acid	0.005% w/v	
Formaldehyde	0.05% v/v	in deionised water

Silver nitrate solution (made freshly)

NaOH 0.36% w/v	63ml	
Ammonia 40%	5.28ml	
$AgNO_3$	4g in 12.0ml	deionised water

Solution for storage of gels

Citric acid 0.05% w/v	200ml	
Methylamine 35%	0.1ml	

Silver Staining solutions for the method of Wray et al. (1981)

Silver Nitrate solution

NaOH 0.36% w/v	21ml	
Ammonia 40%	1.78ml	
$AgNO_3$ 0.8g in	4.0ml	deionised water

made up to 100ml with deionised water.

Developer

Citric acid	0.005% w/v
Formldehyde (40%)	0.05% v/v

made in deionised water

4.1 Radiolabelling with Iodine^{125}

Bands of core proteins A, B, C and D were cut out of preparative slab gels and extracted as described in methods 3.1.2 and 4.4 respectively. The extracted proteins were iodinated and recovered by the method of Tolan et al. (1980). Iodination was

carried out by addition of 40µl of 2.5mM KI and 50 to 100µCi of NaI^{125} to the protein in up to 2ml of extraction buffer and transferring the whole mixture to a glas vial coated with 50µg of Iodogen. After incubating the mixture for 2 minutes at 0°C with gentle mixing, the reaction was stopped by transferring the contents of the vial into a clean tube. Residual iodine was removed by the addition of 40µl of 2-mercaptoethanol.

Proteins were recovered after iodination by precipitating with 0.1 volumes of 3M potassium acetate (Bray and Brownlee, 1973). After 30 minutes at 0°C, the precipitate was recovered by centrifugation, washed twice with 2.0ml acetone containing 0.1M HCl. The precipitate was resuspended in 10% trichloroacetic acid and kept at 0°C for 30 minutes. The trichloroacetic acid precipitate was pelleted by centrifugation and washed once with acetone. The pellet was then dried in a vacuum desiccator over sodium hydroxide.

4.2 Phosphorylation

4.2.1 In vitro with [γ^{32}P] ATP

4.2.1.1 Qualitative pattern

Endogenous Kinase

hnRNP particles collected from sucrose density gradients (method 1.3.1) were dialysed against 10mM MOPS, pH 6.5, containing 10mM $MgCl_2$. Particles (0.1--> 0.2mg/ml) were incubated at 30°C with ATP^{32}/ATP at 100 Ci/mole for 40 minutes. Reaction was stopped by cooling to 0°C in ice and precipitating protein in acetone. Proteins were then subjected to 2-dimensional electrophoresis (method 3.3), the gels dried and autoradiographed.

Exogenous Kinases

hnRNP particles were incubated with [γ^{32}P]ATP and various exogenous kinases (details in appropriate figure legends) as described for the endogenous kinase except that the incubation was in 10mM Tris.HCl, pH7.6 and 10mM $MgCl_2$.

4.2.1.2 Ratio of ^{32}phosphate to protein (quantitative analysis)

hnRNP particles were incubated with ATP^{32}/ATP as described in Section 4.2.1 for the phosphorylation with endogenous kinase, except that the specific activity of ATP^{32}/ATP used was increased to 400 to 800 Ci/mole. The reaction was stopped by cooling in ice, and 9 volumes of unlabelled carrier hnRNP at the same concentration was added to the incubation mixture. The total hnRNP particles were precipitated in 4% $w/_v$ polyethylene glycol 6000 and run on preparative slab gel (method 3.1.2). The protein in the gel was stained and the band of core protein A cut out and extracted (method 4.4). Extracted protein was precipitated in acetone, and dissolved in lysis buffer (see method, section 3). Concentration of the protein was determined by the Bradford assay (Bradford, 1976), in comparison with bovine serum albumin and histone standards, both of which were prepared in lysis buffer.

The recovered protein A was then subjected to 2-dimensional electrophoresis (method 3.3) and the various charged isomers of the protein detected by staining. The charge isomeric spots of core protein A were assayed densitometrically (method 6.3). From the densitometric data and total concentration of core protein A, the relative concentration of the charge isomers A_1 to A_6 were determined. The 2-dimensional gel was then dried, the

stained spots cut out and their cerenkov counts determined (method 6.8). Cerenkov counts of the ATP^{32}/ATP used was also determined, so that the counting efficiency of the machine could be ignored. From the known specific activity of the ATP and comparison of its cerenkov reading with those of the charge isomers, picomoles of phosphate ion per mole of charge isomer was calculated.

4.2.2 Phosphorylation with ^{32}Phosphate in vivo

Buffalo Rat Liver (BRL) cells were grown in Eagle's medium (Glasgow modification) (Busby et al., 1964) and 10% new born calf serum. The medium was removed from one roller-bottle and replaced by 25ml phosphate-free medium. To this roller bottle, 1mCi of carrier-free ^{32}phosphate was added and it was then incubated for 17 to 20 hours at 37°C. Cells were harvested from this and four other unlabelled roller bottles of cells by means of a rubber "policeman", washed in ice cold Eagle's medium and resuspended in 1% Triton X-100 in RSB (see method, Section 1). BRL cell nuclei were purified as described in method 1.1.2, lysed as desrcibed in method 1.2.2, and hnRNP purified from the lysate as described in method 1.3.1.

Proteins from the sucrose density gradient fractions were acid-precipitated, dissolved in analytical SDS-gel sample buffer and run on SDS-polyacrylamide gels (method 3.2). For analysing the precipitated proteins on 2-dimensional gels, the proteins were first dissolved in 2% SDS before dilution in lysis buffer (see method 3.3). The SDS helped ensure that residual ^{32}P did not cause unacceptable background problems when the proteins were subsequently run on analytical and 2-dimensional gels.

In some experiments BRL cell ^{32}P labelled proteins were mixed with cold Rat Liver hnRNP proteins before electrophoresis. In this case, the lysed nuclear sonicate from BRL cells (method 1.2.2) was treated with DNase (100µg/ml) and RNase (50µg/ml) for 1 hour at 37°C. The proteins in the nuclear sonicate were then acid precipitated and mixed with precipitated Rat Liver hnRNP proteins from dialysed sucrose density gradient fractions (method 1.3.1).

4.3 Deglycosylation (Karp et al., 1982)

The proteins to be deglycosylated were thoroughly dried first. Ovomucoid was used as a standard glycosylated protein to monitor deglycosylation.

Total hnRNP proteins from sucrose density gradients (method 1.3.1) and total core proteins from 8.75% polyacrylamide preparative rod gels (method 3.1.1) were dialysed against 10mM Tris.HCl, pH7.6, then precipitated with 3 volumes of acetone at -20°C. The precipitates were dried under nitrogen and weighed.

To the dry proteins, 180µl anhydrous trifluoromethane sulphonic acid and 20µl of anisole per mg of protein, was added. The mixture was kept on ice for two hours and then added to 4ml of pyridine/diethyl ether (1:9 $v/_v$) in a dry ice-acetone bath. The proteins and pyridinium salt of the acid coprecipitated and were collected by centrifugation at $10,000g_{av}$ for 10 minutes. The pellets were suspended in 0.1M NH_4HCO_3 and dialysed extensively against this solution. The neutralized proteins may form a flocculent precipitate inside the dialysis bag in which case these were recovered by centrifugation. In cases where no precipitate

was formed the contents of the dialysis bag were lyophilized. The dried precipitate/lyophilized residue was dissolved in sample buffer and electrophoresed on analytical SDS-polyacrylamide gels as described in Section 3.2.

4.4 Extraction of Protein from Polyacrylamide Gels

Proteins were extracted from polyacrylamide gels by a slight modification of the method of Tolan et al., (1980). Stained, protein spots from 2-dimensional (method 3.3) or bands from preparative slab gels (method 3.1.2) were cut out and chopped finely. The gel pieces were rinsed with distilled water and dried for 1-2 hours in a vacuum desiccator over sodium hydroxide to remove the acidic destaining solution. The gel pieces were then swollen in extraction buffer and homogenised. In the case of the protein spots from 2-dimensional gels, the homogenisation was carried out in 1.5ml eppendorf tubes with about 100µl of extraction buffer, using a hand-held teflon pestle. To extract the proteins, the contents of the eppendorf tubes were vortexed at regular intervals over a period of 2 hours. The tubes were then centrifuged and the supernatant was recovered. The protein from preparative gels were extracted as above using 2-3ml of extraction buffer for a gel band of 8x0.2x0.2cm. For complete extraction of the protein, the homgenised gel slurry was left stirring in extraction buffer overnight. The extracted proteins were recovered by passing the slurry over glass wool in a 5ml syringe and acetone precipitating the filtrate overnight at -20°C.

4.5 Partial Peptide Maps on One and Two Dimensional Gels

Partial peptide mapping of the core proteins was carried out by the method of Cleveland et al., (1977) on analytical SDS-polyacrylamide gels. Digested peptides were also subjected to 2-dimensional electrophoresis (method 3.3). Partial peptide maps were prepared of:

a) Pure fractionated core proteins A, B, C and D eluted from preparative rod gels (method 3.1.1). Lyophilised proteins were dissolved in Cleveland sample buffer, digested and subjected to analytical SDS-polyacrylamide gel electrophoresis. Proteins A, B, and C were eluted from 15% polyacrylamide gels (figure 4.6) and protein D from 12% polyacrylamide gels (figure 4.5).

b) Charge isomers of A and B after extracting them from gels (method 4.4).

The extracted charge isomers in 20 to 40µl of extraction buffer were digested and subjected to:

I. One dimensional SDS-polyacrylamide gel electrophoresis

II 2-dimensional electrophoresis.

In all cases described above, the digestion of the proteins with S. aureus V8 protease and chymotrypsin were done in eppendorf tubes. After stopping the reaction by the addition of 2-mecaptoethanol and SDS to final concentrations of 10% $v/_v$ and 2% $w/_v$ respectively, the samples were boiled, made 10% $v/_v$ with respect to glycerol and contents of the tubes loaded directly onto gels. In case of samples subjected to 2-dimensional analysis the

glycerol was omitted and samples were made 9M with respect to urea and 2% $w/_v$ with respect to ampholines (1.6% pH5-7 and 0.4% pH3.5-10). Larger amounts of V8 protease were required for digestion of the charge isomer proteins, (which were extracted from polyacrylamide gels), compared to that of pure protein (eluted from preparative gels) presumably because of the higher SDS content of extraction buffer. The same amount of V8 protease as that required for digestion was always loaded on a spare track of the polyacrylamide gel to ensure that the band pattern of V8 protease could be differentiated from the band pattern of the digested proteins. The one dimensional SDS-polyacrylamide gels and 2-dimensional gels were silver stained as described in Section 4.6.1 and 4.6.2.

4.6 Silver Staining

4.6.1 One dimensional SDS-polyacrylamide gels (method of Steck et al. (1980) and Eschenbruch and Burk (1982)

Gels were fixed and first stained with Coomassie Blue using the reagents described in Section 4. They were shaken for 1 hour in solution A and 1 to 3 hours in solution B. Background destaining was by diffusion overnight in several changes of solution B without Coomassie Blue. Gels were washed free of formaldehyde with deionised water for at least 3 hours and then shaken in ammoniacal silver nitrate at 27°C in the dark. Stained gels were washed for an hour in six changes of deionised water and developed for approximately 8 minutes. The gels were washed once in deionised water and placed in solution for storage of gels.

4.6.2 Silver staining two-dimensional gels (method of Wray et al., 1981)

This method is suitable for silver-staining 2-dimensional polyacryalmide gels where background tends to be much darker.

Unstained gels were soaked in 50% $v/_v$ reagent grade methanol for one hour before transfer to deionised water, in which they were allowed to swell overnight. Several changes of water are desirable. The gels were then returned to 50% methanol and washed for 3 hours with 3 changes.

Gels were then stained for 15 to 20 minutes in the dark at 27°C with constant shaking in silver nitrate solution. The gels were then washed in six changes of deionised water over one hour. The gels were developed, washed with deionised water and stored in 50% methanol. The gels could be dried without heat overnight in a gel drier.

5.0 IMMUNOLOGICAL TECHNIQUES

Composition of solutions used in section

Phosphate buffered saline (PBS)

NaCl	0.14M
KCl	2.7mM
KH_2PO_4	1.5mM
Na_2HPO_4	8.1mM

PBS/Tween

0.1% $v/_v$ Tween in phosphate buffered saline

Mcllvanies buffer

Citric acid 0.1M

Na_2HPO_4

pH6.0

Substrate solution for enzyme in ELISA

Orthophenyl diethylamine (OPD)	0.4mg
Mcllvanies buffer	9ml
Hydrogen peroxide (22.8% solution)	1ml

Blotting buffer

Tris.HCl	25mM
Glycine	0.19M
Methanol	20%$v/_v$
SDS	0.02% $w/_v$

Amido black stain and destain

Amido black	0.25%$w/_v$
Acetic acid	7.5%$v/_v$
Methanol	50%$v/_v$

Destain

Methanol	90%$v/_v$
Acetic acid	2.0%$v/_v$

Washing buffer

Tris.HCl	20mM pH7.2
NaCl	0.15M
Tween 20	0.5%v/v
Sodium azide	0.5mg/ml

5.1 Preparing and Injecting Antigen

5.1.1 Total core protein

Total core proteins were eluted from 8.75% polyacrylamide-SDS preparative rod gels (method 3.1.1) and dialysed against PBS without NaCl. Core proteins were then acetone-precipitated and taken up in PBS. The primary injection was given with 1mg total core protein in 0.5ml PBS and 0.5ml of Freund's complete adjuvant. Injections were given subcutaneously at 4-5 sites on the back. Booster injections were given subcutaneously at 3-4 sites at 2-weekly intervals with 0.2mg total core protein in Freund's incomplete adjuvant. The rabbits were bled for the first time two weeks after the second booster injection and from then on at monthly intervals. Sera was heated at 55°C for 10 minutes to inactivate complement and stored frozen at -20°C in 1ml aliquots.

5.1.2 Core proteins A and B

Core proteins A and B from preparative slab gels (method 3.1.2) were cut out and washed extensively. The gels were macerated, frozen and then ground to a fine powder in a mortar and pestle. Water was added to the powdered material (1ml/g of gel powder) to make a fine suspension and to it was added an equal

volume of Freund's complete or incomplete adjuvant. The injection and bleeding protocol was exactly as for total core proteins as described in the previous section.

5.2 Enzyme Linked Immunoabsorbent Assay (ELISA)

Dilutions of $2x10^{-3}$ to $2.5x10^{-4}$ of total core proteins (1mg/ml) in PBS were used as the antigen. One hundred microlitres of the antigen solution was added to each well of a microelisa plate (Falcon). The plates were incubated overnight at 4°C or at room temperature for 1 to 2 hours. After discarding the antigen solution, the wells were washed in PBS/Tween. The remaining protein-binding sites in each well were then blocked with 200µl of 5% bovine serum albumin in PBS for 1 hour at room temperature. The wells were then rewashed as above and 100µl of different dilutions ($2x10^{-3}$ to $2.5x10^{-4}$) of antibody in PBS/Tween were added and incubated for 1 to 2 hours at room temperature. The wells were again washed as above and 100µl of horseradish peroxidase-bound goat antirabbit immunoglobulin (of dilution $2x10^{-3}$) was then added to each well for 45-60 minutes. Once again the wells were washed before 100µl of substrate solution for horseradish peroxidase was added to each well. After leaving the substrate solution in the wells for 30 minutes in the dark, 50µl of 4N sulphuric acid was added to each well and readings taken in an automated microelisa reader at a wavelength of 492nm.

5.3 Immunoblotting

Proteins were transferred electrophoretically onto nitrocellulose as described by Towbin et al. (1979) in blotting buffer employing an electrophoretic destaining tank run at 400mA for 2.5 hours. The transfers were washed in washing buffer for 60 minutes at 37°C. The Tween in washing buffer blocked the protein-free spaces on the nitrocellulose. Blots of molecular weight markers and spare proteins were stained with amido black to monitor the blotting procedure.

Transfers for immunoanalysis were incubated for 90 minutes at 37°C with a mixture of inactivated horse serum (5% $v/_v$) and 1.5ml of antibody in 60ml washing buffer. The transfers were washed at 37°C with 5 x 60ml of washing buffer over a period of 30 minutes. The bound antibodies were then decorated for 1-2 hours with I^{125}-protein A (0.5µl/ml) in washing buffer and then washed as above before drying and analysis by autoradiography.

6.0 MISCELLANEOUS

Composition of solutions used in section

Coupling buffer

$NaHCO_3$	0.1M
Na_2CO_3	0.1M
NaCl	0.5M
	pH8.3

Blocking buffer

Glycine	0.1M pH8.0 with NaOH

Acetate buffer

Sodium acetate 0.1M

acetic acid to pH 4.0

NaCl 0.5M

Bradford reagent

Coomassie Blue G250	100mg
Ethanol 95%	50ml
phosphoric acid, 85% $w/_v$	100ml

Made up to 1000ml and filtered

Reagents for Lowry assay

Solution A: 2% Na_2CO_3 in 0.1M NaOH

Solution B: 0.5% $CuSO_4.5H_2O$ in 1.25% sodium potassium tartarate adjusted to pH7.0.

Solution C: Solution A, 50ml

Solution B, 1ml

6.1 Affinity Chromatography of Snake Venom Phosphodiesterase I

One gram of cyanogen bromide-activated sepharose was swollen for 15 minutes in 1mM HCl and then washed with 200ml of the same solution on a sintered glass filter. The sepharose beads were then placed in coupling buffer with 30mg of RNase in the same buffer. The RNase solution was mixed with gel suspension in an end-over-end mixer for 2 hours at room temperature. The gel was then transferred to blocking buffer for a further 2 hours at 20°C. Excess adsorbed protein was washed away with coupling buffer followed by acetate buffer and then coupling buffer again.

The RNase-sepharose conjugate was placed in solution containing 10mM Tris.HCl, pH 7.0 and 0.5M NaCl and poured into a 5ml syringe. Commercial phosphodiesterase enzyme (5mg/ml) was then repeatedly passed through the RNase-sepharose column (10 times). Eluate was collected and used as protease-free phosphodiesterase, since the protease present in phosphodiesterase is capable of degrading RNase. Protein-sepharose columns may be used to retard the protease activity of commercial phosphodiesterases (Whish, W. J. D., personal communication). I chose RNase since this is a commonly available basic small MW protein.

6.2 TLC on Polyethyleneimine (PEI) Impregnated Cellulose Plates of hnRNP Particle Products After Phosphodiesterase I Treatment

hnRNP particle proteins in 10mM Tris.HCl, pH7.0 (0.1mg/ml) were treated with affinity-purified snake venom phosphodiesterase I (see method 6.1) at 100µg/ml for 30 minutes at 37°C. The mixture was then filtered through a YM2 Diaflo membrane (with a cut-off molecular weight of 1000) in an Amicon ultrafiltration unit. To 12ml of the filtrate, 0.1µCi [γ^{32}P] ATP was added so that the label could be picked up by a mini-monitor. The filtrate was then passed through a DEAE-sephadex column at pH7.0. The bound radioactivity was washed off with 30ml of 200mM triethyl-ammonium bicarbonate, pH7.0 (made by adding dry ice to ethylamine) collected and lyophilised (Kostka and Schweiger, 1982). The lyophilised residue was dissolved in 20µl of water and 10µl was applied to a PEI plate (1µl at a time). Twenty nmoles of

5'AMP and 20 nmoles of ADP-ribose were used as markers. The plates were developed in 1M acetic acid for 3cm and and then in a solution of 0.9M acetic acid and 0.3M LiCl for a further 15cm without intermediate drying (Rickwood et al., 1977). Spots were looked for under a UV. torch.

6.3 Densitometric Scanning of Polyacrylamide Gels

Stained slab gels were scanned before drying on a LKB 2202 ultroscan laser densitometer. In the case of 2-dimensional gels several readings at different positions were taken of large spots or streaks so that non-uniformity of staining could be taken into account.

6.4 Precipitation of Protein

Polyethylene glycol was used up to 4% ($w/_v$) to precipitate high molecular weight complexes of protein such as hnRNP particles. Precipitation was complete after 2 hours at 0-4°C and hnRNP particle proteins were recovered by centrifugation at $16,000g_{av}$ for 10 minutes in the Sorval HB4 rotor. Proteins were washed once with 2ml 10mM Tris.HCl pH 7.6 and recovered by centrifugation as above.

Trichloroacetic acid (10% $w/_v$) was used to precipitate proteins (5 to 100μg) from aliquots of preparative gel fractions (method 3.1). Proteins were left overnight in 10% trichloroacetic acid at 4°C. Proteins were recovered by centrifugation at $16000g_{av}$ for 5 minutes in the Sorval HB4 rotor. The precipitate was washed once with acetone and proteins recovered by centrifugation at $16000g_{av}$ for 2 minutes.

Large quantities of protein (from 0.1 to 2mg), were usually precipitated with 3-5 volumes of acetone at -20°C overnight. Proteins were recovered by centrifugation at $16000g_{av}$ for 10 minutes. Residual acetone was evaporated in a stream of nitrogen gas.

6.5 Concentrating Proteins

Proteins were concentrated (1) by lyophilisation in a flexi-dry freeze drier attached to a high-vacuum pump; (2) by packing solid polythene glycol 6000 in contact with a dialysis bag containing the protein solution at 4°C for 2-3 hours.

6.6 Dialysis and Microdialysis

Salts were removed by overnight dialysis in 2 x 100 volumes of the appropriate buffer at 4°C.

For the dialysis of small volumes (<200µl) the solution to be dialysed was placed in a 1.5ml Eppendorf tube, which had previously had the centre of its cap removed. The tube was then recapped with a piece of dialysis tube trapped such that the semi-permeable membrane effectively replaced the cut-out segment of the Eppendorf cap. The dialysate was then brought into contact with the dialysis membrane by a brief centrifugation in an inverted position and the inverted tube was suspended in a piece of polystyrene and allowed to float on the dialysis buffer. Any air bubble trapped between the dialysis tubing and buffer surface were carefully removed.

6.7 Sterility Precautions

To eliminate problems with endoribonuclease digestions during the preparation of hnRNP particles, all solutions were sterilized by autoclaving or filtration. Glassware required during the hnRNP preparation was baked in an oven at 200°C for 4 hours. Disposable rubber gloves were worn and all dialysis bags were boiled in sterile distilled water.

6.8 Cerenkov Counting for ^{32}P

Dried gel pieces from polyacrylamide gels were put into scintillation vials. One ml of water was added and vials counted in Beckman LS 6800 automatic liquid scintillation counter.

6.9 Gamma Counting for I^{125}

Aliquots of protein labelled with I^{125} were placed in vials and counted in an LKB 1275 Minigamma.

6.10 Protein Estimation

Protein concentrations were estimated usually by the method of Bradford (1976). Where substantial amounts of sodium dodecyl sulphate was present in protein samples, the Lowry assay was used (Peterson, 1979).

For the Bradford assay, the standard or micro protein assay was used depending on the approximate concentration of protein in samples. For the standard assay, 5ml of protein reagent was added with mixing, to 0.1ml of protein-containing solution and the absorbance was measured at 595nm after 2 minutes. Standard protein used was either Bovine Serium Albumin or Histone

at concentrations of 10-100μg. A micro assay for the analysis of 1-10μg protein employed the same procedure except that 1ml of protein reagent was added to 0.1ml of protein-containing solution.

In the Lowry assay, 1ml of solution C was added to 200μl of protein-containing solution and mixed well. After 10 minutes, 0.1ml of Folin Ciocalteau reagent diluted 1:1 with distilled water was added and test tubes shaken well. Readings were at 680nm after 30 minutes.

6.11 Radiolabelling protein A and molecular weight markers with Iodine^{125}

Proteins were labelled by the method of Fraker and Speck (1978). Twenty microlitre of 1mg/ml Iodogen in chloroform was pipetted into disposable glass vials. The vials were rotated under nitrogen until the solvent evaporated, leaving a film of iodogen. Into one vial was put 1mg of protein A in 0.5ml 20mM Tris.HCl, pH7.2 containing 0.15M NaCl (buffer A). Sodium iodide^{125} (200μCi) was then dispensed into the vial in a fume cupboard. The vial was incubated at 4°C for 3-4 minutes with continuous mixing after which the mixture was removed from the vial (this termiates the production of $^{125}I^{+}$). To separate the iodinated protein from free iodide, the mixture was loaded on to a 10ml G-50 sephadex column previously equilibrated with buffer A. Fractions of 1ml were collected and 5μl aliquots counted. Fractions containing the intial peak of radioactivity were pooled, aliquoted and stored frozen at -20°C.

The protocol for iodinating molecular weight markers was identical except that 1 vial of Pharmacia low molecular weight

calibration markers (approximately 700μg) in 1ml buffer A were used as starting material. After iodination markers were aliquoted to provide 20000 cpm per experiment and stored frozen.

IV RESULTS

1.0 ISOLATION AND FRACTIONATION OF hnRNP CORE PROTEINS

1.1 Isolation of hnRNP

The routine method by which hnRNP particles were isolated was extraction of purified nuclei at pH8 as described by Samarina et al. (1968). This technique has been employed in this and other laboratories for many years. For the extraction of particles from tissue culture cells, however, the method gives low yields unless high temperatures (Pederson, 1974a; Beyer et al., 1977) and higher pH (Quinlan et al., 1974) are used. For experiments with liver tissue culture cells, therefore, hnRNP particles were isolated from lysed nuclei (Pederson, 1974a). Figure 4.1 compares the protein profile of particles isolated from both methods and compares them with other basic low molecular weight proteins, that is, histones and ribosomal proteins. It can be seen that both preparations are dominated by the so-called core proteins arrowed in the figure. The core proteins of the rat liver nuclei have been marked as A, B, C and D according to the nomenclature of Wilks and Knowler (1981a). As described by others (Gallinaro et al., 1975; Thomas et al., 1981b), the extra bands visible in the particles prepared by sonication may belong to histones and non-histone chromatin proteins. Figure 4.1 A, Lanes 1 and 4 also reveal a group of higher molecular weight bands, (MW66000-88000) which show resemblance in arrangment and band spacing to the core proteins. The different techniques used thoughout this work appeared to cause variation in the intensity of these bands. Sometimes a third, ever higher MW set of bands was seen. Data to be presented suggests that they are SDS-resistant aggregates of core proteins (Results, section 2.3).

Figure 4.1

hnRNP Particle Proteins

hnRNP particles were extracted either at high pH or by sonicating nuclei (method 1.2.1 and 1.2.2). The hnRNP particles were then purified on sucrose density gradients as described in method 1.3.1. Aliquots of the purified particles were precipitated, dissolved in sample buffer, run on SDS-polyacrylamide gels (method 3.2) and stained with coomassie blue.

4.1 A A 12% polyacrylamide gel of Rat Liver hnRNP particles extracted at high pH.
1. hnRNP particle proteins 60μg
2. Histones 80μg
3. 80S ribosomal proteins 50μg
4. hnRNP particle proteins 30μg

4.1 B A 8.75% polyacrylamide gel of Buffalo Rat Liver Cell Line hnRNP particles isolated from lysed nuclei.

Lanes 1-6 represent consecutive fractions of the sucrose density gradient on which the particles were purified. Each fraction was approximately 6ml. Three ml of each fraction was precipitated and run on the gel. Proteins in Lanes 3 and 4 represent a very small peak of the gradient at 260nm.

Core proteins and markers have been labelled on the left of the gel.

Fig. 4.1 A

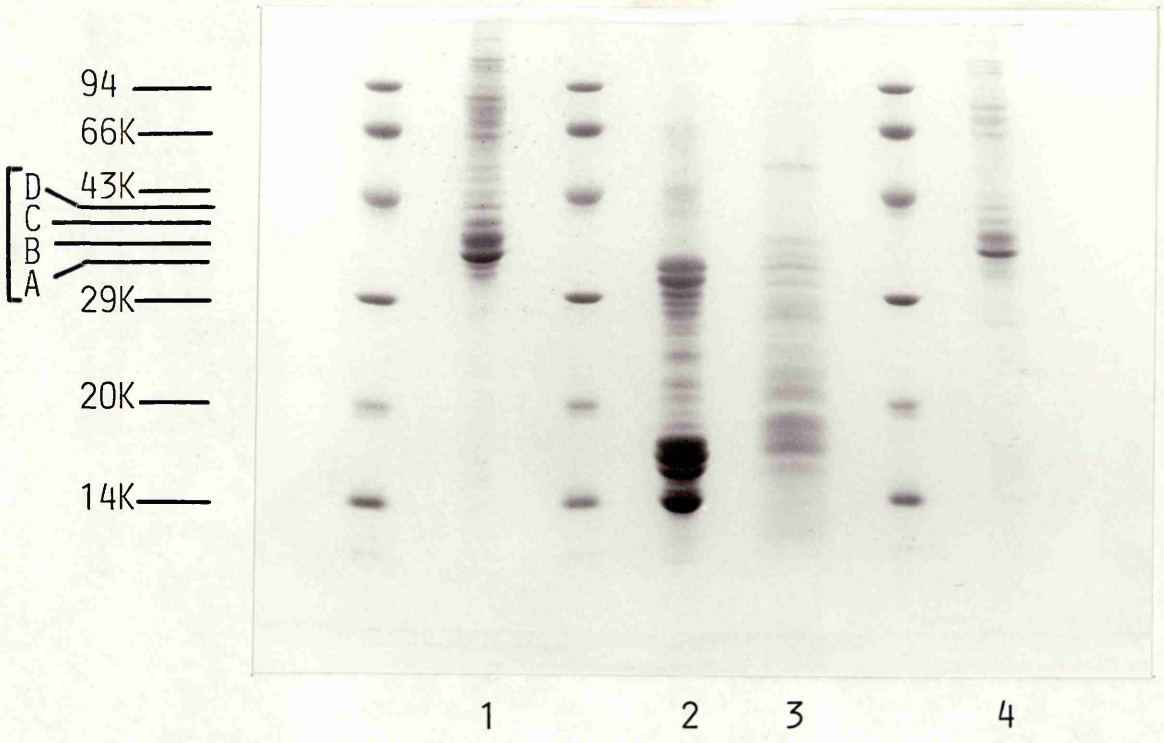
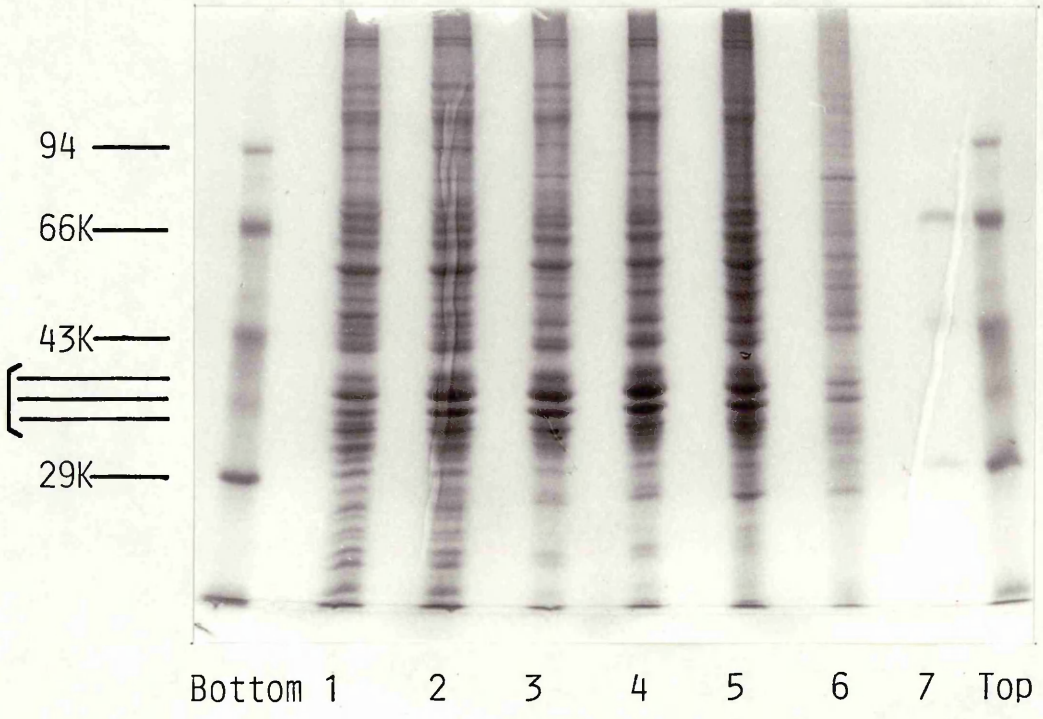


Fig. 4.1 B



Particles made by both of the above methods were recovered as peaks from 15 to 30% sucrose density gradients (Samarina et al., 1968). This technique suffers from the disadvantage that particles are isolated in relatively large volume in approximately 22% sucrose and have to be dialysed and concentrated before further analysis. As an alternative method for purification of hnRNP particles, therefore, Sephacryl S-1000, a gel filtration support with an exclusion limit of 300-400nm was tried. Figure 4.2 shows that hnRNP could be recovered from Sephacryl. However, aggregation appeared to be a greater problem when the particles were recovered in this way and recoveries were poor. Further more, the particles were eluted in an even greater volume than those recovered from sucrose density gradients and the method was not adopted.

1.2 Fractionation of hnRNP Proteins

Many techniques were employed in early attempts to fractionate hnRNP proteins. Various ion-exchange resins were tried and rejected. The major reason for this was aggregation of the particles in any buffer continaing low salt concentrations which led to considerable losses and abnormal elution profiles. Such aggregation due to hnRNP particle proteins has been reported before (Leser et al., 1984; Pullman and Martin, 1983; Wilk et al., 1983). Gel filtration on Biogel P-60 or sephadex G-100 could fractionate hnRNP particle proteins as long as it was peformed in a high salt (1M NaCl) buffer (figure 4.3). However losses were large (table 1) and although the system could separate core proteins from other hnRNP polypeptides, it could not separate core

Figure 4.2

Purification of hnRNP Particles

hnRNP particles extracted by the method of Samarina et al. (1968) (method 1.2.1) were purified either on sucrose density gradients or Sephacryl S-1000 gel filtration column (see method 1.3). Aliquots of appropriate fractions from the sucrose gradient and Sepharcyl S-1000 were precipitated and run on a 8.75% polyacrylamide SDS-gel.

1. 80µg from the 40S peak of sucrose density gradient.
2. Total pooled proteins of fractions 1-10 of Sephacryl.
3. Total pooled proteins of fractions 11-20 of Sephacryl.
4. 1/5 pooled proteins of fractions 21-26 of Sephacryl.
5. 1/5 pooled proteins of fractions 27-32 of Sephacryl.

Lanes 6 to 12 contain total pooled proteins of fractions 33-38, 39-51, 52-61, 62-71, 72-81, 82-91 and 92-101 respectively of the Sephacryl column.

Fig. 4.2

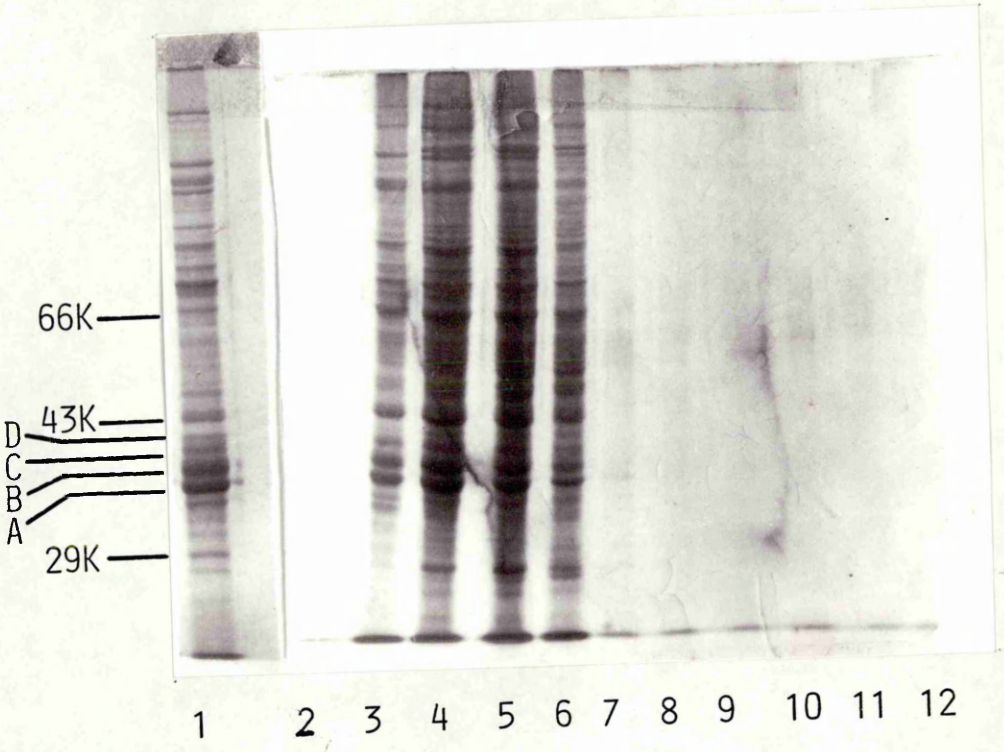


Figure 4.3

Fractionation of hnRNP Proteins on Sephadex G-100

hnRNP particles were purified on sucrose density gradients (method 1.3.1) and 2mg of the proteins fractionated on a Sephadex G-100 filtration column (method 2.1). 2ml fractions were collected. Appropriate fractions were pooled. Proteins in the pooled fractions were precipitated and analysed on a 8.75% polyacrylamide SDS gel. Fraction numbers are indicated at the bottom of the gel.

Fig. 4.3

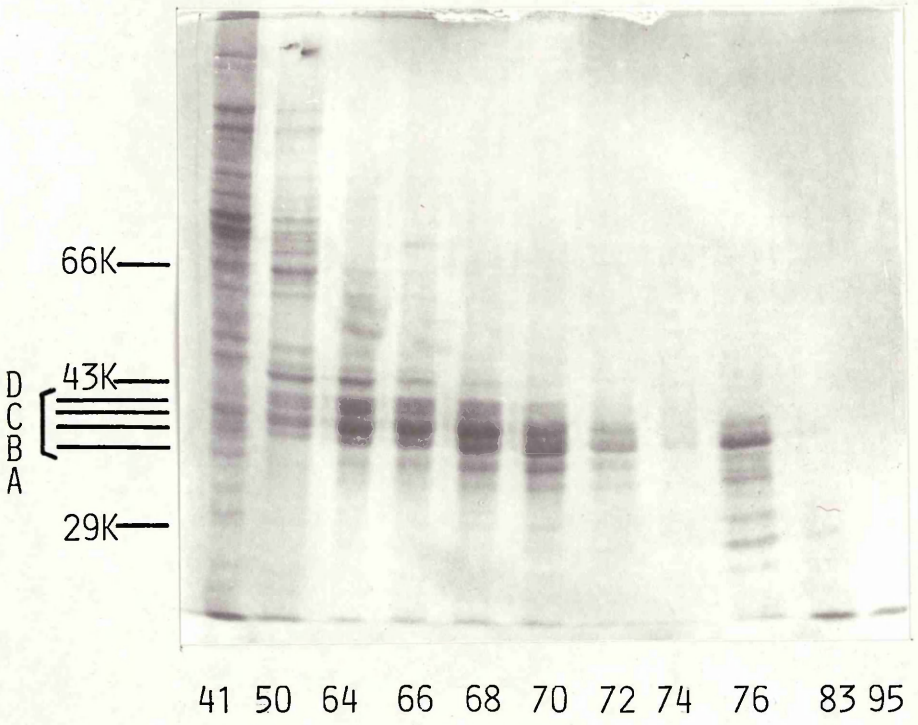


TABLE 1

COMPARISON OF TOTAL CORE PROTEIN RECOVERY BETWEEN GEL FILTRATION AND PREPARATIVE GEL ELECTROPHORESIS

Isolation steps	Gel Filtration Sephadex G-100 Protein concn.	Preparative gel Electorphoresis on 8.75% polyacrylamide. Protein concn.
1. Total HnRNP protein from Sucrose gradient	3.7mg	3.7mg
2. Removal of Sucrose by dialysis	3.0mg	3.0mg
3. P.E.G. concentration (only for gel-filtration)	2.0	--
4. P.E.G. precipitation (only for preparative gel electrophoresis)	--	2.8mg
5. Recovery of fractions containing core proteins	0.550mg	2.0mg

proteins from each other. Even fractionation of the core proteins by HPLC on a Biorad reverse phase column was given up because the system could not handle the salt which was needed to keep the proteins disaggregated.

The only system that was found to be capable of fractionating core proteins was preparative SDS gel electrophoresis; this also gave high yields (table 1) although it did suffer from the fact that protein was recovered as an SDS-complex and the system could only handle 3mg at a time. In practice, it was found desirable to isolate core proteins free from other hnRNP proteins by preparative electrophoresis on 8.75% polyacrylamide (fig 4.4) and to then fractionate the pooled and concentrated total core protein into its four components by a second preparative electrophoretic fractionation on 12 or 15% polyacrylamide (figures 4.5 and 4.6). Even with this method, however, relatively few of the fractions contained single core proteins free of detectable contamination with the others (figures 4.5 and 4.6). Hence although recovery of core protein was good, yields of pure core protein species was low. Table 2 gives the amount of core proteins A to D obtained starting from 10 rat livers.

Where larger quantities of pure proteins were required, for example, for raising antibodies and in some phosphorylation experiments, bands of core proteins were cut out from stained 15% polyacrylamide preparative slab gels (method 3.1.2). For the antibody experiments, suspension of powdered protein-containing-gel with adjuvant was injected (method 5.1.2). For the phosphorylation experiments, however proteins had to be extracted from strips of gel (method 4.2.1.2).

Figure 4.4

Fractionation of Total hnRNP Proteins by Prepartive Gel Electrophoresis

hnRNP proteins from sucrose density gradients (method 1.3.1) were dialysed against 10mM Tris.HCl, pH 7.6. 3mg of hnRNP particle proteins were precipitated by polyethylene glycol (method 6.4) and dissolved in sample buffer before loading them on an 8.75% polyacrylamide preparative rod gel (method 3.1.1). 1ml fractions were collected from the preparative gel and proteins in 150µl aliquots of these fractions were precipitated and analysed on a 8.75% polyacrylamide analytical gel. Fraction numbers are indicated on the bottom of the gel.

Fig. 4.4

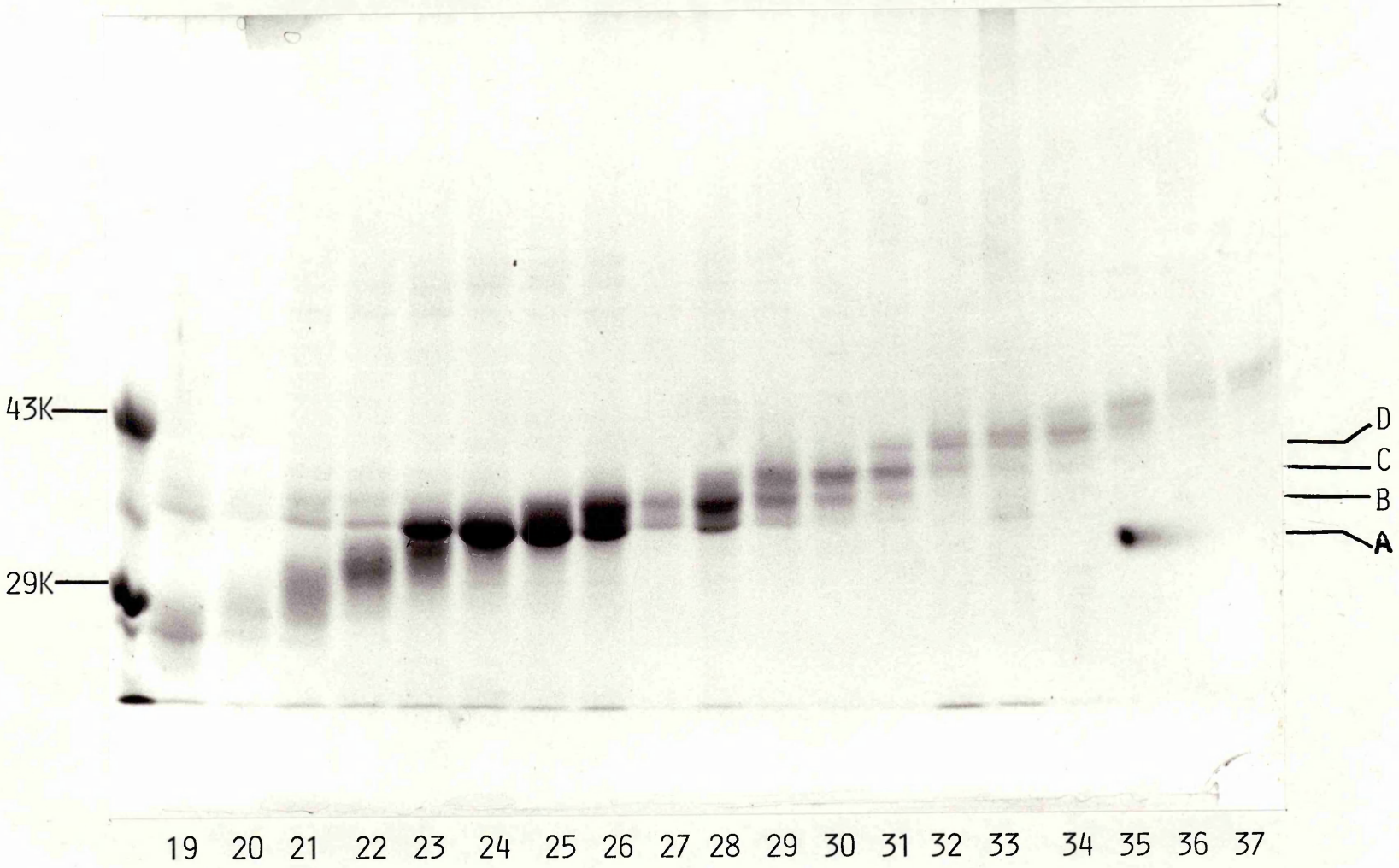


Figure 4.5

Core Protein Fractionation by 12% Polyacrylamide Preparative Gel

Total core proteins were collected from 8.75% polyacrylamide preparative rod gels (method 3.1.1) and dialysed against 10mM Tris.HCl, pH 7.6. The core proteins were precipitated and reloaded on a 12% preparative rod gel (method 3.1.1). Aliquots of 150µl from 1ml fractions indicated in figure were analysed for their protein content on 8.75% polyacrylamide slab gel.

Fig. 4.5

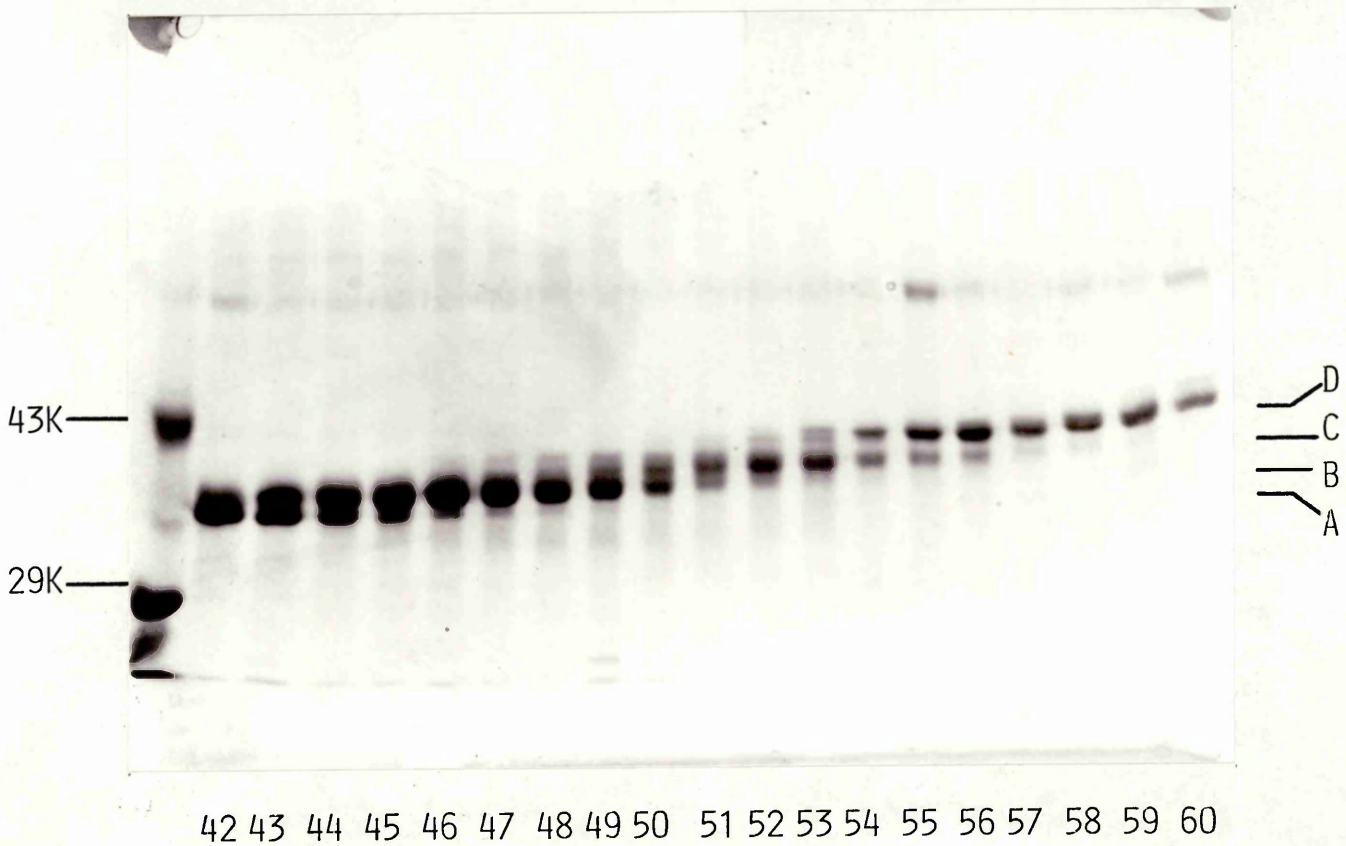


Figure 4.6

Fractionation of Core Proteins on 15% Polyacrylamide Preparative Gel

Total core proteins were collected as described in legend to Figure 4.5 and loaded on a 15% polyacrylamide preparative rod gel (method 3.1.1). 1ml fractions were collected from 1 to 160. Protein in selected fractions were analysed on a 15% polyacrylamide-SDS gel. These selected fractions are indicated on the bottom of the gel. In case of fractions 87 to 91, 1/3rd of each fraction was analysed. For fractions 109 to 135, half of each fraction and for fractions 142 to 152 whole fractions were precipitated for analysis.

Fig. 4.6

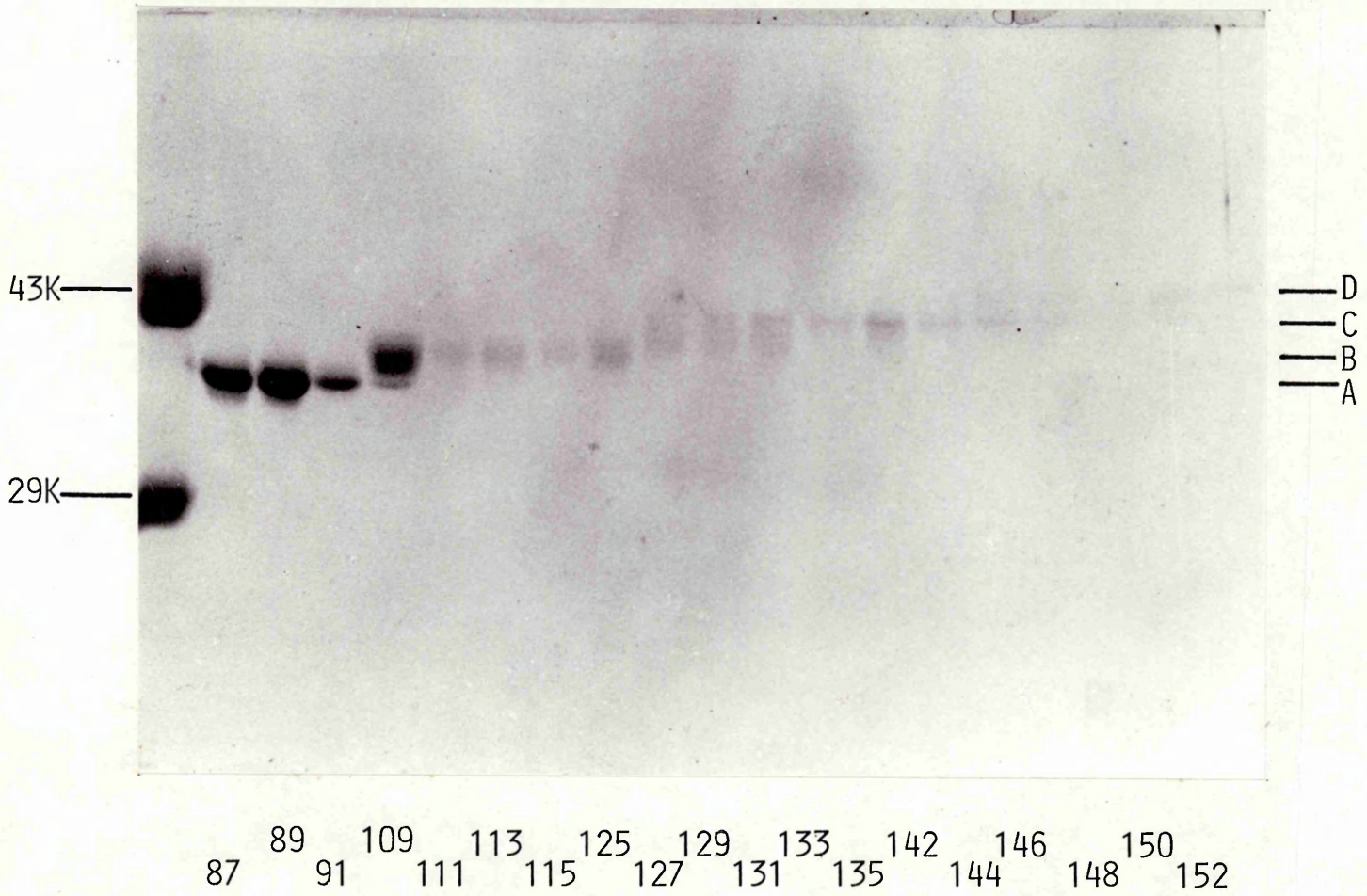

43K
29K
D
C
B
A
87
89
91
109
111
113
115
125
127
129
131
133
135
142
144
146
148
150
152

TABLE 2

YIELD OF CORE PROTEINS A, B, C, AND D AFTER FRACTIONATION BY PREPARATIVE GEL ELECTROPHORESIS

Isolation steps	Yield of protein
1. 10 rat livers	
2. 15ml pH 8 nuclear extract	34.0mg
3. 40ml hnRNP dialysate after sucrose density gradients	4.0mg
4. Eluate containing total core proteins from 8.75% polyacrylamide preparative gel	2.5mg
5. Pure fractions recovered from 12 or 15% polyacrylamide preparative gels	core protein A - 80μg core protein B - 60μg core protein C - 35μg core protein D - 20μg

Proteins A to D were judged pure when aliquots of isolated fractions showed a single band on polyacrylamide gels stained with Coomassie Blue. An example of how the proteins were collected from a typical fractionation is shown below:

Protein A Fractions 89-91 in fig 4.6

Protein B Fractions 113-115 in fig 4.6

Protein C Fractions 142-143 in fig 4.6

Protein D Fraction 59 in fig 4.5

See fig 4.8 lanes 3, 5, 7 and 9 for a silver stain of isolated fractions containing A, B, C and D respectively.

2.0 STRUCTURAL INTER-RELATION OF THE FOUR CORE PROTEINS OF RAT LIVER hnRNP PARTICLES

2.1 Cleveland Maps: Comparison of Partial Peptide Maps of Core Proteins A, B, C and D on Analytical SDS-Polyacrylamide Gels

The peptide structure of the four core proteins was investigated in an attempt to understand their relationship to each other. Previous two-dimensional tryptic peptide mapping in this laboratory (Wilks and Knowler, 1981a) were rather inconclusive and in an extension of their studies, the partial digest method of Cleveland et al. (1977) was adopted because of its sensitivity and ability to differentiate between closely related proteins (Goodman and Karn, 1983; Easley et al., 1983; Caballero et al., 1983; Grirgera and Tisminetzky, 1984). Cleveland maps of purified A, B, C and D from preparative gels (results 1.2) were carried out using Staphylococcus aureus V8 protease and α-chymotrypsin (see method 4.5). Figures 4.7 and 4.8 show the partial peptide maps of core proteins A and B and core proteins A, B, C and D with V8 protease. The digested peptide pattern of the four proteins are very similar indicating their close relationship to each other. The minor differences have been marked by arrows; for example the peptide of 18000 in the digested pattern of A is absent or faint in the digested peptides of B. This 18000 peptide is also absent in peptides of C and D. The series of peptides below 10000MW in A are fainter in B; perhaps indicating that the same peptide bonds are in some way less accesible to the enzyme in the latter proteins. A peptide of 25000 is present only in the peptides from core protein C.

Figure 4.7

Partial Peptide Map of Core Proteins A and B with V8 Protease

Core proteins A and B were eluted from a 15% polyacrylamde preparative gel (method 3.1.1 and results 1.2). Fractions containing pure protein A and B were separately microdialysed against 10mM Tris.HCl, pH 7.6 (method 6.6). The proteins were then lyophilsed, dissolved in Cleveland sample buffer (see method section 4) upto a volume of 40µl in eppendorf tubes and subjected to digestion with 2.5µg of V8 protease at 37°C (method 4.5). Reaction was stopped with 2-mercaptoethanol and SDS to final concentration of 10% and 2% respectively. Samples were boiled for 2 minutes and loaded directly on to a 15% polyacrylamide SDS-gel. Stacking gel was longer than normal with larger wells. The gel was silver-stained (method 4.6.1).

Lane 1	V8 protease with 2-mercaptoethanol and SDS
Lanes 2 and 4	Core protein A and B respectively. 5µg each
Lanes 3 and 5	5µg of core protein A and B with 2.5µg of V8 protease at zero time digestion
Lanes 6, 8, 10	15µg of core protein A with 2.5µg of V8 protease
and 12	at 10, 20, 30 and 40 minutes respectively
Lanes 7, 9, 11	15µg of core protein B with 2.5µg of V8 protease
and 13	at 10, 20, 30 and 40 minutes respectively.

The bands at about 66K appearing in almost all the lanes are probably due to non-proteinaceous components of the sample or polyacrylamide gel that stain with silver. It is possible that this arises from the mercaptoethanol (Wray et al. 1981).

Fig. 4.7

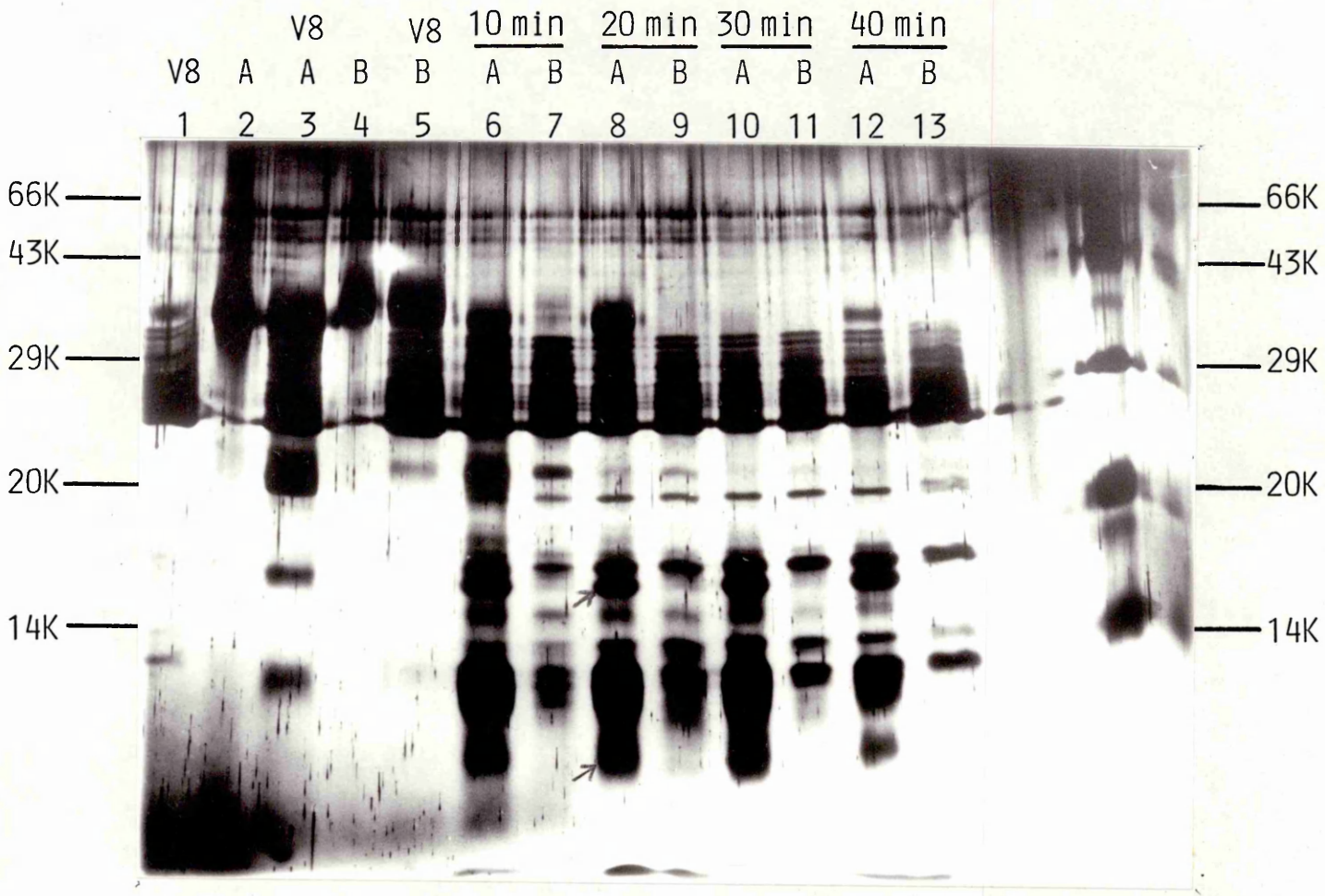

V8
V8 A
V8 A B
V8 B
10 min A B
20 min A B
30 min A B
40 min A B
1 2 3 4 5 6 7 8 9 10 11 12 13
66K
43K
29K
20K
14K
66K
43K
29K
20K
14K

Figure 4.8

Partial Peptide Map of Core Proteins A, B, C and D with V8 Protease

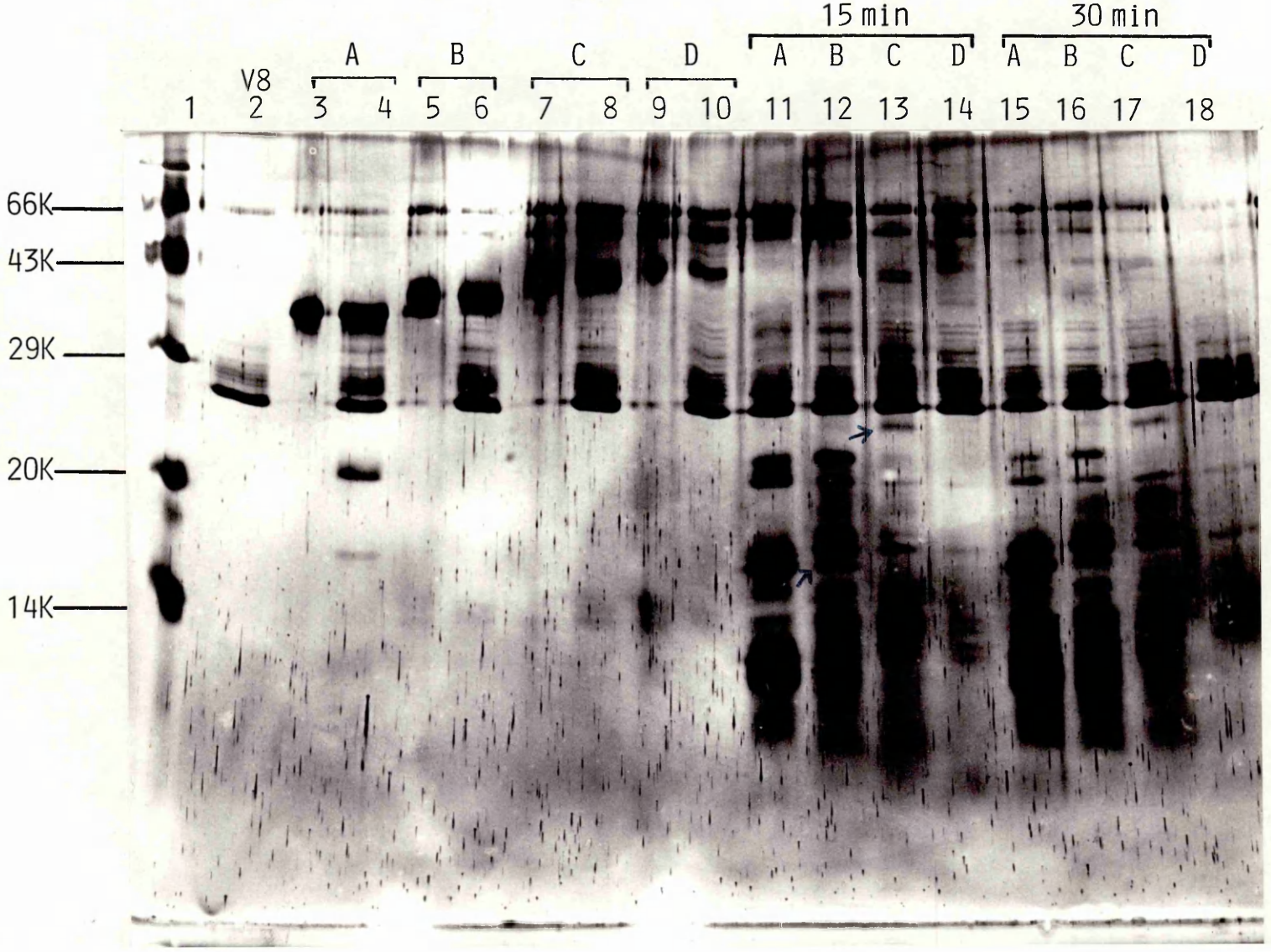
Core proteins A, B, C and D were isolated and digested exactly as described in legend to figure 4.7. Digestion products were analysed on a 15% polyacrylamide SDS gel and silver stained (method 4.6.1.).

Lane 1	Molecular weight markers
Lane 2	V8 Protease with 2-mercaptoethanol and SDS
Lanes 3, 5, 7 and 9	5µg of purified polypeptide A, B, C and D respectively
Lanes 4, 6, 8 and 10	Purfied A, B, C and D in the presence of 2.5µg V8 at zero time digestion
Lanes 11, 12, 13 and 14	Proteins A, B, C and D digested for 15 minutes with 2.5µg V8 protease
Lanes 15, 16, 17 and 18	Proteins A, B, C and D digested for 30 minutes with 2.5µg V8 protease

This result was reproducible in 5 experiments.

The bands at about 66K are probably artefacts (see legend to fig 4.7).

Fig. 4.8


15 min
30 min
A
B
C
D
V8
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
66K
43K
29K
20K
14K

The Cleveland map using ∝-chymotrypsin reinforces the relatedness of the four core proteins (figure 4.9). Minor differences are present; peptide of 25000 from core protein A is absent in B, C, and D. A peptide of 27000 is present in digestion patterns of A, B and C but absent from D.

In an attempt to simplify the complexity of the digestion products obtained in the Cleveland maps of figures 4.7, 4.8 and 4.9, conditions of digestion were varied. Iodinated core proteins were digested with V8 protease so that only the I^{125}tyrosine containing fragments were observed when the SDS-polyacrylamide gels were analysed (figure 4.10). A few peptides with identical mobilites were seen in the digestion products of core protein A, B and C. Core protein D did not digest well under these conditions. In general the Cleveland map of figure 4.10 exhibited greater variation between core proteins A, B and C than apparent in those of figures 4.7 to 4.9. This probably reflects different degrees of digestion of the iodinated proteins because of the difficulty in accurately quantifying the proteins after iodination.

To obtain the Cleveland digests of the iodinated core proteins, the proteins had to be extracted from polyacrylamide gels (method 4.4) and then iodinated in a strongly oxidising reaction (method 4.1). To ascertain that the structural identity of the proteins was not affected by the extraction and iodination procedures, a control was run. Extracted proteins A and B were iodinated and digested with V8 protease. Their digestion products were then examined by autoradiography and by a silver-staining (figure 4.11). It can be seen that the digestion pattern detected

Figure 4.9

Partial Peptide Map of Core Proteins A, B, C and D with α-Chymotrypsin

Core proteins A, B, C and D were isolated as described in legend to figure 4.7. Proteins were digested with 150ng of α-chymotrypsin. Reaction was stopped and products analysed on a 15% polyacrylamide gel as described in figure 4.7.

Lane 1	Chymotrypsin with 2-mercaptoethanol and SDS
Lanes 2, 4, 6, 8	5μg of purified polypeptides A, B, C and D respectively
Lanes 3, 5, 7, 9	Polypeptides A, B, C and D with Chymotrypsin at zero time digestion
Lanes 10, 11, 12, 13	15μg of purified proteins A, B, C and D digested for 15 minutes
Lanes 14, 15, 16, 17	15μg of proteins A, B, C and D digested for 30 minutes
Lane 19	Molecular weight markers

This result was reproducible in 5 experiments.

The bands at about 66K are probably artefacts (see legend to fig 4.7).

Fig. 4.9

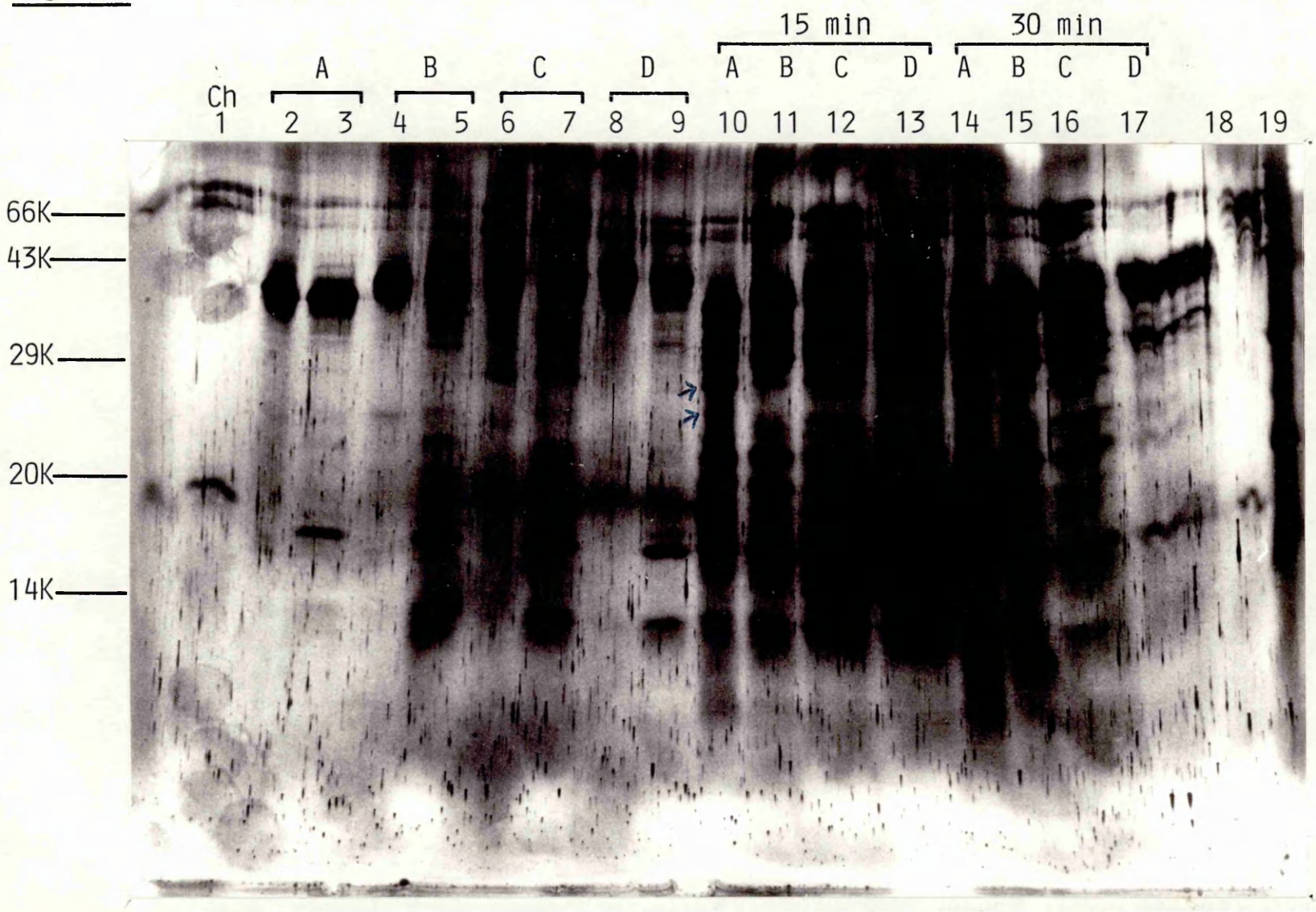

15 min
30 min
A
B
C
D
A
B
C
D
A
B
C
D
Ch
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
66K
43K
29K
20K
14K

Figure 4.10

Partial Peptide Map of Iodinated Core Proteins A, B, C and D

Core Proteins A, B, C and D were extracted from a preparative polyacrylamide slab gel (method 4.4) and iodinated in their respective tyrosine residues (method 4.1). Precipitated proteins were dissolved in Cleveland sample buffer (see method, Section 4). Aliquots of the purifed core protein A, B, C and D (with sufficient counts to be detected on a gel) were digested with approximately 50ng of V8 protease in eppendorfs at 37°C. Reaction was stopped by addition of 2-mercaptoethanol and SDS to concentrations of 10% and 2% respectively (see method 4.5). The samples were boiled, loaded on to a 15% polyacrylamide gel and electrophoresed at 25mA for 4 to 5 hours. Gels were dried and autoradiographed. Exposure was for 1 hour, with a Kodak x-omat film and Dupont intensifying screen.

Lanes 1, 3, 5 and 7	Purified and iodinated core proteins A, B, C and D
Lanes 2, 4, 6 and 8	Iodinated proteins A to D after incubation V8 Protease at zero time
Lanes 9, 10, 11 and 12	Proteins A to D digested with V8 protease for 15 minutes
Lanes 13, 14, 15 and 16	Proteins A to D digested with V8 protease for 30 minutes

This result was reproducible in 2 experiments.

Fig. 4.10

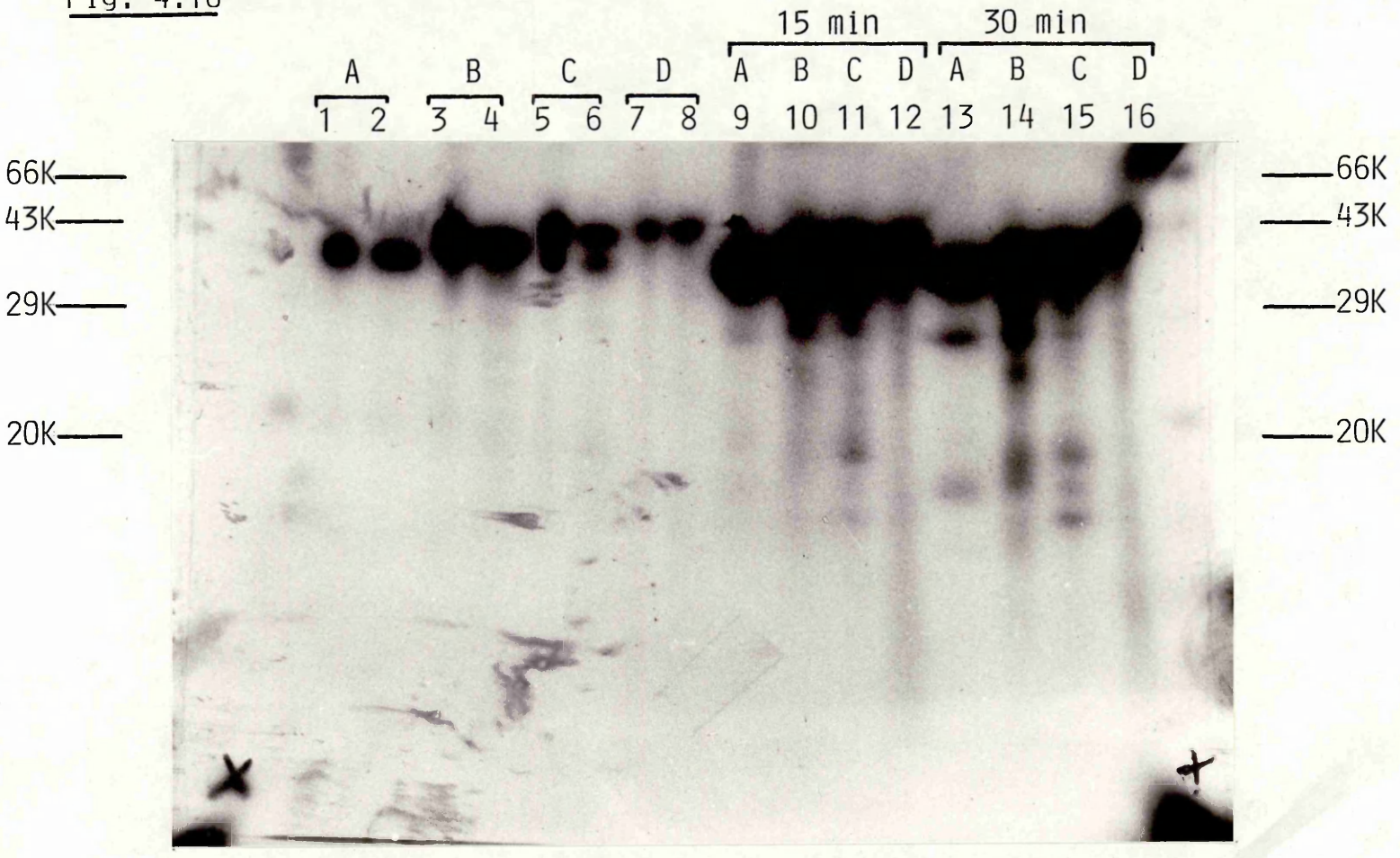

15 min
30 min
A B C D A B C D A B C D
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
66K
43K
29K
20K
66K
43K
29K
20K

Figure 4.11

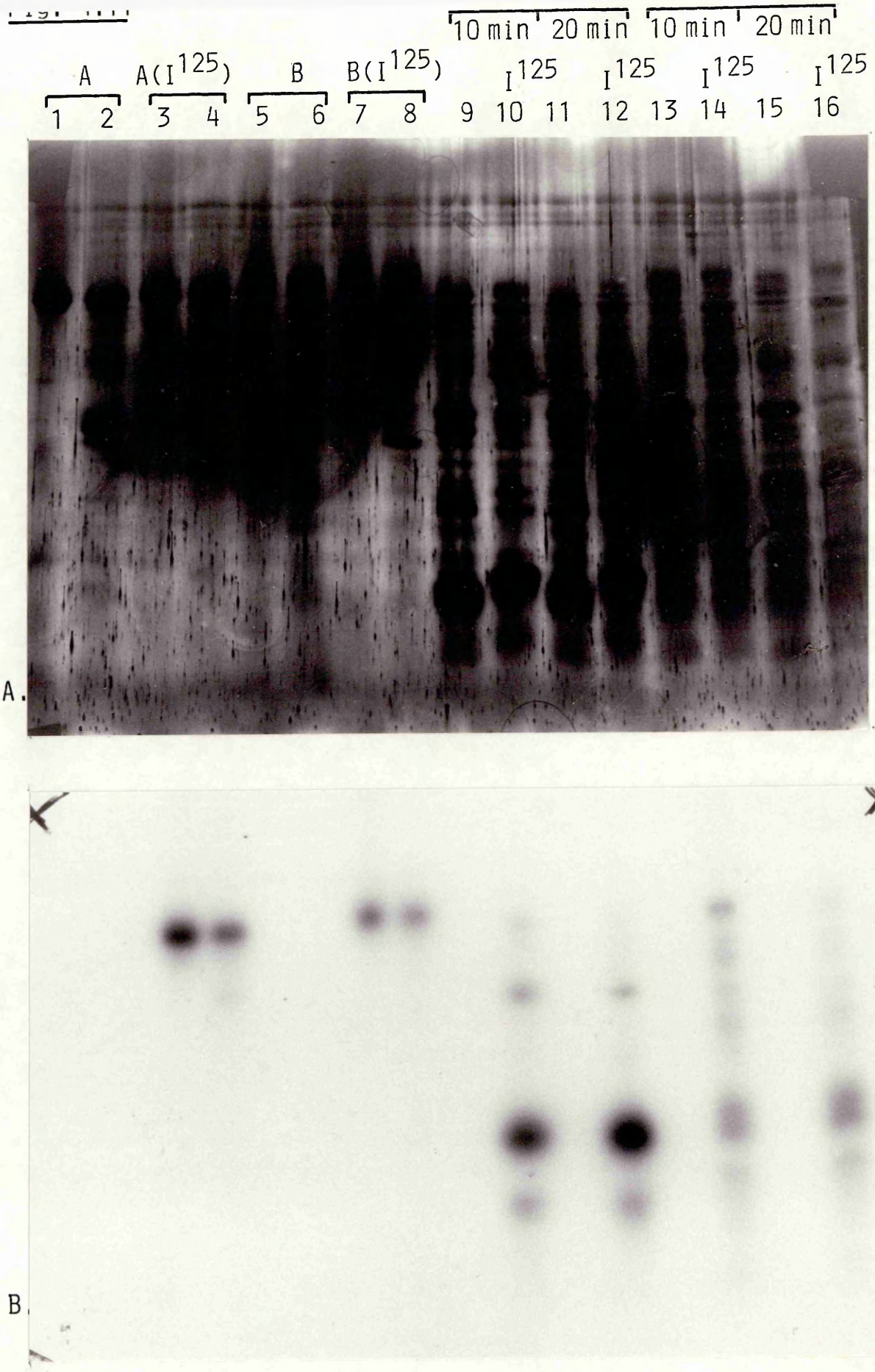
Analysis of the Effects of Iodination and Extraction from Gels on the digestion of Core Proteins A and B by V8 Protease

Core proteins A and B were 1) extracted from preparative slab gels (method 4.4) and 2) extracted from preparative slab gels and iodinated (method 4.1). The precipitated proteins were dissolved in Cleveland sample buffer (see method, section 4) and digested with 4µg V8 protease. Reactions were stopped with 10% 2-mercaptoethanol and 2% SDS (method 4.5). Samples were boiled, analysed on a 15% polyacrylamide gel and silver-stained. Gel was sealed in a polythene bag and autoradiographed.

A: Silver Stained Gel:

Lanes 1 and 5	5µg extracted core proteins A and B
Lanes 3 and 7	5µg extracted and iodinated core proteins A and B
Lanes 2 and 6	Extracted core proteins A and B with V8 protease at zero time
Lanes 4 and 8	Extracted and iodinated core proteins A and B with V8 protease at zero time
Lanes 9 and 11	15µg extracted core protein A digested with V8 protease for 10 and 20 minutes respectively
Lanes 10 and 12	15µg extracted and iodinated core protein A digested with V8 protease for 10 and 20 minutes respectively
Lanes 13 and 15	15µg extracted protein B digested with V8 protease for 10 and 20 minutes respectively
Lanes 14 and 16	15µg extracted and iodinated protein B with V8 protease for 10 and 20 minutes respectively

B: Autoradiograph of Gel in A


10 min
20 min
10 min
20 min
A
A(I125)
B
B(I125)
I125
I125
I125
I125
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
A.
B.

by both forms of analysis corresponds to the digestion pattern obtained for pure core protein A and B eluted from preparative rod gels (compare with figures 4.8 and 4.10).

2.2 Deglycosylation of Total Core Proteins

Given the considerable similarity in the peptide maps of the four rat core proteins, it remains possible that their differing molecular weights arise from post-translational modification rather than polypeptide length. Of the various modifications possible, that which causes the most dramatic changes in both molecular weight and apparent molecular weight on SDS-polyacrylamide gels is glycosylation. The molecular weight of many proteins is substantially increased by glycosylation; one example which, like the core proteins is a small basic protein, is pancreatic RNase, where the A and B forms of the enzyme differ only in their glycosylation.

There are conflicting reports about whether core protein stain with the schiff-glycoprotein reagent, periodic acid. Fuchs et al. (1980) reported positive staining but Wilks (1980) reported negative results. However, this technique of glycoprotein detection is not very sensitive. Alternative staining techniques such as lectin-binding would require the testing of many lectins as they exhibit specificity towards different sugar residues. They might therefore give a negative reaction because of the absence of suitably placed recognition sugar rather than the absence of glycosylation. Another approach which has been employed to detect glycoproteins is based on deglycosylation and detection of the consequent change in molecular weight (Edge et

al., 1981; Karp et al., 1982). Trifluoro methane sulfonic acid (TFMS) treatment of proteins under totally anhydrous conditions completely cleaves O-glycosyl groups as well as most N-acetyl glycosyl groups whilst leaving the protein backbone intact (Edge et al., 1981).

Figure 4.12 shows and SDS-polyacrylamide gel of core proteins before and after deglycosylation using TFMS acid (method 4.3). Deglycosylation of a standard glycoprotein ovomucoid (Lanes 1 and 2) under identical conditions was used as a control to monitor deglycosylation. Absolutely no change in molecular weight of the core proteins was detected after TFMS acid treatment, indicating that core proteins are not glycosylated.

2.3 Antibodies to Rat Liver Core Proteins

Antibodies can provide a tool to investigate the structural relationship between closely related proteins. Antibodies to total core proteins from rat liver were thus raised in rabbits with little difficulty. This despite the report by Martin and Okamura (1981) that effective titres of antibodies to mouse hnRNP polypeptides could not be raised in rabbit but only in chicken, because of the conserved nature of the core proteins. Figure 4.13 shows an immunoblot (method 5.3) where the antisera to total core proteins recognise core proteins from nuclear extracts. The antibodies also recognise two other groups of proteins of higher molecular weight. These groups of bands which show considerable resemblance in arrangement and spacing to the core proteins have been referred to in section 1.1 and appear to be SDS-resistant core protein aggregates. High molecular weight

Figure 4.12

Deglycosylation of Total Core Proteins

Total hnRNP proteins, core proteins and a standard glycosylated protein ovomucoid were deglycosylated with trifluoromethane sulphonic acid and the proteins recovered as dry precipitates as described in method 4.3. The protein precipitates were dissolves in sample buffer and subjected to electrophoresis on a 10% polyacrylamide SDS gel (see method, section 3 and 3.2). The gel was stained with Coomassie Blue.

Lane 1	50µg ovomucoid
Lane 2	Ovomucoid after deglycosylation
Lane 3	30µg total hnRNP proteins
Lane 4	hnRNP proteins after deglycosylation
Lane 5	50µg total core proteins
Lane 6	Core proteins after deglycosylation

This result was reproducible in 2 experiments.

Fig. 4.12

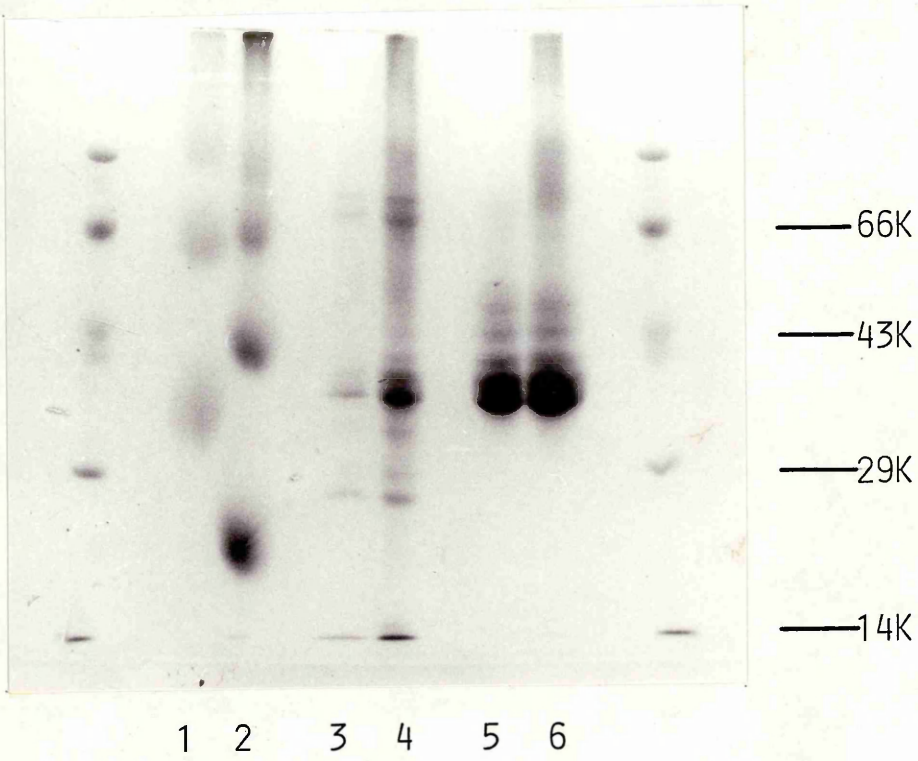
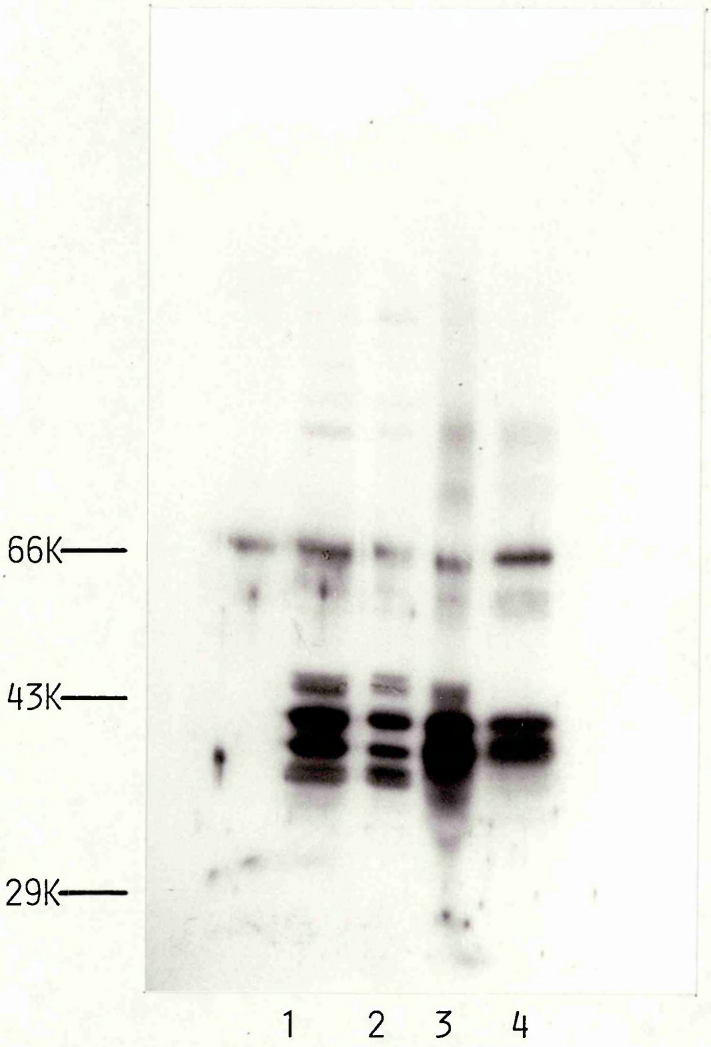


Figure 4.13

Immunoblot of hnRNP Particle with Polyclonal Antisera to Total Core Proteins

Core proteins at different stages of purification were run on a 8.75% polyacrylamide gel. Tracks on the gel were of: lane 1, 100 μg of hnRNP particle proteins obtained after extracting nuclei at elevated pH (method 1.2.1); lane 2, 100 μg of hnRNP particle proteins recovered after sonicating rat liver nuclei (method 1.2.2); lane 3, 100 μg purified hnRNP particle proteins from sucrose density gradients (method 1.3.1) and lane 4, 50 μg total core proteins from a 8.75% preparative gel (method 3.1.1). The proteins in the polyacrylamide gel were blotted onto nitrocellulose, incubated with antisera and iodinated protein A as described in methods 5.3 and 5.1.1. The dried nitrocellulose was then autoradiographed for 2 days wth a Kodak X-omat film and Dupont intensifying screen.

Fig. 4.13



oligmers of core proteins have been reported before (Leser et al., 1984). Once it was ascertained that antibodies to core proteins could be raised in rabbit, attempts were made to raise antibodies to the individual core proteins. Core proteins A and B were cut out of preparative slab gels (method 3.1.2), and used to prepare separate antibody preparations (method 5.1.2). The immunoblots obtained from antisera to core proteins A and B are shown in figure 4.14. The individual antisera were of low titre. Both were non-specific in that each cross-reacted with both A and B and possibly with C and D, though the sensitivity was too low to be sure that these less abundant proteins interacted. At this time, the Cleveland map data that was being generated indicated that it was probably futile to hope for a polyclonal antibody specific for one core protein and it was considered that specific monoclonal antibodies would prove insufficiently valuable to justify the work of raising them. Subsequent to this work, Leser et al. (1984) have produced monoclonal antibodies to mouse core proteins and despite the fact that they will recognise different epitopes, most of them cross-react with all of the core proteins in this species. Work with antibodies to hnRNP core proteins was thus discontinued.

3.0 STUDY OF THE CHARGE ISOMERS OF THE CORE PROTEINS

3.1 Charge Heterogeneity of the Core Proteins

Each of the four rat liver hnRNP core proteins seen on SDS-polyacrylamide gels, resolve into a series of spots on a pH gradient (Suria and Liew, 1979; Wilks and Knowler, 1980; Comings and Peters, 1981). Most of the spots form a cluster around a

Figure 4.14

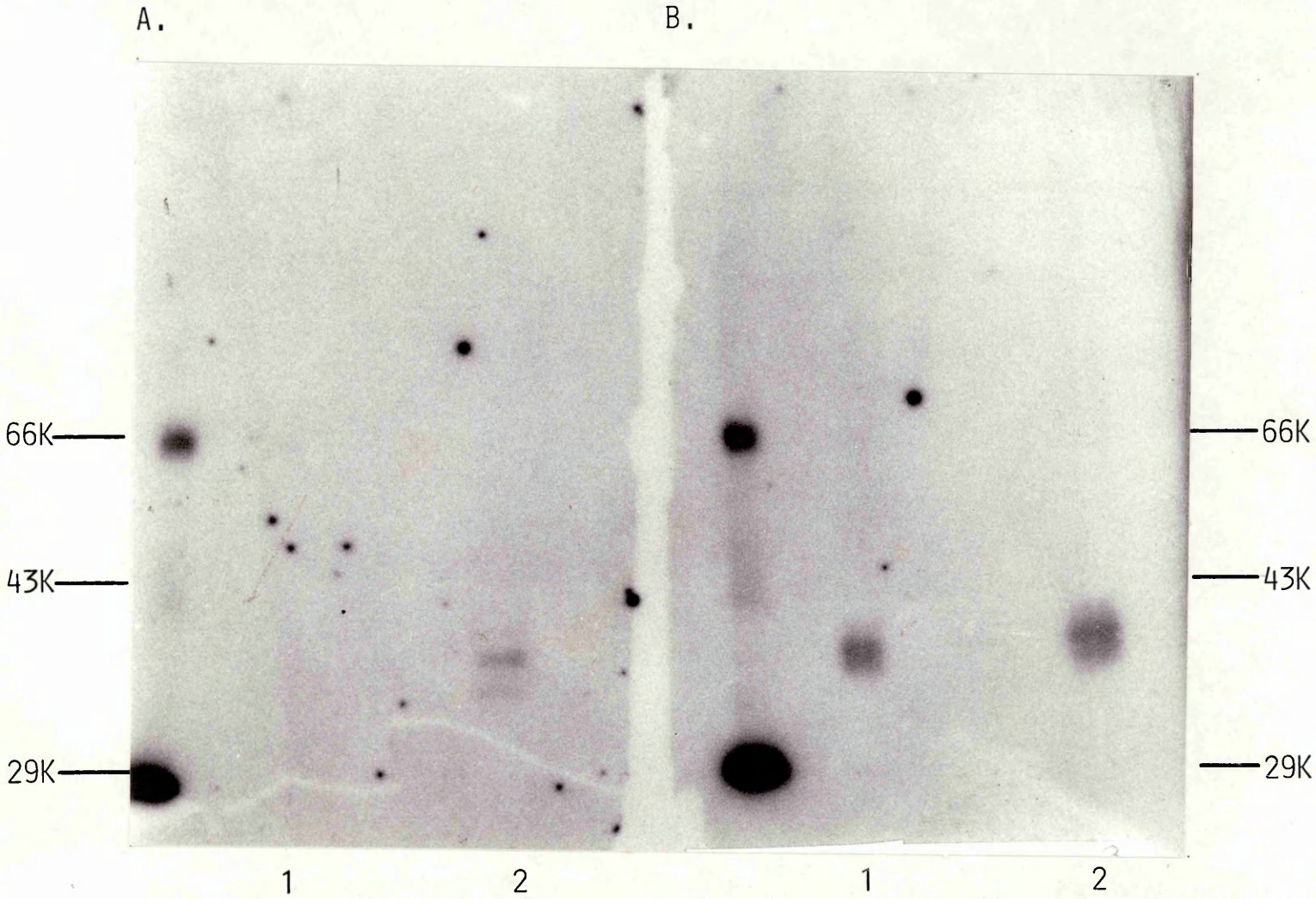
Immunoblot of Core Proteins with Polyclonal Antisera to Core Protein A and Core Protein B

One hundred µg hnRNP particle proteins after extraction from nuclei at elevated pH (lane 1) (method 1.2.1) and 50 µg total core proteins eluted from a preparative gel (lane 2) (method 3.1.1) were run on a 8.75% gel, blotted onto nitrocellulose and incubated with antisera against core protein A and core protein B, as described in methods 5.3 and 5.1.2. After incubation of the nitrocellulose with I^{125}-protein A, it was dried and autoradiographed.

A. Autoradiograph of protein blot incubated with antisera against core protein A.

B. Autoradiograph of protein blot incubated with antisera against core protein B.

Fig. 4.14



marker with isoelectric point of 8.1 but one string of spots spreads between markers of pI 6.7 and 6.1 (Peters and Comings, 1980; Comings and Peters, 1981). Figure 4.15 shows a 2-dimensional fractionation of the core proteins. The first dimensional separation was by non-equilibrated pH gradient electrophoresis (NEPHGE) and the second by SDS-polyacrylamide electrophoresis (O'Farrell et al., 1977). This system of fractionation is better suited to the basic core proteins than a combination of Isoelectric Focusing and SDS-polyacrylamide electrophoresis (Wilks and Knowler, 1980). Nomenclature for the charge isomers of the core proteins in figure 4.15 is that of Wilks and Knowler (1981a). Whether the charge heterogeneity of the core proteins is due to different polypeptides with the same molecular weight or due to post-translational modification of the same polypeptide is not known. There are reports that core proteins may undergo postsynthetic modifications such as phosphorylation (Karn et al., 1977; Holcomb and Friedman, 1984; Wilk et al., 1985), ADP-ribosylation (Kostka and Schweiger, 1982) and glycosylation (Fuchs et al., 1980). That the core proteins are not glycosylated was shown in the last section (Results 2.2). Hence the charge heterogeneity cannot be due to glycosylation.

Before experimentally tackling the problem of what causes the charge heterogeneity of the core proteins, a few control experiments were performed to rule against the possibility that the charge isomers were artefactual. Sample buffers (referred to as lysis buffer) for Isoelectric Focusing or NEPHGE of core proteins contain 9.5 M urea (O'Farrell, 1975; O'Farrell et al., 1977) (See method, section 3). Urea is prone to degradation

Figure 4.15

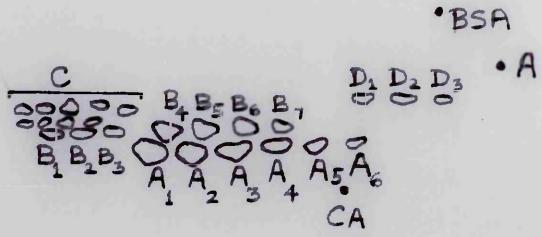
Charge Isomers of the Core Proteins on Two-Dimensional Gels

hnRNP particles were extracted from Rat Liver nuclei (method 1.2.1) and also prepared from the lysed nuclei of Buffalo Rat Liver Cells (method 1.2.2). The particles from both sources were purified on sucrose density gradients (method 1.3.1). Sucrose was removed from the particle preparations by dialysis against 10 mM Tris.HCl, pH 7.6 containing 10 mM $MgCl_2$ and particle proteins were precipitated by acetone. The proteins were dissolved in lysis buffer (see method, section 3) and subjected to 2-dimensional electrophoresis as described in method 3.3.

A. Two dimensional analysis of rat liver hnRNP protein (300 μg). The overlay indicates the numbering system of Wilks and Knowler (1981a) used throughout the remainder of this thesis. It also indicates the position of the marker proteins that were used to derive the molecular weights and isoelectric points (IpH) plotted on the ordinate and abscissa of the figures. A = actin, BSA = bovine serum albumin, CA = carbonic anhydrase, SIn = Soybean Trypsin inhibitor.

   To the right of the 2 D-gel, a one dimensional gel containing hnRNP proteins (40 μg) is shown for comparison. This has been marked as 1D.

B. Two dimensional analysis of Buffalo Rat Liver cell hnRNP protein (500 μg).



SIn

BSA

A

CA

Fig. 4.15 A

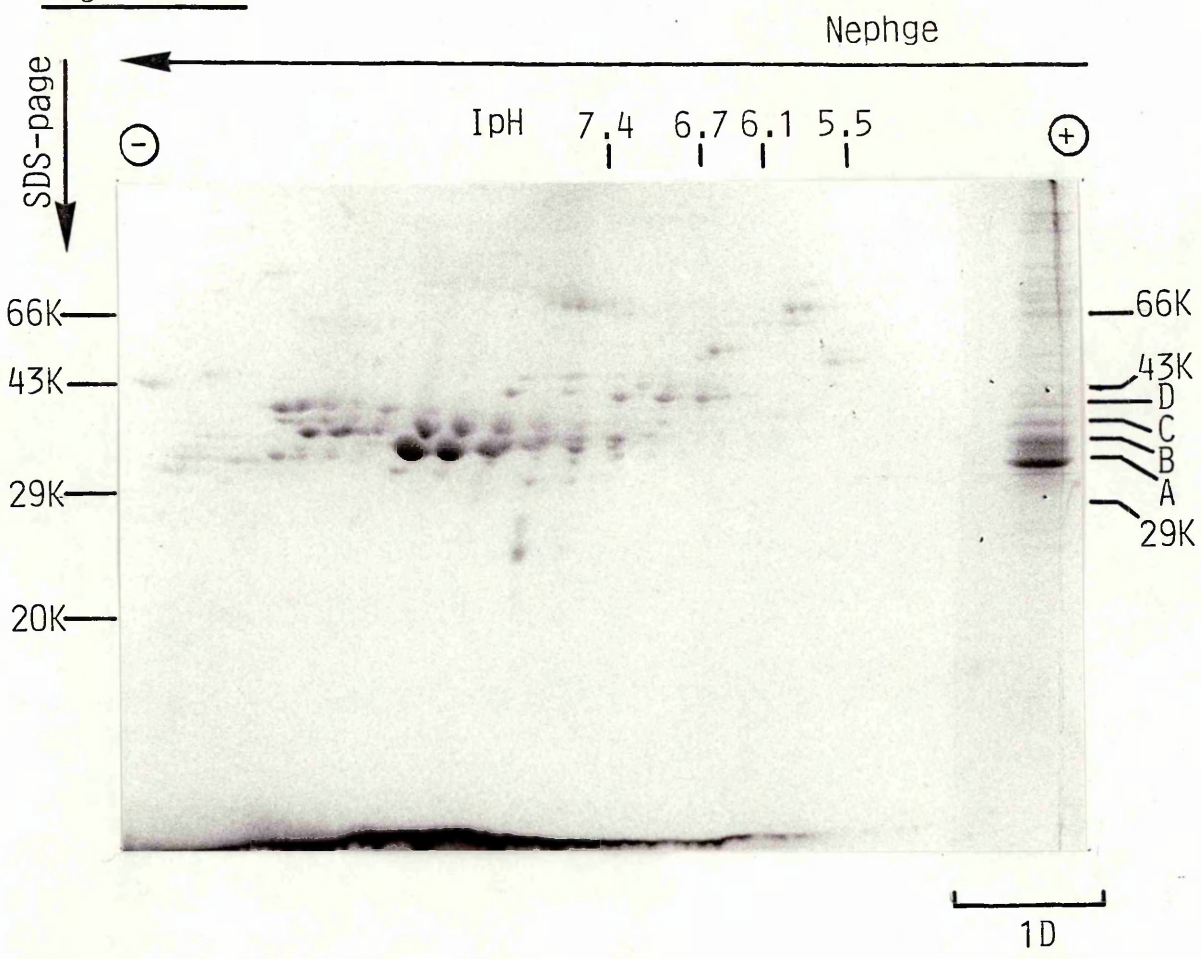
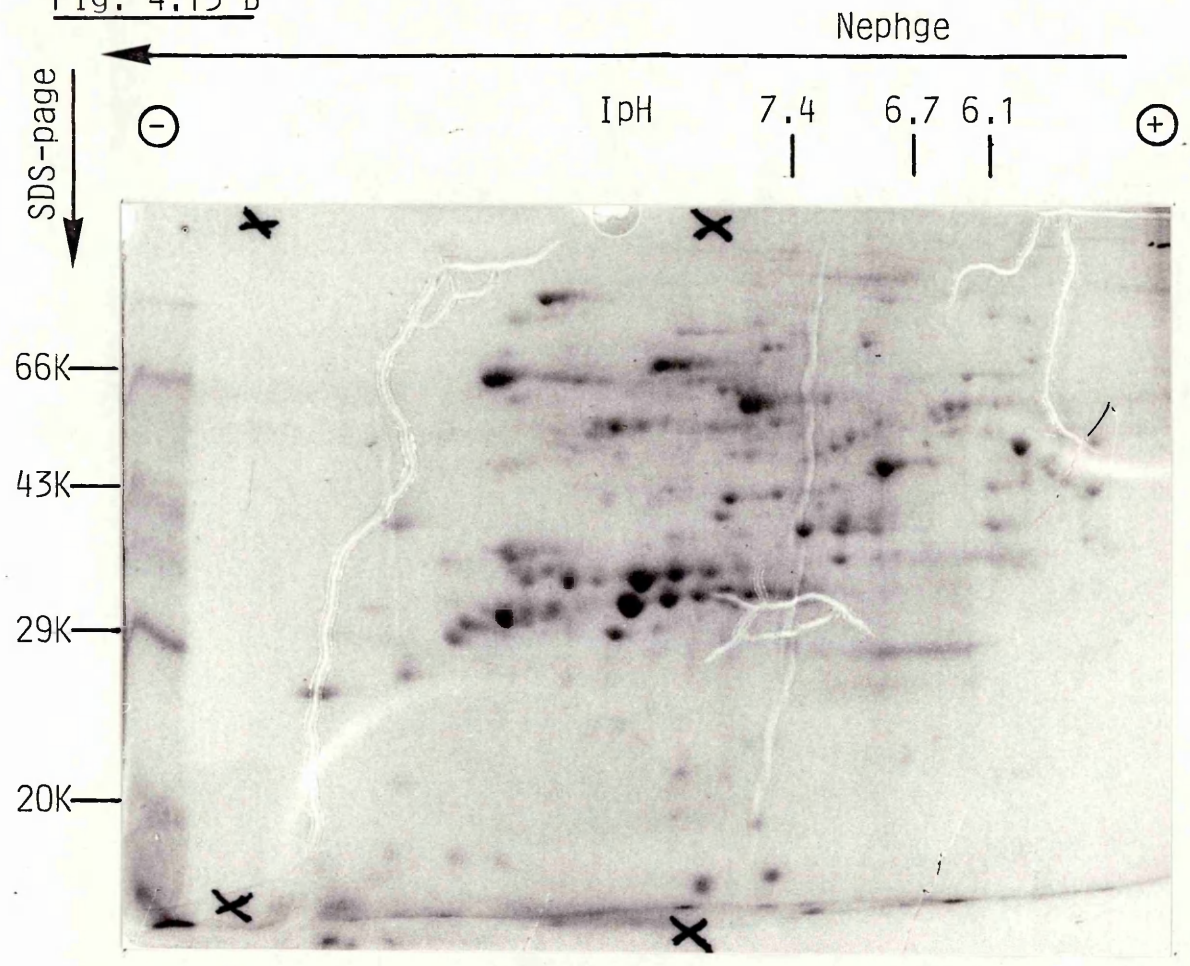


Fig. 4.15 B



forming cyanate ions which can carbamylate lysine residues of a protein (Peterson, 1972) and hence cause charge isomers. Figure 4.16 compares enlargements of the core protein areas of 2-dimensional gel separation using 3 kinds of lysis buffers. In gel A, hnRNP particles were dissolved in lysis buffer which was made up with freshly recrystallised AnalaR urea. For gel B, hnRNP particles were dissolved in lysis buffer which had been repeatedly frozen and thawed over a period of 2 months; and for gel C, the lysis buffer used was freshly made using AristaR urea. As can be seen the charge pattern of the core proteins is exactly the same. It thus seems unlikely that urea degradation gives rise to the charge isomers.

Another possible artefactual cause of charge isomers is deamidation. hnRNP particles were extracted at pH8 from purified nuclei (Samarina et al., 1968). High pH can cause deamidation of asparagine and glutamine residues and cause charge heterogeneity in a protein (Funakoshi and Deutsch, 1969). However such effects are most commonly observed at much higher pH than pH8. Wilks (1980) has shown 2-dimensional fractionation of extracted particles at pH8 and of nuclear extracts after sonication of nuclei at pH7. The charge isomeric pattern obtained was identical and the same as the normal pattern obtained for purified hnRNP particles from sucrose density gradients by many workers (Suria and Liew, 1979; Peters and Comings, 1980). This characteristic charge pattern for the hnRNP proteins, that is the core proteins reproduces in figures 4.15 and 4.16.

It remains possible that deamidation or a related artefact is a product of isoelectric focusing or NEPHGE, as the

Figure 4.16

Two-Dimensional Gel Electrophoresis of Core Proteins in Different Lysis Buffers

hnRNP particle proteins from sucrose density gradients were dialysed against 10 mM Tris.HCl, pH 7.6 containing 10 mM $MgCl_2$. Proteins were acetone precipitated. Ten µg of hnRNP proteins were separately dissolved in 3 different lysis buffers and all three subjected to 2-dimensional electrophoresis as described in method 3.3. The gels were silver-stained. Enlargements of the core protein regions of the gels are shown.

A. Proteins were dissolved in lysis buffer made with freshly recrystallised AnalaR urea.

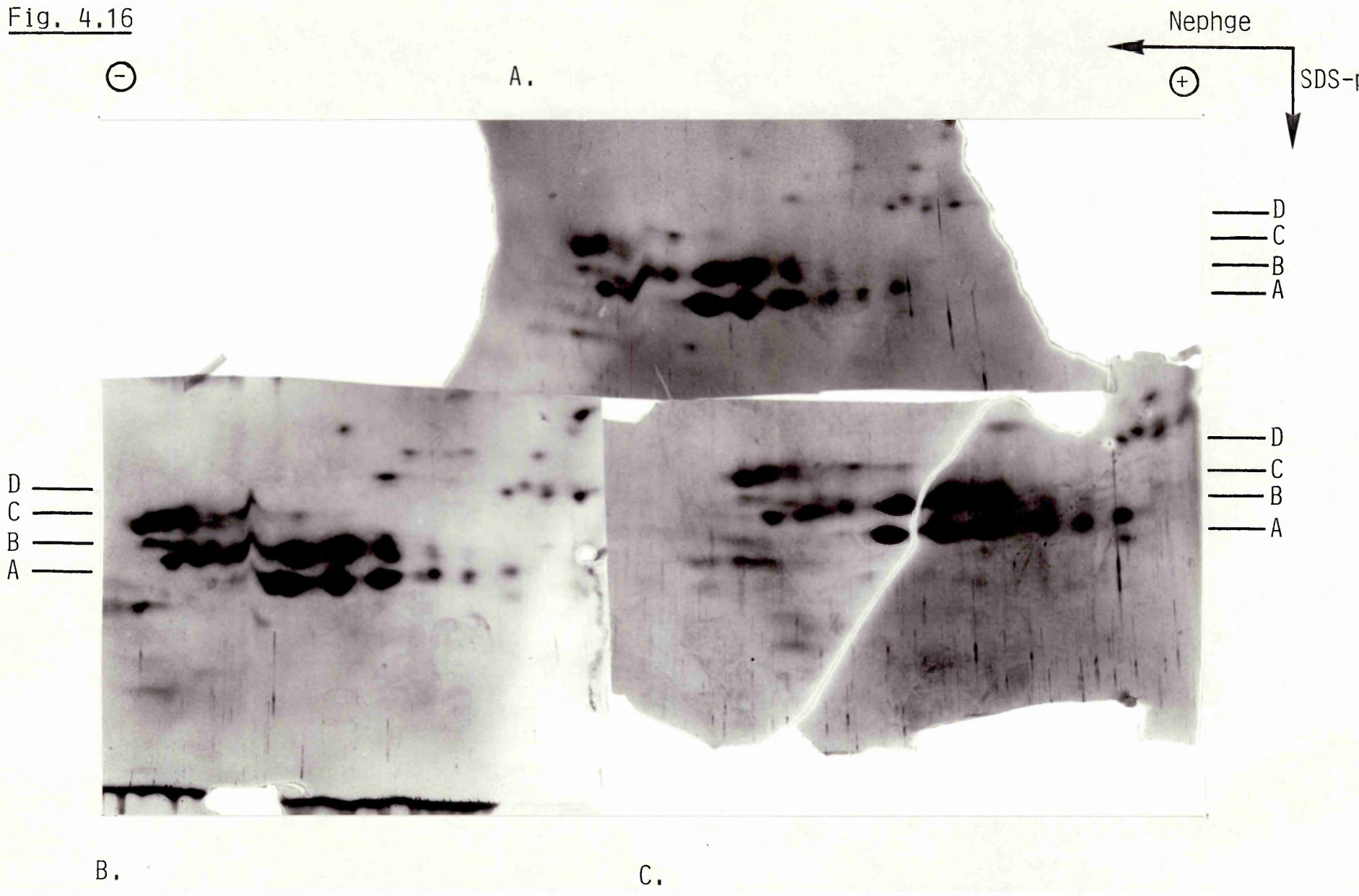
B. Proteins were dissolved in lysis buffer which had been repeatedly thawed and frozen over a period of 2 months.

C. Proteins were dissolved in freshly made lysis buffer using AristaR urea which had been stored at room temperature.

Position of core proteins A, B, C and D have been marked. Migration of proteins in the 1st dimension by non-equilibrated pH gradient gel electrophoresis (NEPHGE) and 2nd dimension by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) has been shown by arrows.

This result was reproducible in 3 experiments.

Fig. 4.16



proteins are by definition moving through a pH gradient. That this might lead to deamidation is difficult to exclude and no satisfactory methods for the measurement of deamidation have been published. It can be assumed however, that if a chain of charge isomers is created by deamidation, then the more basic spots are less deamidated. If therefore such spots are recovered and resubmitted to charge gradient electrophoresis, some of the polypeptide should be further deamidated and give rise to further heterogeneity. This is not observed as shown in figure 4.17 where core proteins A_2 and A_3 are seen to rechromatograph as single entities. Core protein A_1 however seems to rechromatograph as A_1 and a more acidic species. It is difficult to ascertain why A_1 produces two spots on refocusing while A_2 and A_3 refocus as single entities.

3.2 Cleveland Mapping of the Charge Isomers of Core Proteins

The relationship between polypeptides of the same molecular weight but of different charge observed on 2-dimensional gels of core proteins was investigated wtih the help of Cleveland mapping (Cleveland et al., 1977). In order to ease the detection of the digestion products of the charge isomers, core proteins A and B were extracted and iodinated in the tyrosine residues (see method 4.1 and 4.4). Core protein A was then subjected to 2-dimensional electrophoresis, charge isomers A_1, A_2, A_3 and A_4 were cut out and subjected to Cleveland mapping (method 4.5). Figure 4.18 shows the digestion pattern of the charge isomers using S. aureus V8 protease on a 15% polyacrylamide-SDS gel. The digestion pattern of the charge isomers is near identical. However since

Figure 4.17

Rerunning Focused Spots of the Charge Isomers of Core Protein A on 2-Dimensional Gels

Core proteins A_1, A_2 and A_3 were cut from 2-dimensional gels (see Figure 4.15) and extracted as described in method 4.4. Each of the three 50 µl protein extracts were made 9.5 M with respect to AristaR urea and 2% with respect to ampholines (comprising 1.6% of pH range 5-7 and 0.4% of pH range 3.5-10). The proteins A_1, A_2 and A_3 were then separately subjected to 2-dimensional electrophoresis as described in method 3.3. 1, 2 and 3 show re-electrophoresis of A_1, A_2 and A_3 respectively. Position of A_1, A_2 and A_3 been indicated. The other spots are those of isoelectric points and molecular weight markers.

This result was reproducible in 2 experiments.

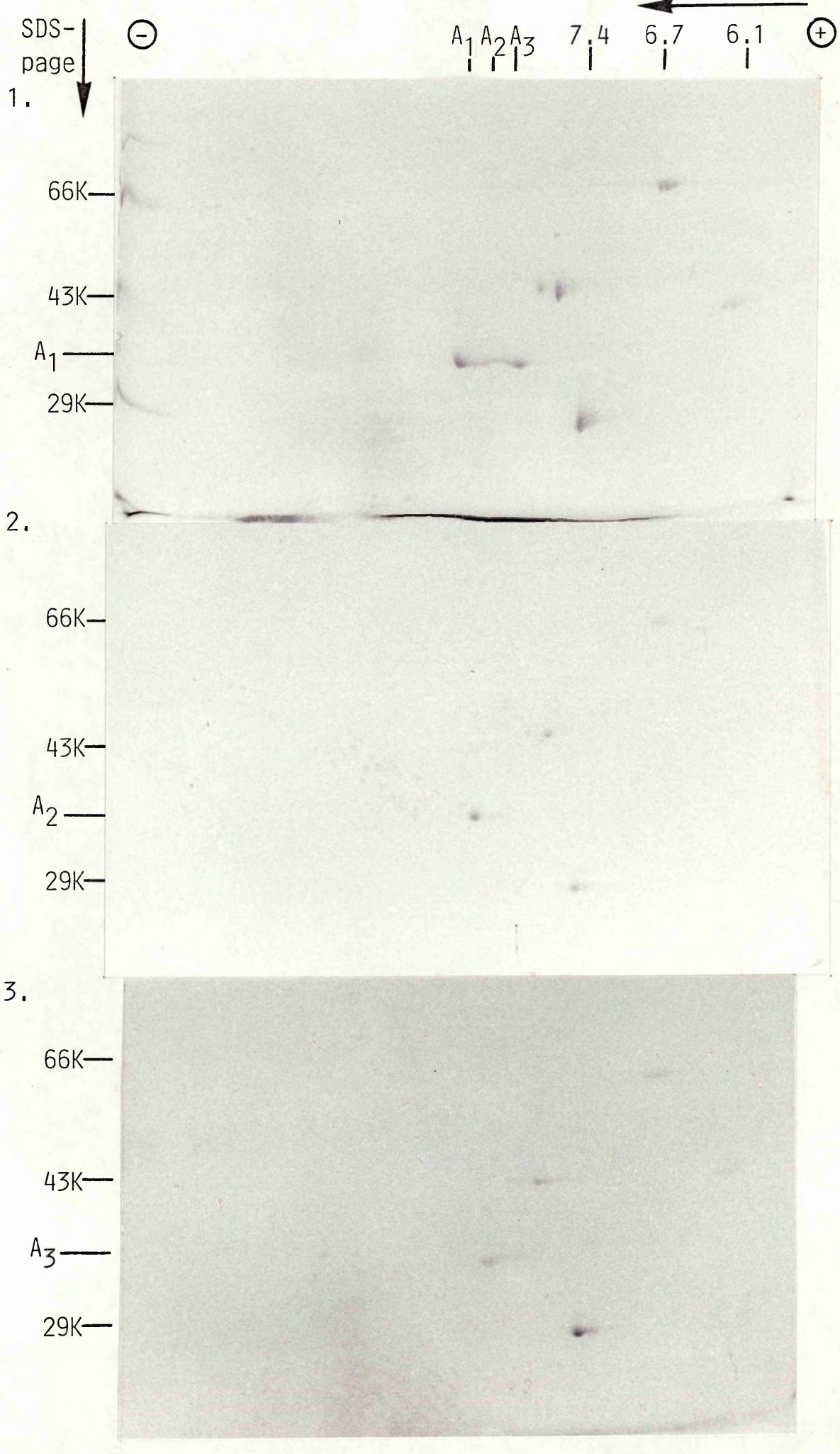

SDS-
page
⊖
A1 A2 A3
7.4
6.7
6.1
⊕
1.
66K
43K
A1
29K
2.
66K
43K
A2
29K
3.
66K
43K
A3
29K

Figure 4.18

Partial Peptide Mapping of the Iodinated Charge Isomers of Core Protein A

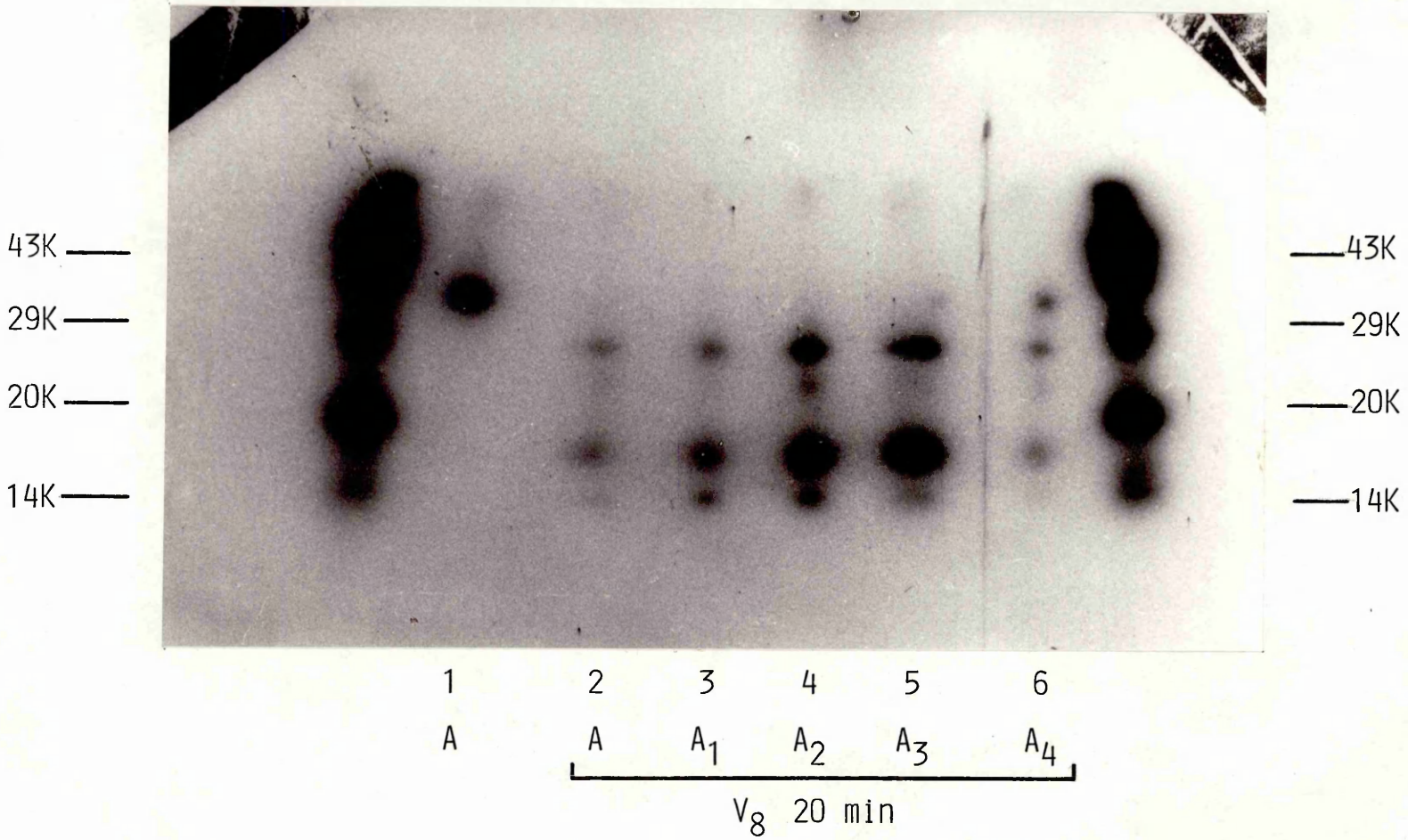
Core protein A was extracted from a preparative slab gel (method 3.1.2 and 4.4), iodinated and recovered as a precipitate (method 4.1). The protein was subjected to 2-dimensional electrophoresis (method 3.3) and its charge isomers namely A_1, A_2, A_3 and A_4 (see Figure 4.15) were cut out and extracted in 40 µl of extraction buffer (see method 4.4). A band of total core protein A was similarly extracted from a one-dimensional SDS-polyacrylamide gel. The proteins were then digested with V8 protease and the products of digestion analysed on a 15% polyacrylamide gel. After electrophoresis the gel was dried and autoradiographed.

Lane 1: Core protein A, extracted and re-electrophoresed.

Lanes 2-6: Core proteins A, A_1, A_2, A_3 and A_4 after digestion with 5 µg V8 protease.

This result was reproducible in 5 experiments.

Fig. 4.18



only few digestion products were obtained, comparison of the peptide pattern is not very meaningful. Digestion products are seen to be few probably because there are not many tyrosine residues in core protein A (Le Stourgeon et al., 1978).

Contrary to initial indications, it seemed probable that Cleveland mapping combined with silver-staining was sufficiently sensitive to examine the charge isomers without their iodination at least for proteins A and B. The charge isomers needed to be extracted from 2-dimensional polyacrylamide gels first and figure 4.19 shows that the extraction procedure used (method 4.4) did not affect the general charge pattern of core protein A. Proteins extracted from gels did however require higher levels of V8 protease than those eluted from preparative gels. Because of this, in the Cleveland maps of the charged isomers of core proteins A and B (figures 4.20 - 4.24) some of the most intense bands arise from V8 protease and care must be taken over the interpretation of the often fainter bands arising from the core proteins. Figure 4.20 and 4.21 compares digestion products of the charge isomers of core protein A. Bearing in mind that some of the most intense bands belong to V8 protease, A_1, A_2 and A_3 seem identical, except perhaps one small peptide of about 17 K which seems more intense in case of the products of A_3 after 20 min digestion with V8 protease (figure 4.21). Comparison of digestion products of A_2 with A_4, A_5 and A_6 (figure 4.20) reveals one peptide of about 17 K which is present in digestion patterns of A_4, A_5 and A_6 but is absent from digestion products of A_2. Another peptide of about 12 K is more intense in patterns of A_4, A_5 and A_6 when compared with pattern of A_2 (figure 4.20).

Figure 4.19

Two Dimensional Electrophoresis of Core Protein A After Extracting it From a SDS-Polyacrylamide Gel

Core protein A was extracted from a preparative slab gel (method 4.4). The protein was acetone-precipitated and subjected to 2-dimensional electrophoresis (method 3.3). The gel was stained with Coomassie blue. Position of the charge isomers of core protein A are marked on the bottom of the gel and the numbers on top of the gel are the iselectric points of marker proteins (see Figure 4.15). See Figure 4.16 for details of the other labels.

This result was reproducible in 2 experiments.

Fig. 4.19

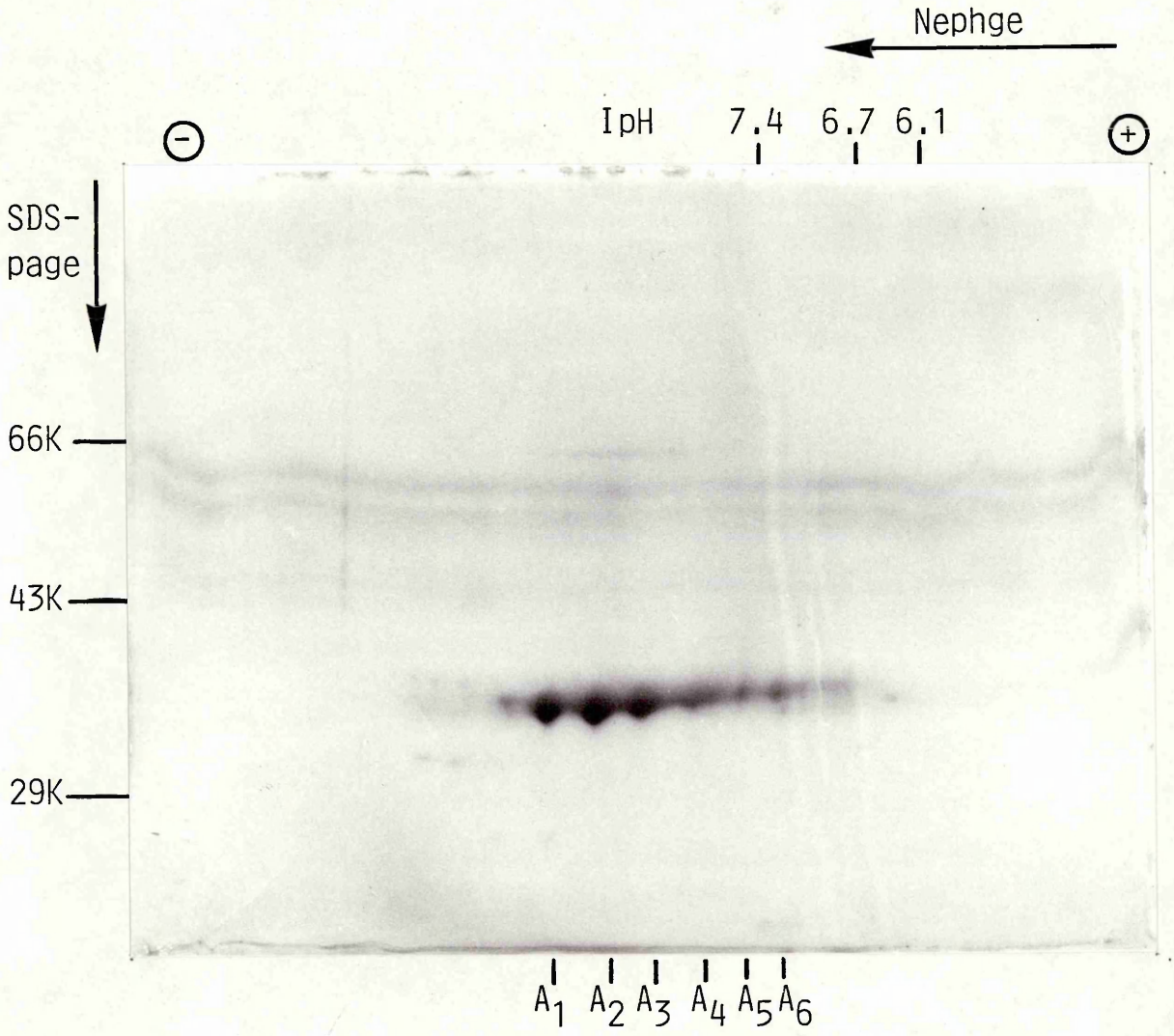


Figure 4.20

Partial Peptide Mapping of Core Protein A and Its Charge Isomers A_2, A_4 A_5 and A_6

hnRNP particle proteins from sucrose density gradients (method 1.3.1) were dialysed against 10 mM Tris.HCl containg 10 mM $MgCl_2$. One mg protein was precipitated with acetone and subjected to 2-dimensional electrophoresis (method 3.3). Gels were stained briefly and destained. Charge isomers of core protein A and B (see Figure 4.15) were cut out and extracted (method 4.4). Twenty to 30 µg of each of the charge isomers were obtained in 50 µl of extraction buffer. Core proteins A and B were also extracted from SDS-polyacrylamide gels. In each case, 2 to 3 bands of the respective protein was used for extracting. The proteins were digested with 5 µg V8 protease and reaction stopped with 10% 2-mercaptoethanol and 2% SDS (method 4.5). The products of digestion were analysed by 15% polyacrylamide SDS-gels. The gels were then silver-stained (method 4.6.1).

Lanes 1-5: 1 µg each of proteins A, A_2, A_4, A_5 and A_6 before digestion.

Lanes 6-10: 12 to 14 µg each of proteins A, A_2, A_4-A_6 after digestion with 5 µg V8 protease for 10 min.

Lanes 11-14: 12 to 14 µg each of proteins A, A_2, A_4-A_6 after digestion with 5 µg V8 protease for 20 min.

Lane 15: 5 µg V8 protease incubated on its own after 20 min. 2-mercaptoethanol and SDS were added as above.

Fig. 4.20

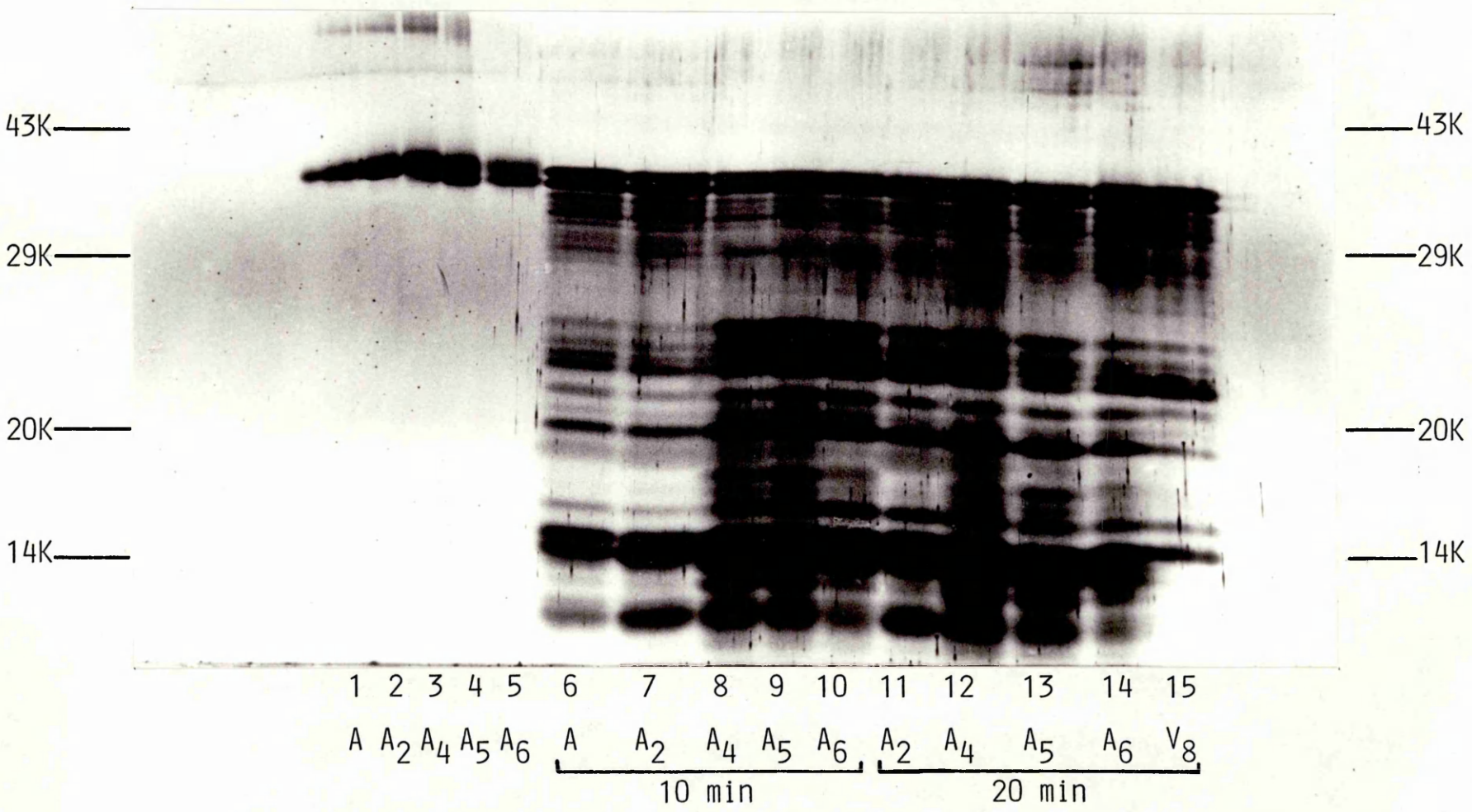

43K
29K
20K
14K
43K
29K
20K
14K
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
A A2 A4 A5 A6 A A2 A4 A5 A6 A2 A4 A5 A6 V8
10 min
20 min

Figure 4.21

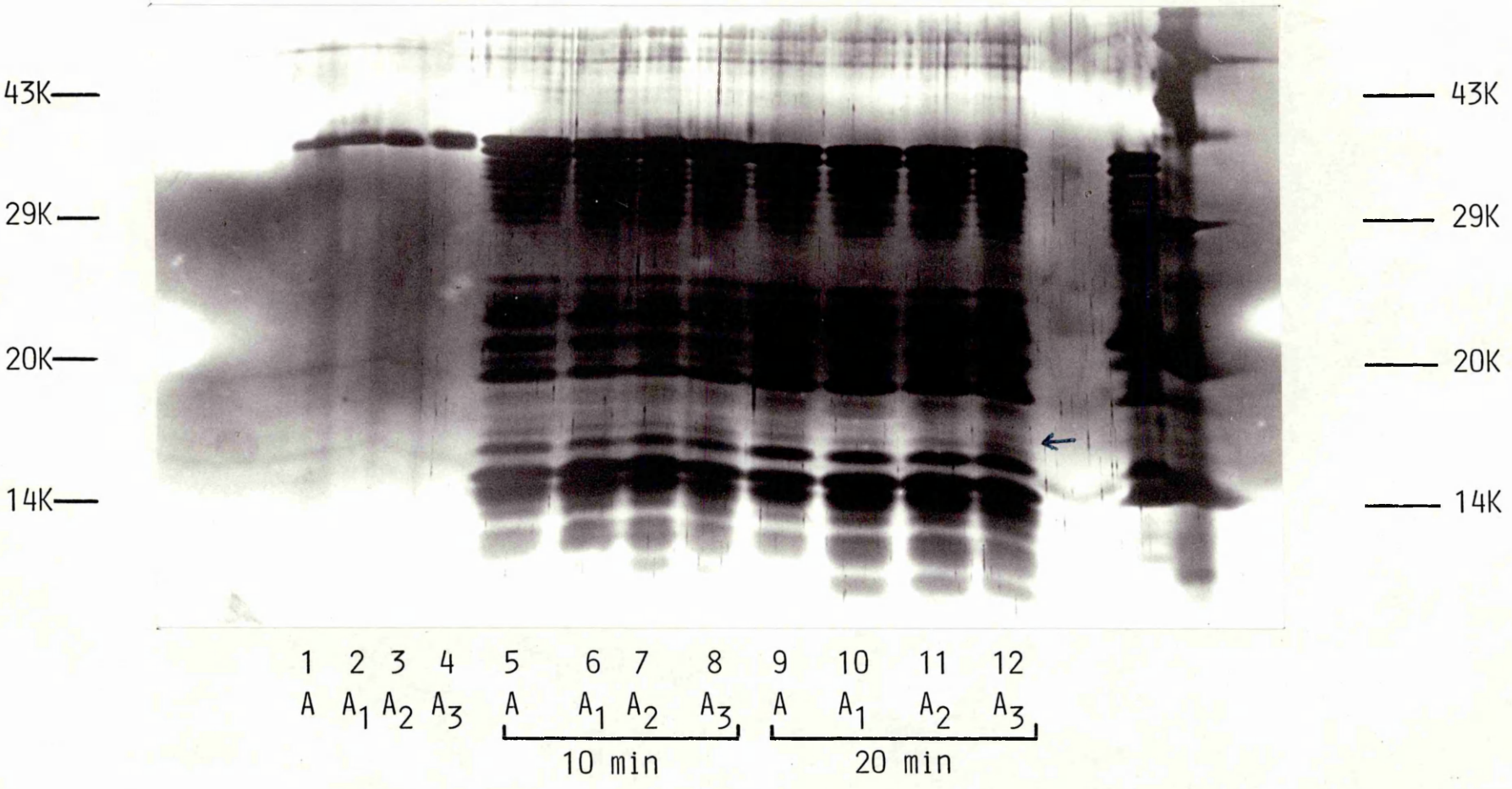
Partial Peptide Mapping of the Charge Isomers of Core Protein A, A_1, A_2 and A_3

See legend to Figure 4.20 for experimental details and labelling used.

This result was reproducible in 2 experiments.

The bands at about 66K are probably artefacts (see legend to fig 4.7).

Fig. 4.21


43K
29K
20K
14K
43K
29K
20K
14K
1 2 3 4 5 6 7 8 9 10 11 12
A A1 A2 A3 A A1 A2 A3 A A1 A2 A3
10 min
20 min

When partial peptide maps of the core protein B charge isomers were compared (figures 4.22 - 4.24), B_1, B_2 and B_3 seemed near identical and so did B_4 and B_5, although V8 again contributes so much to the total pattern that core protein derived peptides are difficult to unambiguously identify. When peptide pattern of B_1 was compared to that of B_5 (Figure 4.24), a doublet of about 16-17 K was more intense in case of B_5. Also a peptide of about 14 K was present only in the digestion product of B_1.

To summarise the results of the partial peptide maps discussed above, the general pattern of the digestion products of the charge isomers of core protein A is similar to those of B. The digestion products of the acidic charge isomers of core protein A, from A_3 to A_6 contain a peptide of about 17 K which is absent from products of A_1 and A_2. Similarly, the digestion products of the acidic charge isomers of core protein B, B_4 and B_5 contain a doublet of 16-17 K absent from B_1 and B_2. Therefore it seems that the acidic species of the charge isomers of both core proteins A and B are less susceptible to digestion with V8 protease, since these contain one to two peptides of higher molecular weight in their digestion pattern than the basic species. This pattern of digestion could be due to a charge modification of the acidic species which makes digestion slower. This possibility seems stronger than the alternative probability of the peptide backbone being different because of the general similarity in the patterns of digestion of the acidic species of both core protein A and B. The charge isomers of the B series could however be charge variants of two proteins (figure 4.24) with the isomer B_1 to B_3 being derived from one protein and B_4 and B_5 from another.

As already pointed out, the figs. 4.20 and 4.21 are confused because of the difficulty of distinguishing V8 peptides

Figure 4.22

Partial Peptide Maping of the Charge Isomers of Core Protein B, B_1, B_2 and B_3

Experimental details exactly as described in legend to Figure 4.20 except that the experiment was with protein B and its charge derivatives. For explanation of labels also see Figure 4.20.

This result was reproducible in 2 experiments.

The bands at about 66K are probably artefacts (see legend to fig 4.7).

Fig. 4.22

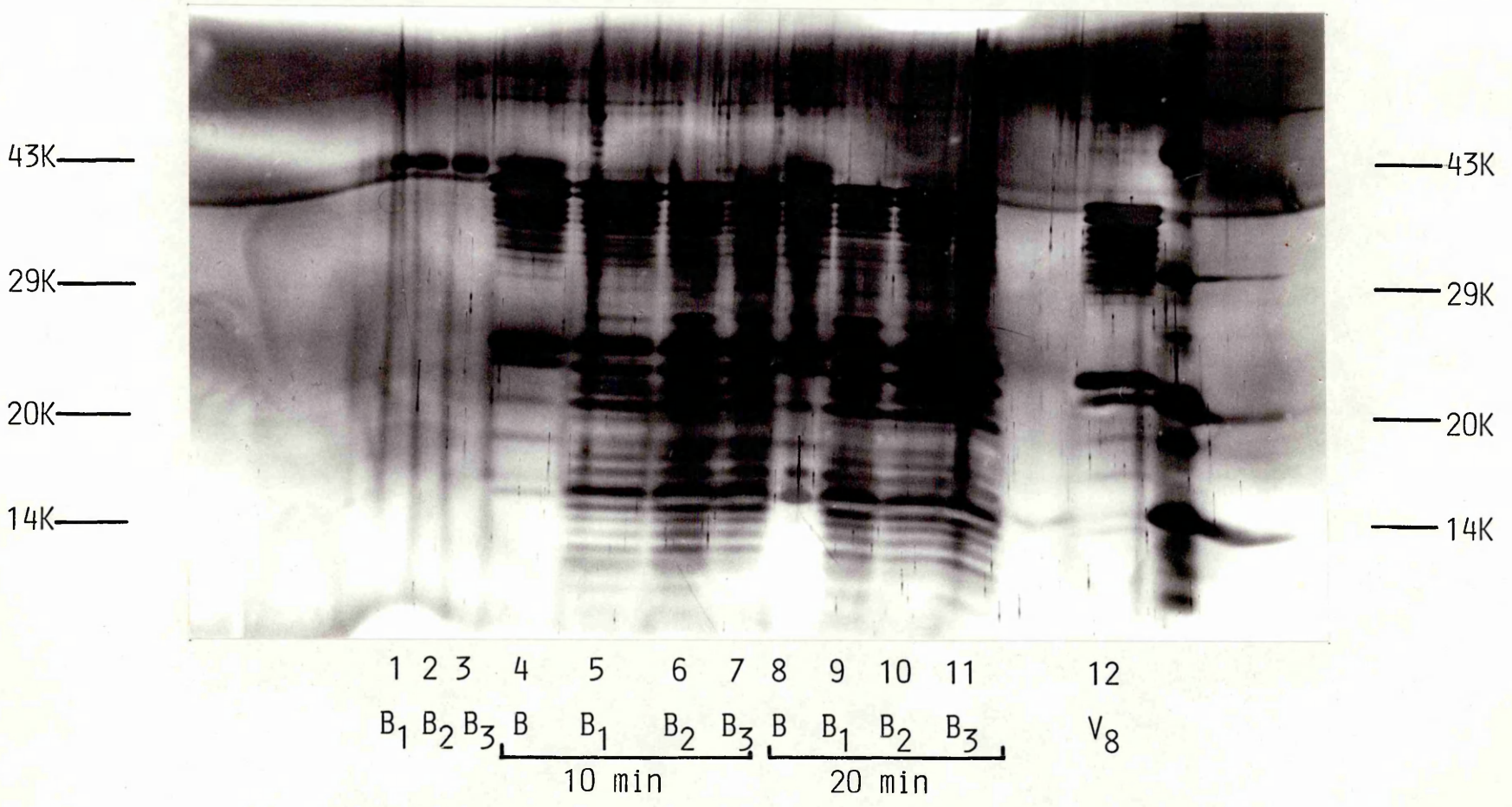

43K
29K
20K
14K
43K
29K
20K
14K
1 2 3 4 5 6 7 8 9 10 11 12
B1 B2 B3 B B1 B2 B3 B B1 B2 B3 V8
10 min
20 min

Figure 4.23

Partial Peptide Mapping of the Charge Isomers of Core Protein B, B_4 and B_5

See legend to Figure 4.22 for experimental details and labelling used.

This result was reproducible in 2 experiments.

The bands at about 66K are probably artefacts (see legend to fig 4.7).

Fig. 4.23

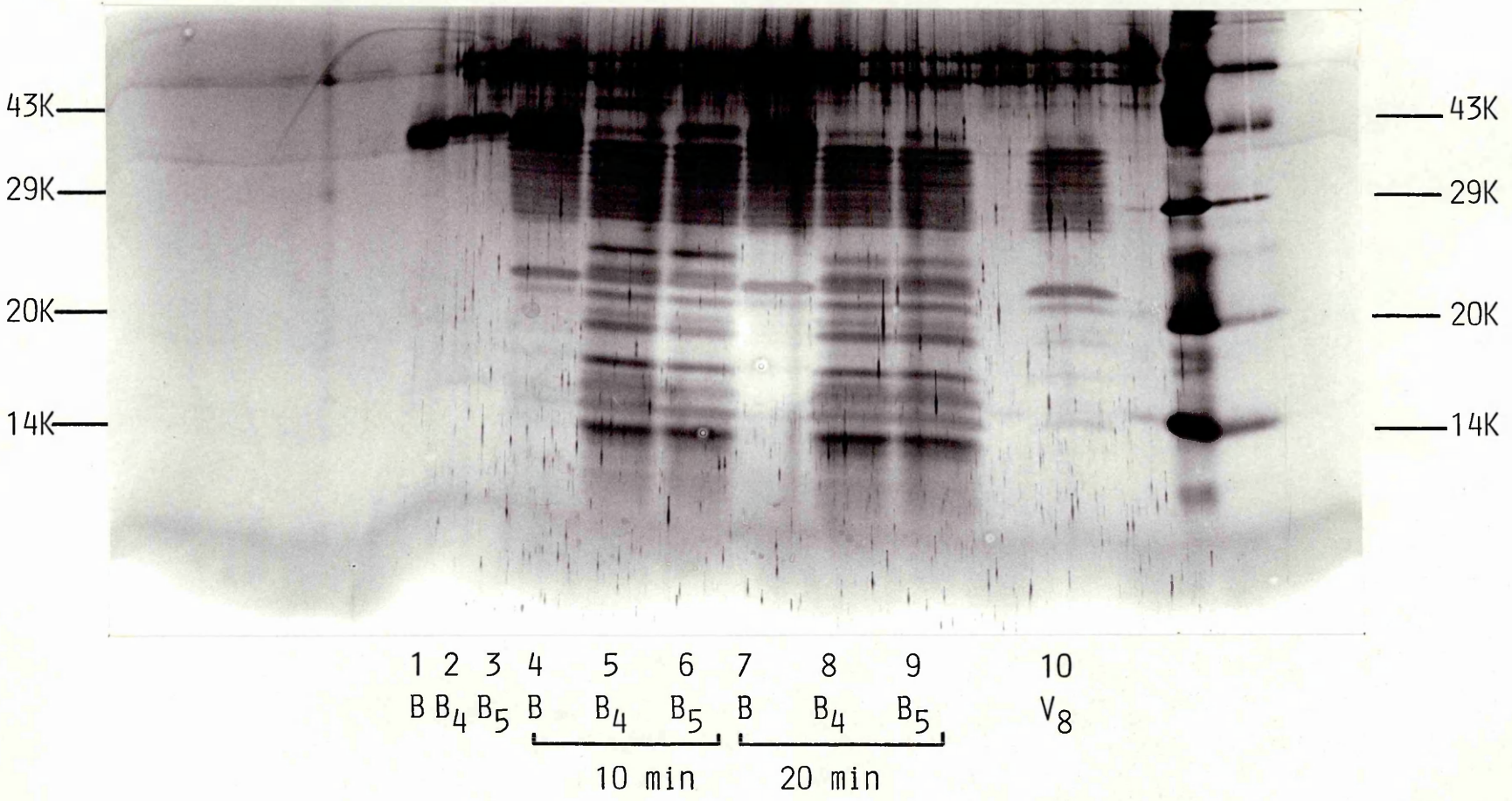

43K
29K
20K
14K
43K
29K
20K
14K
1 2 3 4 5 6 7 8 9 10
B B4 B5 B B4 B5 B B4 B5 V8
10 min
20 min

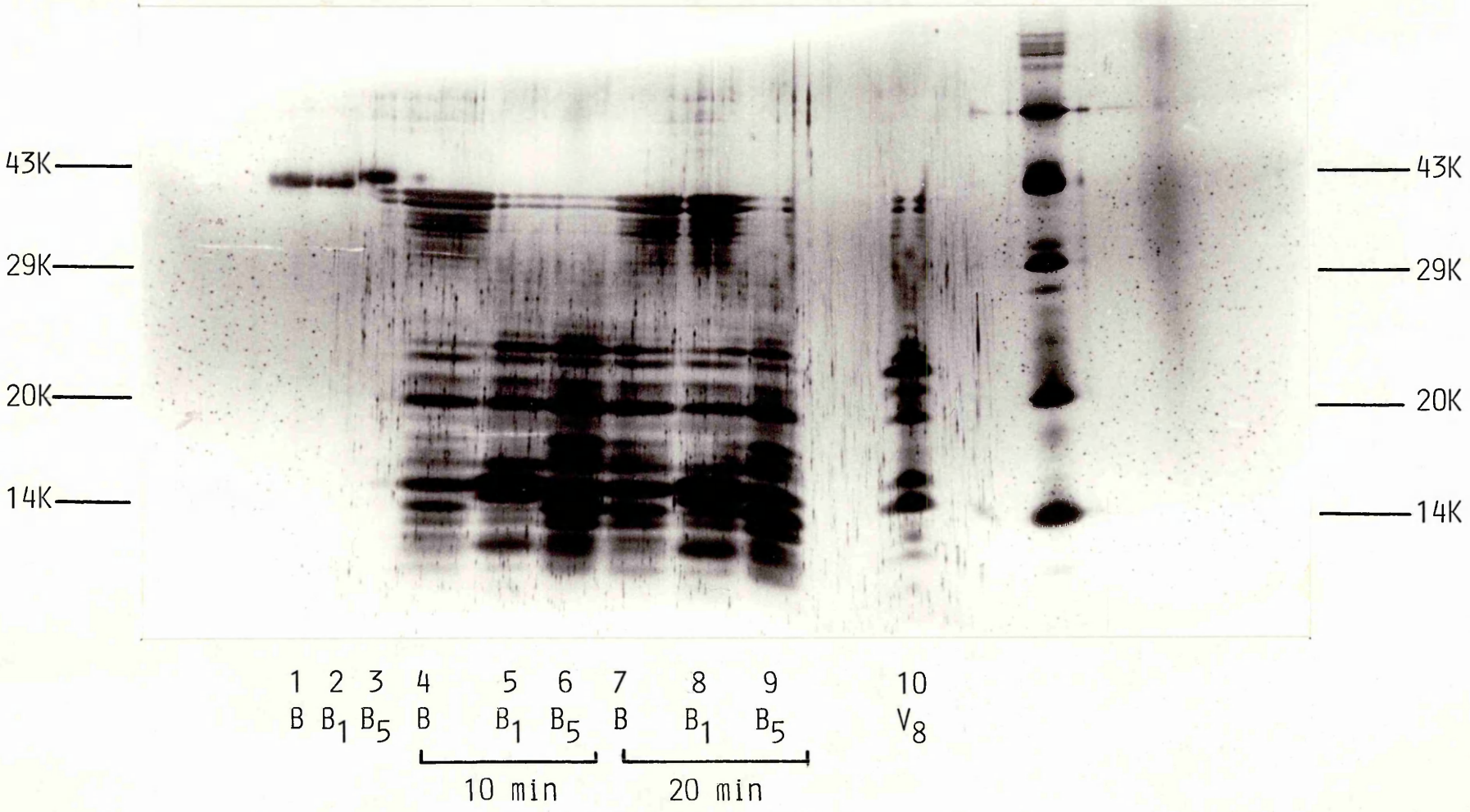
Figure 4.24

Partial Peptide Mapping of Core Protein B Charge Isomers, B_1 and B_5

Experimental details and labels as in legend to Figure 4.22.

This result was reproducible in 2 experiments.

Fig. 4.24


43K
29K
20K
14K
43K
29K
20K
14K
1 2 3 4 5 6 7 8 9 10
B B1 B5 B B1 B5 B B1 B5 V8
10 min
20 min

from protein A peptides. It was found however that, if the digestion products were fractionated on 2-dimensional gels, although the peptides then stained very weakly, those arising from V8 were all clumped at the acid end of the gel while those derived from A protein were more basic (figure 4.25). As seen in these 2-dimensional gels, the pattern of the digested peptides of A_1, A_2 and A_3 are very similar and reinforce the information of the 1-dimensional peptide maps of the same charge isomers (see figure 4.21). However, the peptide of about 17K which was present only in the one dimensional digestion products of A_3 and absent from A_1 and A_2 is not seen in figure 4.25. It is possible that this peptide was too acidic to enter the charge-gradient gel, although this possibility was not investigated. It was noted that in all the three 2-dimensional peptide maps there were 3 to 4 peptides of different charges of the same molecular weight of about 12-13K (spots numbered 1, 2 and 3 in figure 4.25). The same peptide spots are probably the ones seen as a dense broad band in the one-dimensional maps of A_1, A_2 and A_3 (figures 4.20 and 4.21). Why a 34K polypeptide of a particular isoelectric point should produce 3-4 peptides of 12-13K with different charges is open to question. It was also surprising to find that the peptide map of the most basic of the three polypeptides A_1 contained an acidic peptide which was absent from peptide maps of A_2 and A_3 (spot numbered 4 on the overlay of figure 4.25A). However, basic A_1 has been shown to be susceptible to charge modification after extraction and refocusing on a charge-gradient (figure 4.17). This acidic peptide of 12-13K present only in 2-dimensional map of A_1 may simply be the peptide portion of A_1 which has this susceptibility.

FIGURE 4.25

Partial Peptide Mapping of Core Protein A Charge Isomers A_1, A_2 and A_3 on 2-Dimensional Gels

Core protein A charge isomers A_1, A_2 and A_3 were digested with V8 protease exactly as described in legend to figure 4.20 (see also method 4.5). Reaction was stopped with 10% 2-mercaptoethanol and 2% SDS. The digestion products were then made 9.5M with respect to urea and 2% with respect to ampholines (1.6% of pH range 5-7 and 0.4% of pH range 3.5-10). They were then subjected to 2-dimensional gel electrophoresis (method 3.3). The gels were silver-stained by the method of Wray et al., (1981).

Key:

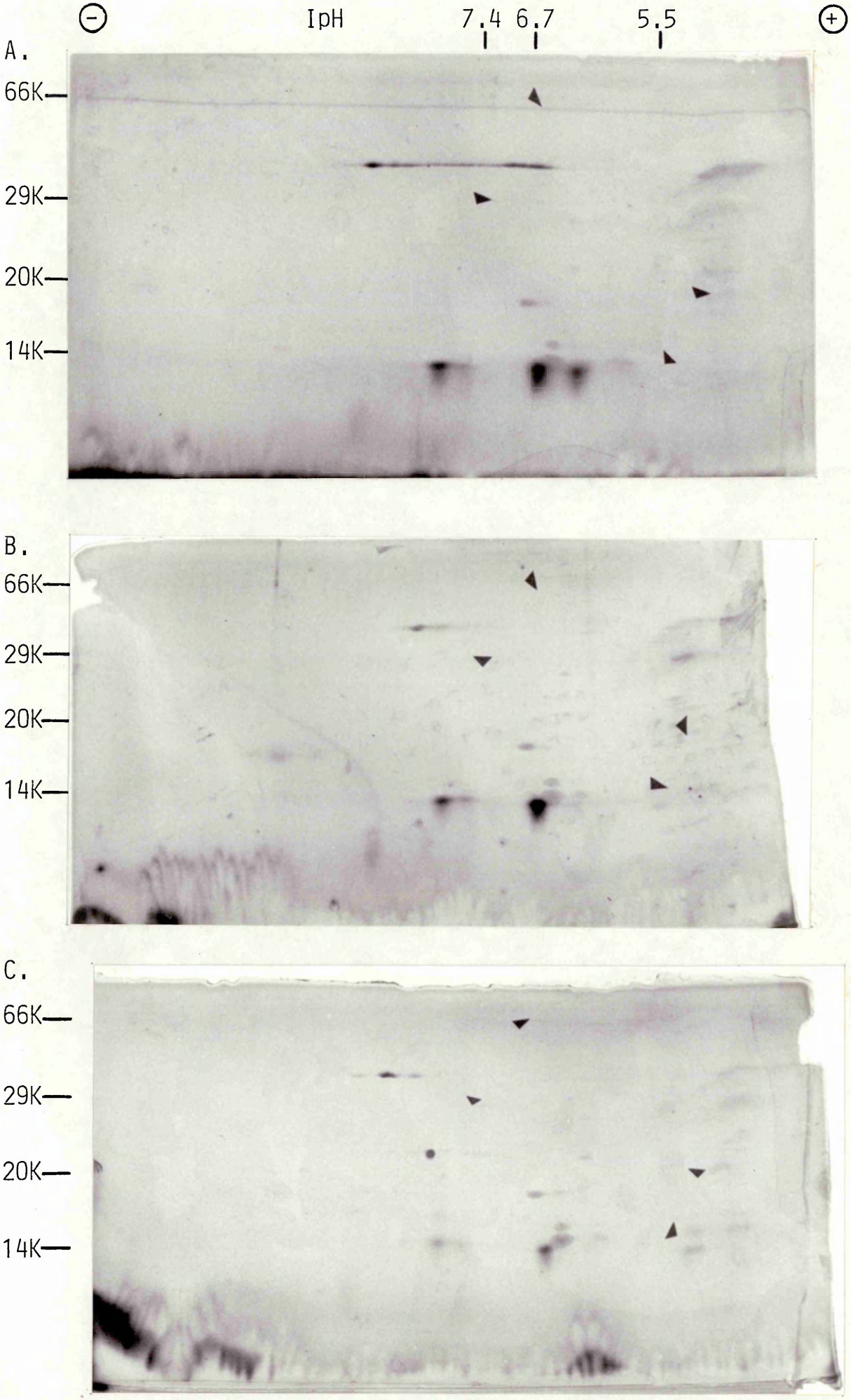
- Shaded spots on overlay indicate digestion products of V8 protease.
- Open spots on overlay indicate digestion products of A_1, A_2, and A_3 on Figure 4.25 A, B and C respectively. Numbered spots are referred to in the text.
- Closed triangles mark the position of marker proteins.
- Striped spots are the undigested charge isomers of core protein A. Figure 4.25 A, B and C indicate undigested A_1, A_3 and A_2 respectively.

This result was reproducible in 3 experiments.

1 2
3
4

1 2
3

1 2
3


⊖
IpH
7.4
6.7
5.5
⊕
A.
66K
29K
20K
14K
B.
66K
29K
20K
14K
C.
66K
29K
20K
14K

3.3 Phosphorylation of Core Proteins

Different states of phosphorylation of one basic polypeptide could account for the charge pattern of core proteins observed on 2-dimensional gels. The core proteins were first phosphorylated *in vitro* because previous attempts to incorporate ^{32}Phosphate into rat liver hnRNP particle proteins *in vivo* were unsuccessful (Wilks, 1980). Apart from the endogenous kinase of hnRNP particles (Karn *et al.*, 1977; Wilks and Knowler, 1981b; Holcomb and Friedman, 1984), exogenous kinases were also used for the *in vitro* phosphorylation of the core proteins with [γ-^{32}P]ATP under appropriate conditions (see method 4.2.1). The exogenous kinases used were

1) the catalytic subunit of c-AMP dependent kinase,
2) calcium and phospholipid dependent kinase C and
3) Pseudorabies virus induced kinase of Baby Hamster kidney cells (BHK).

Excess amounts of the kinases were used so that all available phosphorylation sites could be saturated. Figure 4.26 compares the 2-dimensional pattern of phosphorylation of the core proteins by all the four kinases. Table 3 indicates which of the core protein charge derivatives were phosphorylated. It is not known whether the labelling of a given derivative, say A_3, represents the conversion by the kinase of another derivative, say A_2 to A_3 or whether previously existing A_3 is not significantly changed in its properties by the addition or exchange of phosphate. No previously unobserved core protein derivatives were created by phosphorylation and it is noteworthy that the endogenous kinase produced a phosphorylation pattern different from that of the

FIGURE 4.26

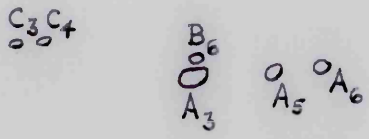
Pattern of phosphorylation of core proteins with various kinases

hnRNP particle proteins (0.8ml of 0.2mg/ml) were incubated with 100 Ci/mole ATP^{32}/ATP in the presence of the appropriate kinase, 10mM $MgCl_2$ and other reaction constituents in a total volume of 1.2ml at 30°C for 40 minutes (see method 4.2.1). Proteins were precipitated and subjected to 2-dimensional gel electrophoresis (method 3.3). Gels were dried and autoradiographed (see figure 4.15 for nomenclature of the charge isomers of core proteins shown).

4.26A Proteins were incubated in the presence of 20 units of catalytic subunit of cAMP dependent protein kinase, 10mM Tris/HCl, pH 7.6, 1mM dithiothreitol, 25mM KCl and 0.1mM EGTA. The gel was autoradiographed for 1 hour.

4.26B Proteins were incubated in the presence of 50 units of pseudorabies virus-induced kinase of BHK cells (Katan *et al.*, 1985), 10mM Tris. HCl, pH 7.6, 10mM 2-mercaptoethanol, 100mM KCl.
(1 unit = 1pmol of phosphate incorporation/min Katan *et al.*, 1985). Gel was autoradographed for 24 hours.

The experiment was performed once only.


C3 C4
B6
A3
A5
A6

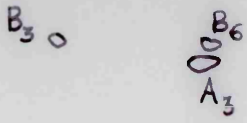

B3
B6
A3

Fig. 4.26 A

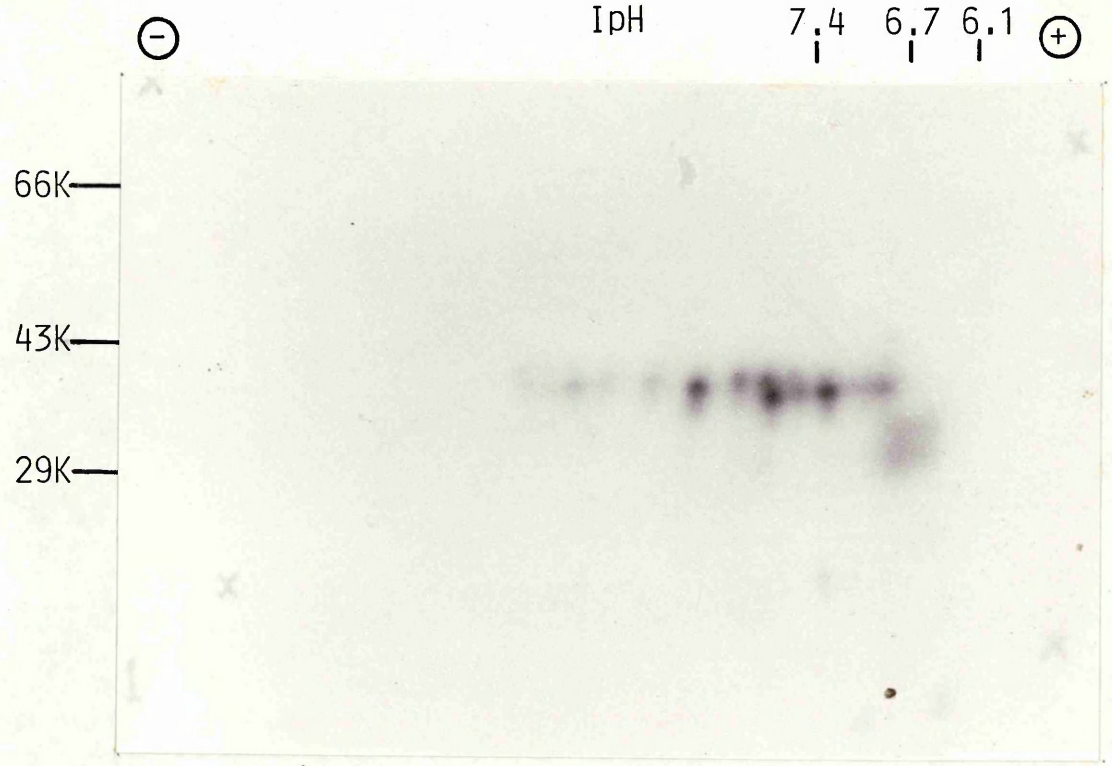


Fig. 4.26 B

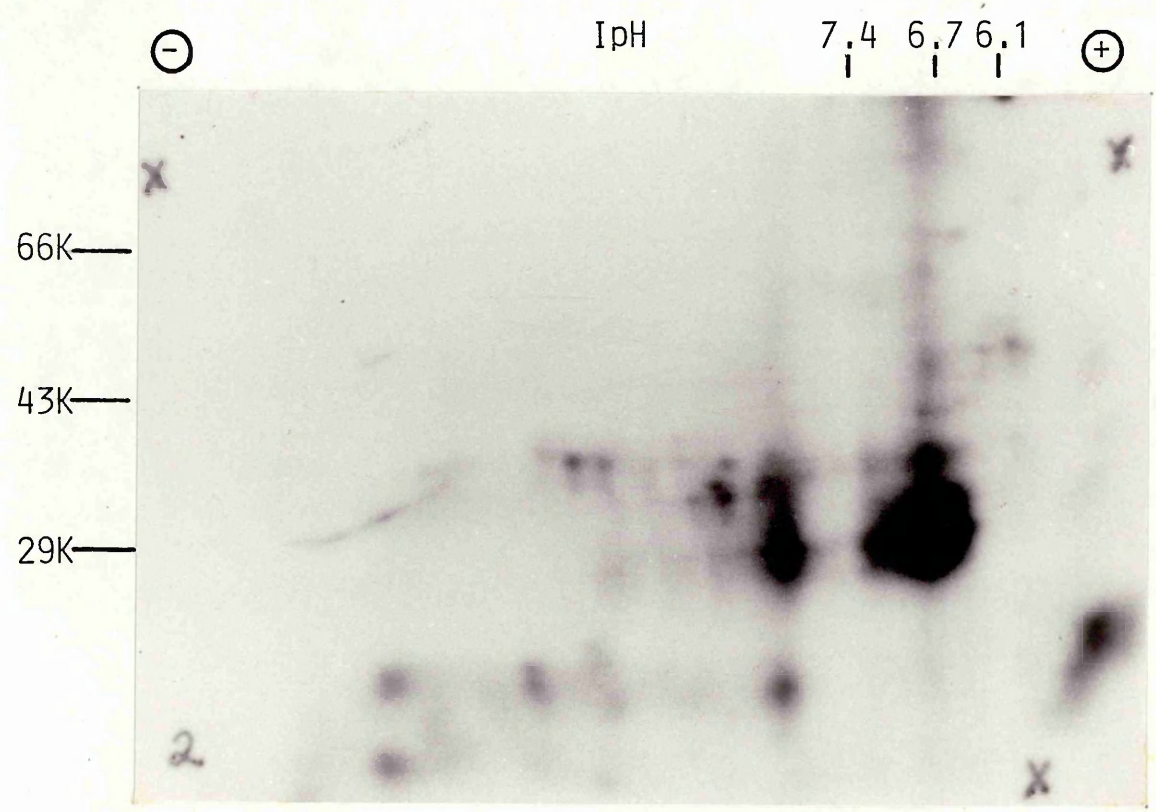
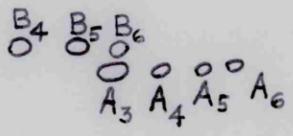


Figure 4.26 (continued)

4.26C Proteins were incubated in the presence of 0.9 units of proteolytically activated Kinase C, 10mM Tris. HCl, pH 7.6, 25mM KCl, 0.15mM EGTA, 5mM 2-mercaptoethanol and 1mM EDTA. Gel was autoradiographed for 2 hours. Before use, the enzyme was proteolytically activated by incubating 2.4U kinase C in 10mM Tris-HCl, pH8, containing 5mM 2-mercaptoethanol, 0.8mg Bovine serum albumin and 1.6µg trypsin in a total volume of 0.4ml for 3 minutes at 30°C. The reaction was stopped by the addition of 16µg soybean trypsin inhibitor which gave a tenfold (w/w) excess (Del Grande and Traugh, 1982).

4.26D hnRNP particle proteins were incubated in 10mM MOPS, pH6.5, containing 10mM $MgCl_2$, with γ^{32} ATP as described above. Gel was autoradiographed for 24 hours.

The experiment was performed once only.


B4 B5 B6
A3 A4 A5 A6

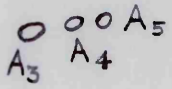

A5
A3 A4

Fig. 4.26 C

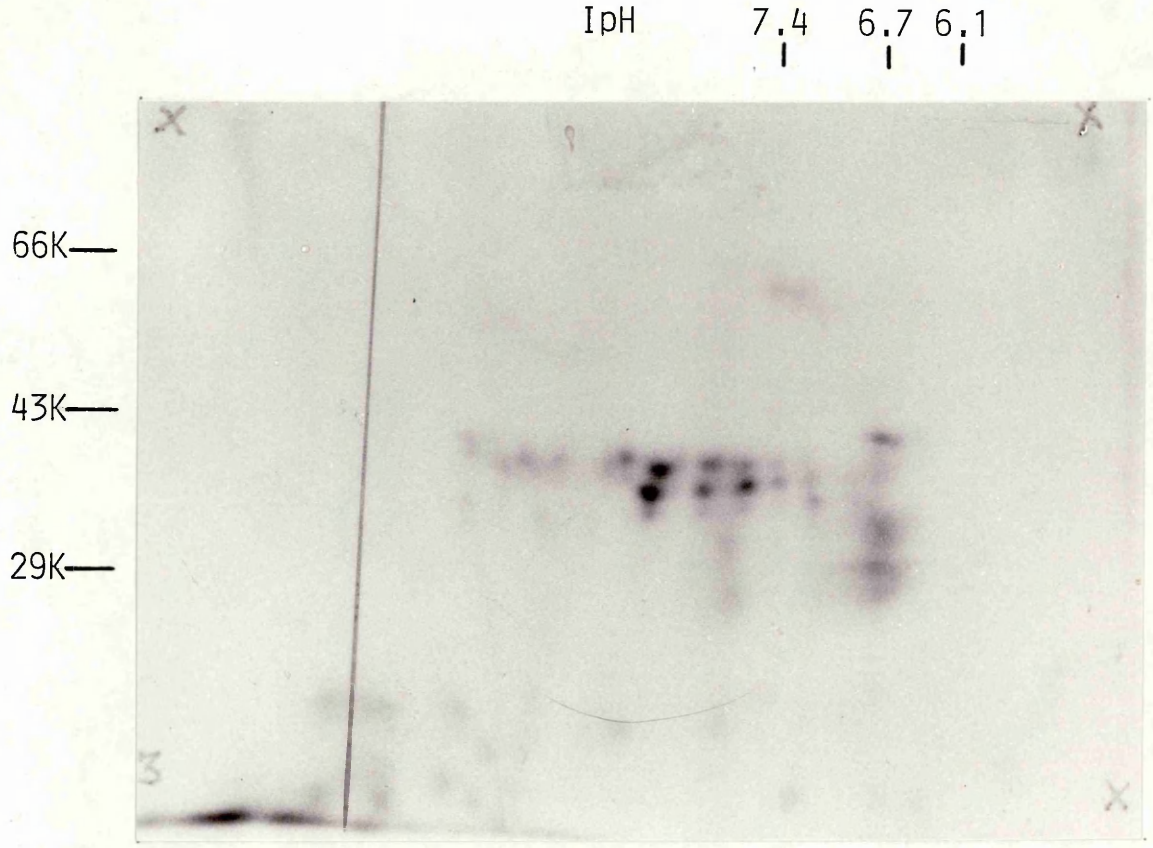


Fig. 4.26 D

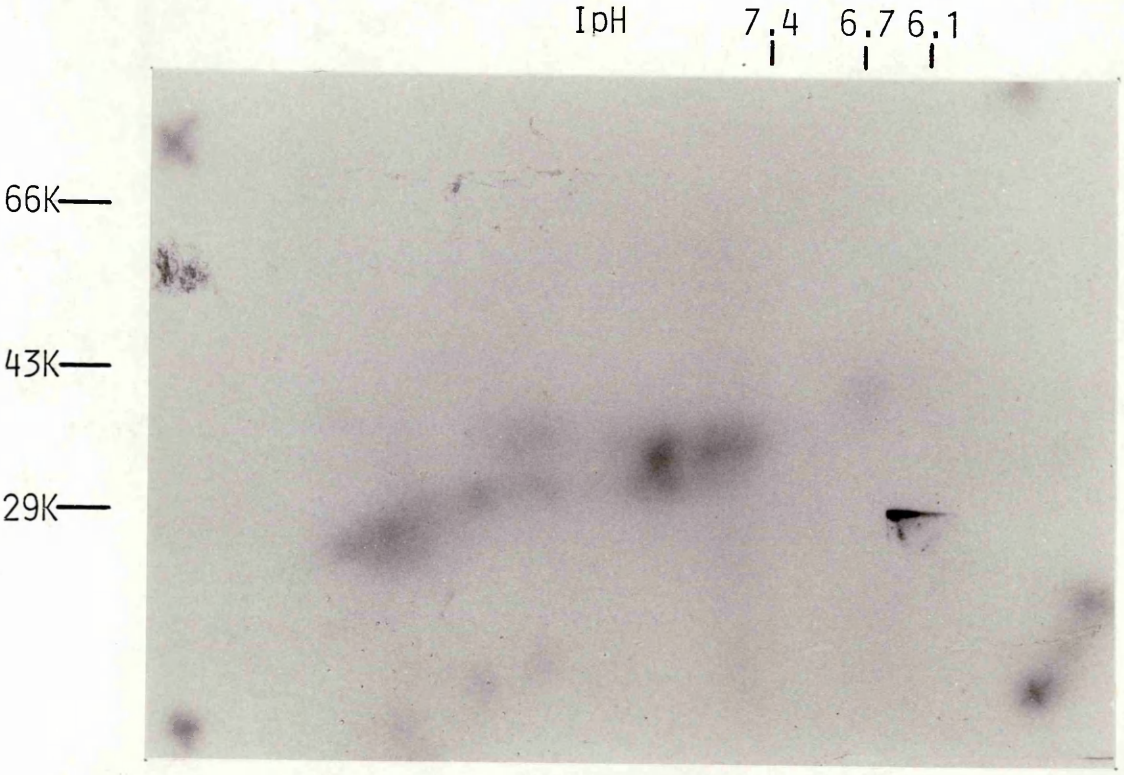


TABLE 3
IN VITRO PHOSPHORYLATION OF CORE PROTEIN
CHARGE ISOMERS

Kinase	Core protein A	Core protein B	Core protein C
C Amp dependent Kinase	A_3 A_5 A_6	B_5 B_6 B_7	C_3 C_4
Pseudorabies induced Kinase of BHK cells	A_3	B_3 B_6	
Kinase C	A_3 A_4 A_5 A_6	B_4 B_5 B_6	
Endogenous Kinase	A_3 A_4 A_5		

other enzymes. The phosphorylation pattern produced by the endogenous kinase was similar to that previously reported by Wilks and Knowler (1981b).

It is clear from figure 4.26 that phosphorylation cannot be the sole cause of the observed charge heterogeneity as some polypeptides, that is A_1 and A_2 are never phosphorylated. The unambiguous demonstration that phosphorylation contributes to the heterogeneity, that is, in the creation of A_3, A_4, A_5 would require much more rigorous proof. It would be necessary to show phosphorylation in vivo and it would be desirable to demonstrate the interconversion between isolated charge isomers by phosphorylation or dephosphorylation, that is, the conversion of A_3 to A_1 or A_2 by dephosphorylating enzymes. Another useful indicator of the extent to which phosphorylation was contributing to the formation of a charge isomer was to look at the stoichiometry of phosphorylation, that is, to ascertain what percentage of the core proteins could be phosphorylated. All three of these experimental approaches have been attempted.

To determine the stoichiometry of ^{32}phosphate incorporation, hnRNP particles were phosphorylated by the endogenous kinase, the core protein A was extracted, run on a 2-dimensional gel and counts for the charge isomers were determined (see method 4.2.1.2). Table 4 shows the values obtained for phosphate incorportation. It is clear that considerably less than one mole of phosphate per mole of protein has been incorporated into any of the derivatives of A. It must be pointed out here that under the conditions used for maximum incorporation of phosphate with the endogenous kinase (10mM $MgCl_2$, pH6.5) the solubility of hnRNP

TABLE 4

STOICHIOMETRY OF ^{32}PHOSPHATE INCORPORATION INTO CHARGE ISOMERS OF CORE PROTEIN A

See method 4.2.1.2 for experimental details

Total protein A loaded on 2-dimensional gel was 6µg of phosphorylated protein mixed with 54µg of unlabelled protein

Charge isomers	Densitometric area X10^7	Ratio to each other	Amount of labelled isomer (µg)	pmol of isomer	pmol of ** phosphate incorporated	nmol phosphate per mole isomer
A_1	6.2	12.4	1.6	47	-	-
A_2	6.6	13.2	1.7	47	-	-
A_3	3.4	6.8	0.88	25	0.01	400
A_4	0.5	1	0.13	4	0.01	2500
A_5	0.6	1.2	0.15	4	-	-
A_6	0.7	1.4	0.18	5	-	-

** Concentration of ATP used to phosphorylate the particles was 2.5mM. 1µl of 2.5mM ATP^{32} had 3 x 10^7cpm. Therefore there were 12 x 10^6 cpm per nmol of phosphate.

- Since cpm for these isomers, A_1, A_2 and A_5 and A_6 were near background, phosphate incorporation was not calculated. The cpm for A_3 and A_4 were 107 and 91 respectively. These values are not much higher than background but A_3 and A_4 consistently phosphorylated much more than other charge isomers.

particles is low and this may be one of the causes for the poor incorporation of phosphate. On the basis of the low quantitative incorporation of phosphate, it appears unlikely that phosphorylation alone could give rise to A_3, A_4 and A_5. Nevertheless, it must be remembered that the experiment was conducted with core protein which may already have been phosphorylated to near equilibrium and thus unable to accept much additional phosphate.

Such a criticism of the data could only be met by attempting to dephosphorylate A_3, A_4 and A_5 hoping perhaps to convert them to A_1 and A_2. Previous attempts in this laboratory to observe such a dephosphorylation with alkaline phosphatase were unsuccessful. Figure 4.27 illustrates a repeat of this experiment using a mammalian alkaline phosphatase rather than the bacterial enzyme used by Wilks and Knowler (1981a). Again no change in core protein pattern or heterogeneity was observed. This experiment might not have detected small changes, that is, partial conversion of A_3, A_4 and A_5 to other derivatives. A better experiment would have been to take pure A_3, A_4 or A_5 and see whether alkaline phosphatase partially converted them to other components. Such experiments were not possible because purified A protein and its charge isomers could only be isolated as SDS- complexes. Holcomb and Friedman (1984) however reported that the C proteins of HeLa cell hnRNP particles become better substrates for endogenous kinase after being treated with E. coli alkaline phosphatase.

Considerable difficulty was experienced by Wilks (1980) in trying to incorporate ^{32} phosphate in vivo into rat liver hnRNP proteins. Further attempts in the present study therefore used

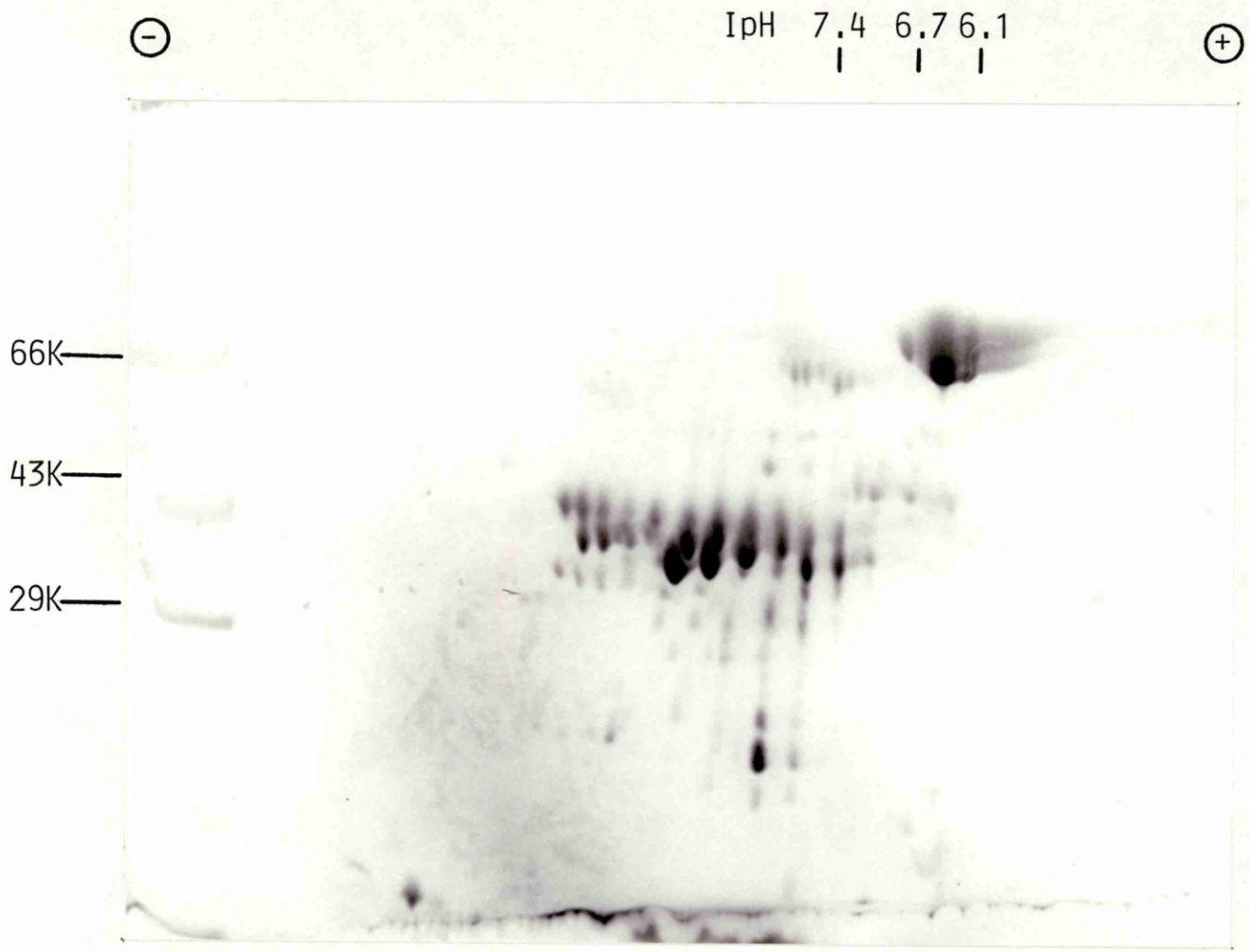
FIGURE 4.27

Treatment of hnRNP Particle Proteins with Mammalian Alkaline Phosphatase

Total hnRNP particle proteins from sucrose density gradients (method 1.3.1) were dialysed against 10mM Ethanolamine-HCl pH 9.5 and 10mM $MgCl_2$ (Morton, 1954). Proteins (concn. 0.1mg/ml) were incubated with 200U/ml of partially purified calf-intestine alkaline phosphatase at 37°C for 20 minutes. Proteins were precipitated by 10% w/v trichloracetic acid in the cold and subjected to 2-dimensional electrophoresis.

This result was reproducible in 3 experiments.

Fig. 4.27



Buffalo Rat Liver (BRL) tissue culture cells (method 4.2.2). Although there are no previous reports about characterisation of the BRL core proteins, the major proteins of the BRL hnRNP particles seem similar to the Rat Liver hnRNP core proteins on one-dimensional SDS-polyacrylamide gels and 2-dimensional gels (figures 4.1 and 4.15 respectively). When BRL hnRNP particles were mixed with Rat Liver particles and together subjected to 2-dimensional fractionation, the core proteins co-electrophores (results not shown).

Figure 4.28A shows proteins precipitated from sucrose density gradient fractions on which the phosphorylated BRL hnRNP particles were purified and figure 4.28B is its corresponding autoradiograph. The major protein bands in figure 4.28A marked as core protein A and B are seen not to incorporate phosphate when compared with figure 4.28B, lane 5. The protein marked C however coincides with a phosphorylated band on the corresponding autoradiograph. Two proteins of molecular weight lower than core protein A and a protein of higher molecular weight of about 50K seem to have incorporated phosphate.

It was noted that the BRL hnRNP proteins were distributed fairly evenly across all fractions of the sucrose gradient which indicated that the hnRNP were polymeric and in contrast to the sharp 40S hnRNP peak corresponding to monomeric hnRNP particles usually obtained from rat liver nuclei. This is typical of hnRNP prepared from tissue culture cells and could be because they contain low levels of endogenous RNase. The heavy background labelling seen in lanes 1 to 5 is presumably caused by coprecipitation of [^{32}P]-hnRNA with the proteins. It is more

FIGURE 4.28

Phosphorylation of hnRNP Particle Proteins in vivo

HnRNP particles were isolated from Buffalo Rat Liver cells labelled with ^{32}Phosphate as described in method 4.2.2. Figure 4.28A shows stained SDS-polyacrylamide gel of proteins acid-precipitated from consecutive fractions of a sucrose gradient on which the hnRNP particles were purified (method 1.3.1). Each fraction was approximately 6ml. Fractions 3 and 4 represent the very small peak from the sucrose gradient at 260nm. 4.28B is the autoradiograph of 4.28A.

This result was reproducible in 3 experiments.

Fig. 4.28 A

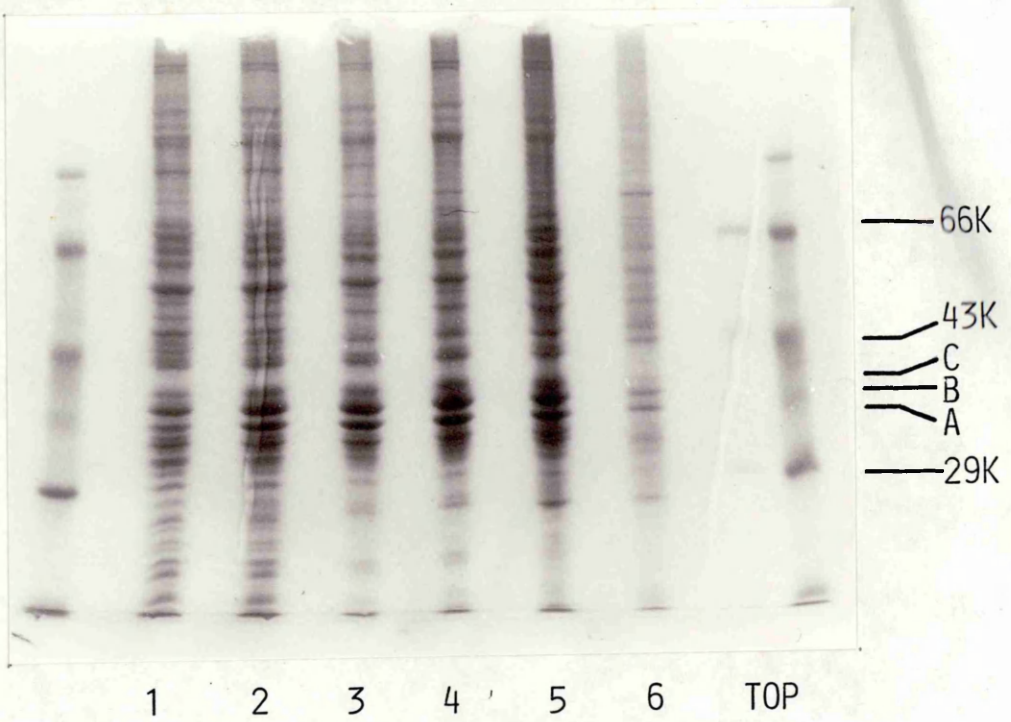
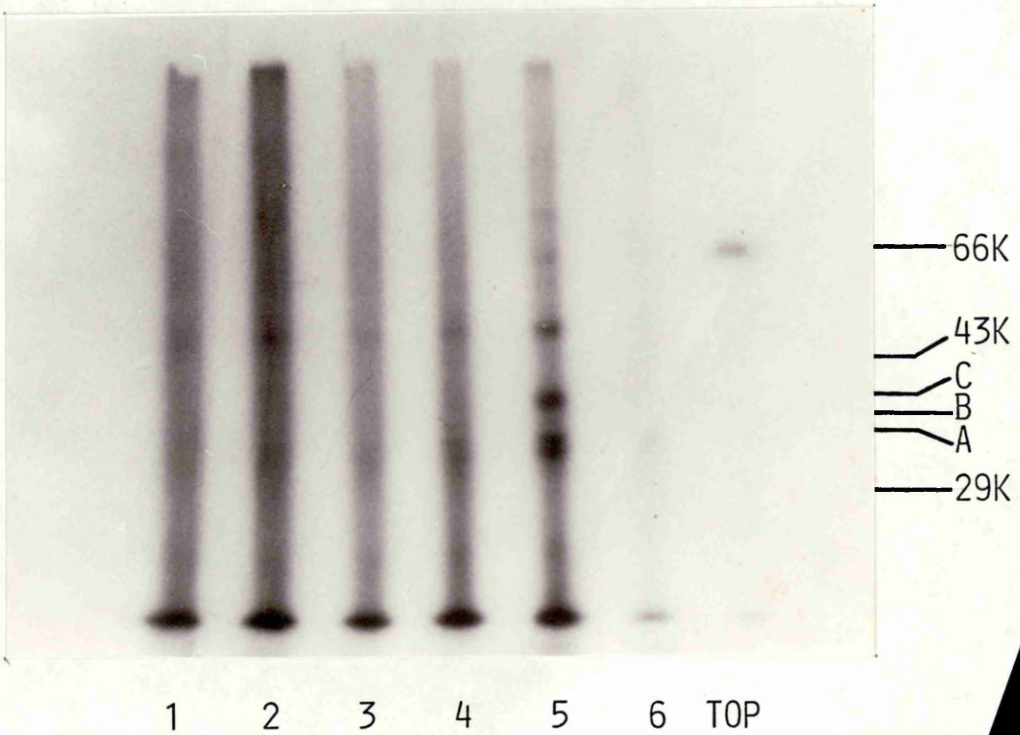


Fig. 4.28 B



difficult to explain why only in lane 5, the phosphorylated bands are seen heavily labelled while in lanes 1-4 they are barely discernable above the background.

The phosphorylation of proteins in lanes 3 and 4 (of figure 4.28A) were seen more clearly when they were subjected to 2-dimensional electrophoresis and autoradiography (figure 4.29C). As had been seen in the one dimensional gel (figure 4.28B), the radioactivity in the 2-dimensional gel corresponded to proteins lower in molecular weight than core protein A and coincided with core protein C. The position of core proteins A, B and C are marked on the molecular weight scale in the figure. Two radioactive spots (marked by arrows) could be due to the acidic charged isomers of core protein A and B, however the protein concentration in the stained gel was too low to match with certainty. These acidic species are however seen in the more heavily loaded (non-radiocative) gel illustrated in figure 4.29A.

In conclusion, the C protein of BRL hnRNP particle seems to be phosphorylated *in vivo* but the evidence for *in vivo* phosphorylation of the acidic charge isomers of core proteins A and B is not conclusive. These results are in agreement with the data of Holcomb and Friedman (1984) who have shown that only the C proteins of HeLa cell hnRNP particles were phosphorylated *in vivo*.

Compiling the data of both the *in vitro* and *in vivo* phosphorylation and phosphatase treatment of core proteins, it seems unlikely that phosphorylation is the sole cause of the charge heterogeneity observed. Holcomb and Friedman (1984) also found no evidence for the differing states of phosphorylation of the C proteins of HeLa cell hnRNP particles which could cause the C-protein charge heterogeneity seen on 2-dimensional gels.

FIGURE 4.29

Phosphorylation of hnRNP Particle Proteins in vivo: 2-Dimensional Picture

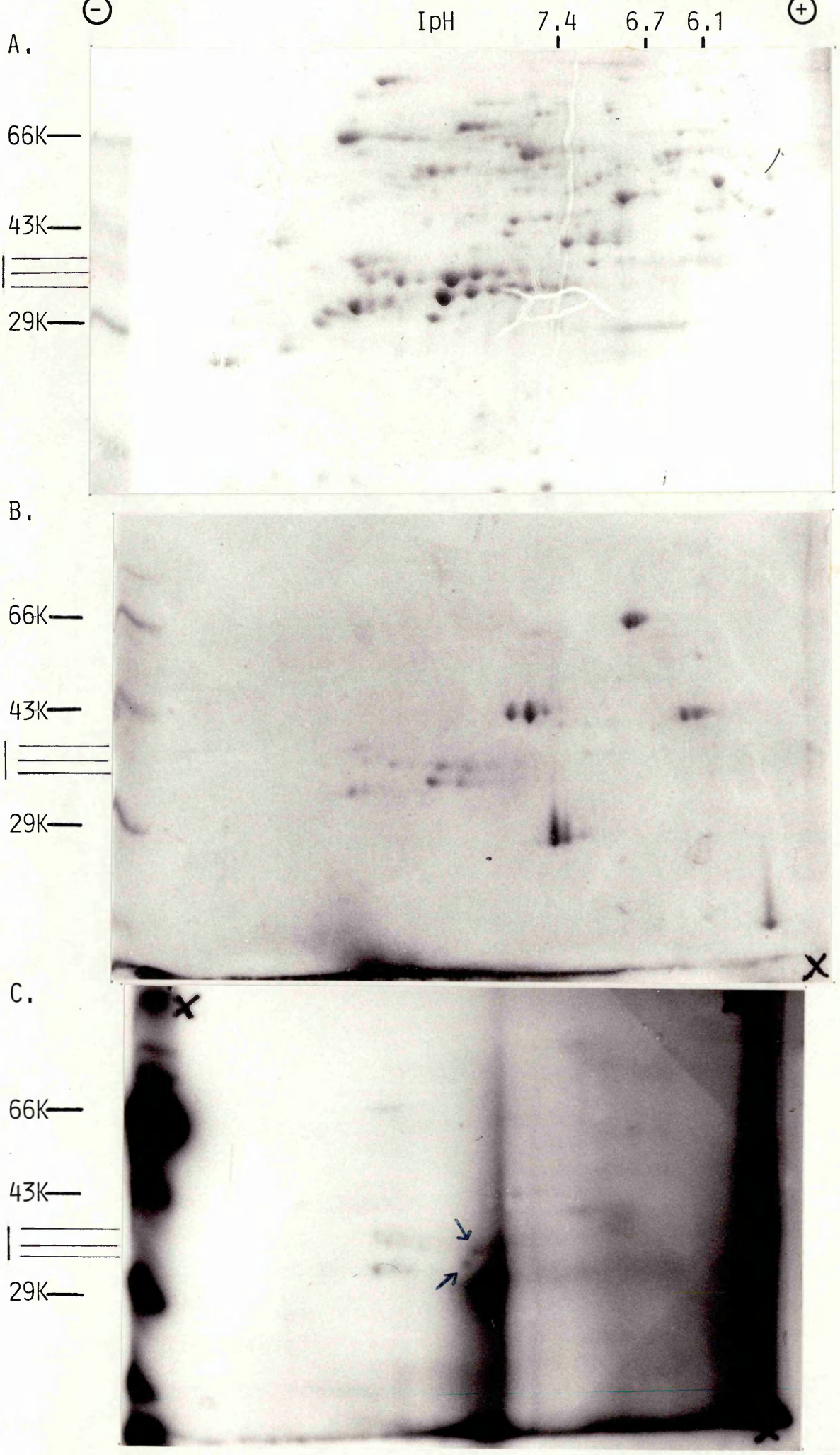
hnNRP particles from ^{32}phosphate-labelled Buffalo Rat Liver (BRL) cells were purified on sucrose density gradients (see method 4.2.2 and figure 4.28). Proteins from the small hnRNP particle peak at 260nm (fractions 3 and 4 in figure 4.28) were dissolved first in up to 2% w/v SDS and then in lysis buffer (see method, Section 3, prior to gel electrophoresis in two dimensions (method 3.3). The gel was dried and autoradiographed for one week.

4.29A High concentration of BRL cell hnRNP particle proteins (500μg) on a 2-dimensional gel.

4.29B BRL cell hnRNP proteins (60μg) phosphorylated in vivo (spots at 29, 43 and 66K are markers whose IpH is shown on top of gel).

4.29C Autoradiograph of 4.29B.

This result was reproducible in 3 experiments.


⊖
IpH
7.4
6.7
6.1
⊕
A.
66K
43K
29K
B.
66K
43K
29K
X
C.
66K
43K
29K
X
X

3.4 Other Possible Causes of Charge Heterogeneity of the Core Proteins

Among previous reports of the modification of core proteins which could cause their charge heterogeneity, the publication by Kostka and Schweiger (1982) of the poly ADP-ribosylation of core proteins was experimentally approached by preliminary analysis. The best test for ADP ribosylation is the incorporation of radioactive NAD. However radioactive NAD is very expensive and, given the difficulties experienced in the *in vivo* and *in vitro* incorporation of ^{32}P, it was decided that the expense of the radioactive derivative was not justified without some other indication that ADP ribosylation was occurring. Snake venom phosphodiesterase I can be used to characterise poly ADP-ribosylated proteins (Kidwell and Purnell, 1984). The enzyme cleaves pyrophosphate linkages in poly ADP-ribosyl groups by the following reaction:-

$$\text{Protein-(ADP-R)}_n + \text{phosphodiesterase I} \longrightarrow \text{Protein-R-P+n-1(ADP-ribose)+5'AMP}$$

Thus after reaction with phosphodiesterase I, the protein should undergo considerable change in charge as well as molecular weight, depending upon the size of the poly ADP-ribose groups. However, commercial preparations of snake venom phosphodiesterases are contaminated with proteases (Gronow and Chapleo, 1979; Pfleiderer and Sumyk, 1961), phosphomonoesterase and 5'nucleotidase (Oka *et al.*, 1978). This makes interpretation of the effects of phosphodiesterase treatment difficult to assess unless specific inhibitors are used.

The proteases in commercial phosphodiesterase I have been reported to be inactive at pH7 (Pfleiderer and Sumyk, 1961) and 1mM PMSF (phenyl methyl sulfonyl chloride) has also been reported

to completely inhibit a protease of snake venom phosphodiesterase that specifically attacks histone H2b (Gronow and Chapleo, 1979). Furthermore, since among common laboratory proteins pancreatic RNase was the only one to be slightly degraded by the phosphodiesterase preparation in the absence of 1mM PMSF, the snake venom enzyme was passed through an RNase-affinity column (method 6.1). The rationale behind this step was that the proteases in the phosphodiesterase should stick to the RNase, letting the phosphodiesterase activity through (Protein-sepharose columns are sometimes used to retard protease activity of commercial snake venom phosphodiesterase, W. J. D. Whish, personal communcation). When core proteins were treated with this partially purified phosphodiesterase I at pH 7.0 in the presence of 1mM PMSF, low molecular weight peptides were produced (results not shown). These low molecular weight peptides did not seem to enter the charge dimension in non-equilibrated pH gradient gel and could have been caused by the action of proteases. The degradation of core proteins could be partially inhibited when 0.4mM EDTA (which inhibits phosphodiesterase I activity, Razzell, 1963) was included in the reaction (results not shown).

As an alternative way of analysing the products of phosphodiesterase I catalysis attempts were made to isolate the adenosine derivatives that would be produced after the enzymic treatment of ADP-ribosylated proteins, that is 5'AMP and ADP ribose. The method used for the isolation of these groups was that of Kostka and Schewiger (1982). The latter workers have described the isolation of labelled charged groups, but since it was not possible to label the proteins in this case, the isolation

procedure was performed with unlabelled protein and the purification followed by the addition of exogenous [γ-^{32}P] ATP (see method 6.2). The isolated groups were subjected to TLC on polyethyleneimine (PEI) coated plates and spots looked for with the help of a UV torch. Twenty nmol of 5'AMP and ADP-ribose were used as markers. A trace of the PEI sheet which was used for the TLC is shown in figure 4.30. There were no spots corresponding to ADP-ribose or 5'AMP in the sample lane.

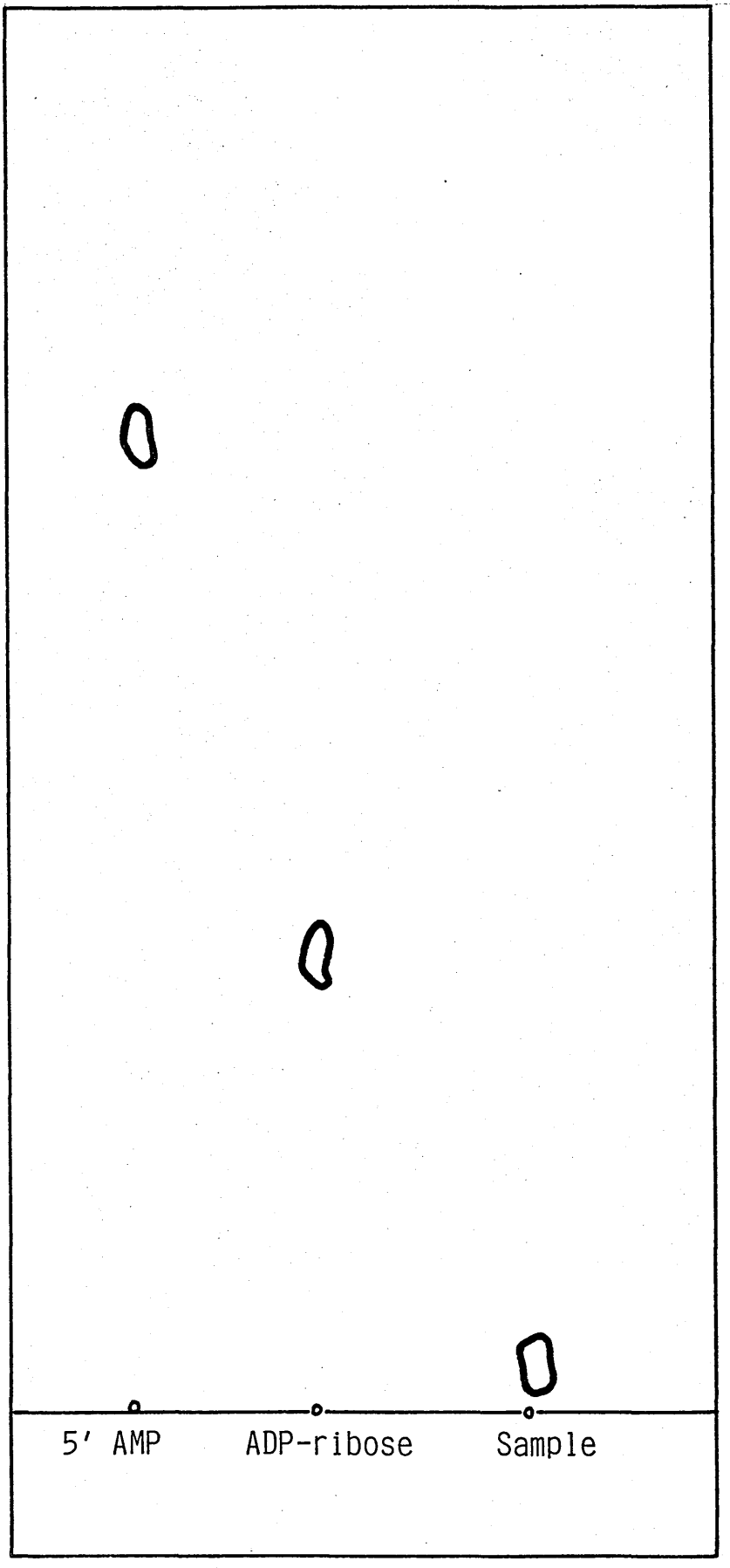
FIGURE 4.30

TLC on Polyethyleneimine (PEI) Coated Plates of hnRNP Protein Products After Phosphodiesterase I Treatment.

Total hnRNP proteins from sucrose density gradients (method 1.3.1) were dialysed against 10mM Tris.HCl, pH7.0. One mg protein solution was incubated with phosphodiesterase I, passed through an Amicon filter and low molecular weight groups in filtrate purified as described in method 6.2. The purified, lyophilsed residue was dissolved in 20µl water and 10µl subjected to TLC on a PEI plate (method 6.2). Figure shows a diagram of the PEI plate as was seen under UV light.

This result was reproducible in 2 experiments.

Fig. 4.30



V. DISCUSSION

In the work presented in this thesis, the four core proteins of rat liver hnRNP particles have been 1) isolated and fractionated, 2) their relationship to each other studied and 3) attempts made to find the cause of their charge heterogeneity.

One of the major difficulties of working with core proteins is their tendency to aggregate above certain concentrations in the absence of salt or an anionic detergent such as sodium dodecyl sulphate (SDS) or deoxycholate (DOC). High salt or anionic detergents are presumably required to stabilize the proteins by charge neutralization in the dissociated state and in the absence of RNA. In the presence of single stranded RNA, core proteins spontaneously reassociate to form native-like RNP complexes (Pullman and Martin, 1983; Wilk et al., 1983). Protein - protein interactions are also important in the formation of such in vitro reconstituted RNP complexes (Thomas et al., 1983). In the present work, antibodies raised to core proteins have been shown to recognise high molecular weight aggregates of core proteins resistant to dissociation by SDS (results 2.3). High molecular weight oligomers of core proteins are also recognised by monoclonals raised against the proteins (Leser et al., 1984). Aggregation of core proteins resulting in the formation of artefactual RNP complexes resistant to dissociation in 2M salt has also been recently reported by Lothstein et al., (1985).

The minor difference in molecular weight between the four core proteins (MW 34000 to 43000) and their property of aggregation makes their separation extremely difficult. Preparative gel electrophoresis of the proteins in the presence of

SDS was found to be the only system capable of fractionating them (results 1.2). The core proteins were isolated in the form of SDS-complexes which meant that they could not be subjected to analysis in their native state. However, the fractionation of the core proteins did allow investigation of the chemical nature of the individual proteins with the help of certain proteases (results 2.1). Only very recently, Wilk et al. (1985) reported the electroelution of the core proteins of HeLa cell from SDS-polyacrylamide gels by using the method of Hunkapillar et al. (1983). However, the preparative fractionation of the core proteins described here gave better quantitative yields of the core proteins, at least in the case of the major core proteins A and B (see table 1.2).

Partial peptide mapping with V8 protease and α-Chymotrypsin of the purified rat liver core proteins A to D on SDS-polyacrylamide have revealed that the four proteins are closely related to each other (results 2.1). When iodinated core proteins were subjected to partial peptide mapping, the similarity between the proteins seemed less but that is probably because, in the latter case, fewer digestion products were observed. Subsequent to this work, Wilk et al. (1985) reported the partial peptide mapping and tryptic peptide mapping of the HeLa cell core proteins. These workers have shown that the core proteins of HeLa cell are a family of closely related species.

The similarity of the four rat core proteins was again made evident when attempts were made to raise polyclonal antibodies to the individual core proteins. Separate antibody preparations to core proteins A and B cross-reacted with both core proteins A and

B and faintly with core proteins C and D (result 2.3). The close relatedness of the core group proteins was further confirmed by Leser et al. (1984), who raised monoclonals to mouse core proteins. A majority of the clones cross-reacted with all the four mouse core proteins, despite the fact that the monoclonals were specific for different antigenic determinants.

The similarity between the four core proteins of rat liver shown in this presentation and the general similarity among the core proteins of HeLa cell and mouse reported by other workers (Wilk et al., 1985; Leser et al, 1984) indicate that core proteins are probably products of a closely related gene family. An example of a similar group of closely related proteins are the vitellogenin proteins of Xenopus laevis. Three distinct vitellogenin monomers with MW of 182000-197000 are known. Four different mRNAs from Xenopus laevis have been isolated and their products which are all recognised by anti-vitellogenin antibody were shown to differ in primary structure (Jaggi et al., 1980). Analysis of the vitellogenin mRNA in Xenopus by cDNA cloning has shown that, in an individual, vitellogenin is encoded in a small family of four related genes (Wahli et al., 1979). Other examples of a family of genes coding for related but not identical proteins are the genes for the multiple keratins of cultured human epidermal cells (Fuchs and Green, 1979) and Dictyostelium actin genes (Kindle and Firtel, 1978). There are recent indications that human genes may contain either moderately repetitive sequences or pseudogenes homologous to an Artemia salina core protein cDNA probe (Cruz-Alvarez et al., 1985). Further proof of the possible repetitive nature of mammalian core protein genes

must await isolation of the genes and their characterization by sequencing studies. The cDNA clones of the Arte mia salina core protein produced by Cruz-Alvarez and coworkers will hopefully provide an efficient probe to locate core protein genes of mammalian cells either in a genomic library or a cDNA library produced from total poly(A) mRNA.

Sequencing studies also have the potential of analysing whether core proteins are produced by alternative processing of a single gene transcript rather than being the products of a multiple gene family. Production of multiple proteins by alternative processing of a single gene has been observed in a few cases like the formation of 3 fibronectins (Schwarzbauer et al., 1983), 2 α A-crystallins (King and Piatigorsky, 1983), 2 myosin light chains (Nabeshima et al., 1984) and 2 myelin basic proteins (Takahashi et al., 1985). In the case of the αA-crystallins one protein called αA^{ins} has an extra 22 amino acid residues when compared to the other, αA_2 crystallin. Rest of the polypeptide chain between the two proteins is identical. The two myosin light chains have a common C-terminal region of 141 amino acid residues. In the case of the two myelin basic proteins, the difference consists of 41 amino acid residues which are encoded by the 5th exon of the myelin basic gene, and are absent in one of the proteins.

The core proteins of any particular species such as rat, mouse and man show extensive homology in their partial peptide maps as discussed earlier. This indicates that the multiple core proteins of any one species may share common domains which may have a functional significance. Such conservation of functionally

important domains are seen in another class of nucleic-acid binding proteins, namely the histones. Despite the existence of subtypes of H1 histones which differ in primary structure, there is a region of about 75 residues which are highly conserved (Isenberg, 1979). The cross-reaction of a number of core protein monoclonal antibodies with all four variants of mouse core proteins (Leser et al., 1984) further suggests that the conserved regions of core proteins within a species are highly antigenic. Another dimension to the problem of core protein variation, their antigenic cross reactivity with each other and the functional significance of such properties has been raised by the recent reports by Valentini et al. (1985) that core proteins of HeLa cross-react with antibodies to mammalian single-stranded (SS) DNA binding proteins. The two types of proteins share one important property, that of binding only to single-stranded polynucleotides, either ss DNA or RNA (Pullman and Martin, 1983). Whether core proteins and ss DNA binding proteins contain common domains having a functional significance can only be discovered by a detailed analysis of the structure and organisation of the gene(s) coding for these proteins (or the protein themselves).

A common posttranslational modification, which quite often results in a change in molecular weight of a protein when examined by SDS-polyacrylamide gel electrophoresis, is glycosylation. That the core proteins are not glycosylated was shown by their failure to deglycosylate (results 2.2). In general, glycosylation is not common in nuclear proteins. Only the nonhistone high mobility group (HMG) proteins have so far been found to be glycosylated (Reeves et al., 1981).

The failure of the rat liver core proteins to be deglycosylated (results 2.2) was monitored by the unaltered mobility of the proteins on SDS-polyacrylamide gels after their treatment with an efficient deglycosylating agent, trifluoromethane sulphonic acid (TFMS). This is a common method for detecting glycosylated prote ins (Edge et al., 1981; Karp et al., 1982; Snow et al., 1985). Usually, after removal of carbohydrate groups from a glycosylated protein, the protein migrates faster on an SDS-polyacrylamide gel. However, in rare cases, no change in mobility of the protein is observed after deglycosylation, for example in the case of HMG 14 and 17 (Reeves and Chang, 1983). One reason for the latter two proteins showing no change in molecular weight after deglycosylat ion could be because they show anomalous migration on SDS gels (Bustin et al., 1978). Anomalous migration of proteins on SDS-polyacrylamide gels can be caused if SDS fails to denature the proteins completely. Wilk et al. (1985) have recently reported the presence of secondary structural elements in core proteins which are resistant to denaturation by SDS. This was indicated because the proteins show an increase in molecular weight when 8M urea is included in the SDS-polyacrylamide gel.

In certain cases therefore deglycosylation of a protein may not result in a change in its mobility on SDS-polyacrylamide gel electrophoresis. Consequently further conclusive proof, that core proteins are not glycosylated is perhaps necessary. Cells grown in culture could be grown in the presence of Tunicamycin which in hibits glycosylation. Alternatively cells could be grown in the presence of radioactive sugar precursors to glycoprotein. Glycos

idases can also be used to remove carbohydrates from proteins and the carbohydrates identified by sugar-specific colorimetric reactions after their separation from the proteins (Reeves et al., 198 1). The disadvantage of using glycosidases is that the right mixture of various endo-and exoglycosidases would be required to effectively remove all glycosyl groups, since the glycosidases are sugar-specific. The disadvantage of the method of glycosidase treatment is shared by lectin-binding studies because the latter are also sugar-specific and a range of lectins would need to be tested.

One final note is made here about the nature of the core protein variants. The formation of SDS-resistant aggregates of core proteins which are observed on one dimensional SDS-polyacrylamide gels has been discussed earlier. These aggregates are not seen on 2-dimensional gels in which the first dimension contains 9M urea (see figure 4.15). The 9M urea does not however change the number of core protein species seen. Therefore it does not seem likely that A, B, C and D represent different SDS-resistant derivatives of core proteins.

Several approaches have been made in this presentation to determine the causes of the charge heterogeneity of each of the in dividual core proteins when they are fractionated through a pH-gradient (see result, section 3.1). The possibility of artefactual formation of the charge isomers has been shown to be unlikely on two counts 1) the charge isomers were not caused by urea degradation 2) re-electrophoresis of some of the charge isomers on a pH-gradient produced single species with original iso-electric points. Although the most basic core protein A, A_1

refocuses as two spots, which could be due to deamidation, the complex pattern of charge isomers of protein A and other core proteins that are normally observed (see figure 4.15) cannot be explained on this basis alone.

The conclusion that all the charge isomers cannot be caused by deamidation alone is in agreement with the partial peptide map of the charge isomers obtained with V8 protease (results 3.2), where the acidic species of the core proteins digest less easily than the basic species. V8 protease cleaves peptide bonds on the carboxyl side of both glu and asp and does not hydrolyse peptide bonds where gln and asn are present (Houmard and Drapeau, 1972). If the acidic species of the core proteins were only due to deamidation, then they should actually digest more readily with V8 protease, and would produce more polypeptide fragments.

Amino acid composition of the core proteins have been reported by a number of workers (Karn et al., 1977; Le Stourgeon et al., 1978; Fuchs et al., 1980; Wilk et al., 1985). However there are no analyses of the number of gln or asn residues in the proteins. Determination of the gln or asn residues are made particularly difficult because of their tendency to spontaneously convert to glu and asp, even under physiological conditions (Robinson and Rudd, 1974). It has been shown that the relative stability of the amides under physiological condition is determined by the nature of the neighbouring amino acids in the polypeptide chain (Robinson et al., 1973). Therefore only a comparison of the mRNA sequence and amino acid sequence would unequivocally establish whether a given glu or asp residue in a protein is a result of direct genetically determined incorporation

of that amino acid or a result of deamidation of the corresponding peptide.

A major criticism of the partial peptide map on one-dimensional SDS-polyacrylamide gels of the charge isomers of core protein A and B (results 3.2) is that the maps are confused because a majority of the bands are actually due to V8 protease. High quantities of V8 protease were required probably due to contamination of the protein samples by SDS and acrylamide that resulted from their extraction from polyacrylamide gel pieces. To reduce confusion, controls containing V8 protease (without sample) were always included for comparison. This method of producing partial peptide maps was followed despite obvious difficulty in interpreting the maps because the alternative of using iodinated core protein was not very informative. Core proteins are low in tyrosine (Le Stourgeon et al., 1978), so produce few iodinated peptides (figure 4.18). With the benefit of hindsight, it would have been worth trying other methods of iodination not dependent on tyrosines and also insensitive to SDS and acrylamide contaminants in the protein. The Bolton and Hunter reagent which conjugates to lysine residues could have proved to be a good choice.

The fractionation of the peptide fragments (produced after V8 digestion) of core protein A charge isomers on 2-dimensional gels succeeded in eliminating contamination by V8 protease and the peptide pattern revealed that the charge isomers tested were very similar. The similarity between the charged isomers of core proteins A and B as shown by their one and two-dimensional partial peptide maps indicate that these are probably postsynthetic

modifications of one protein rather than primary structural variants with the same molecular weight. This conclusion coincides with those of other workers. Wilk et al. (1985) have shown that the peptide pattern of the charge isomers of core protein A_2 of HeLa cell (which has the same molecular weight as the rat liver core protein A) are very similar. Wilks and Knowler (1981a) also reported similar tryptic peptide maps of some of the charge isomers of core proteins of rat liver.

It is possible that the charge heterogeneity of core proteins A, B, C and D are caused by the charge modification of one protein. Among the charge modifications likely, acetylation, ADP-ribosylation, glycosylation, methylation and phosphorylation could cause the observed heterogeneity. Negative results were obtained regarding the possible glycosylation of the proteins, as discussed earlier. Possible acetylation of the core proteins is yet to be demonstrated. The other charged modifications mentioned are discussed in turn.

The core proteins were shown to incorporate phosphate both in vitro and in vivo (results 3.3). However when the stoichiometry of phosphate incorporation in vitro for core protein A isomers was determined, considerably less than one mole of phosphate groups per mole of protein was incorporated. It therefore appeared unlikely that phosphorylation alone could be the cause of derivatives A_3, A_4 and A_5. This statement must be tempered however with the realization that the proteins might already have been phosphorylated to near-equilibrium before the in vitro incubation. This could explain the low specific activity and allow A_3, A_4 and A_5 to be phosphorylated derivatives. If it

were true, however one would expect that phosphatases would be capable of converting A_3, A_4 and A_5 into A_1 and A_2. This was not the case. Furthermore, since core proteins A and B do not seem to be phosphorylated in vivo, phosphorylation cannot be the sole cause of the observed charge heterogeneity of the core proteins. Other workers have also shown that while the C core proteins of HeLa are phosphorylated in vivo, core proteins A and B are not (Le Stourgeon et al. 1981; Wilk et al. 1985). The in vivo phosphorylation of the core proteins in this study were performed with Buffalo Rat Liver Cells (BRL). Although the BRL cell core proteins co-migrate with the rat liver core proteins, it would be more relevant to use the cells of a rat liver rather than a long-established cell line. It would be very difficult, however, to incorporate sufficient ^{32}P into rat liver core proteins during the course of in vivo studies. One alternative was to use cells derived from perfused rat liver. This was tried on several occasions but adequate incorporation was not observed.

Preliminary investigations were carried out to find whether the core proteins are ADP-ribosylated (results 3.4). However, when the products which should be produced after treatment of a poly-ADP ribosylated protein with phosphodiesterase I, were looked for with the help of a UV torch, none were observed. It is possible that the UV light was not sensitive enough to detect the presence of the charged groups, ADP-ribose and 5'AMP. To determine whether the ADP-ribose group, if present, could actually be detected by the UV light, a rough calculation was attempted.

Assuming that ADP-ribosylation is limited to A_4, A_5 and A_6 with 2, 3 and 4 moles of ADP-ribose per mole of charged isomer

respectively:

	A_4	A_5	A_6
Proportion of the charge isomers with respect to core protein A (from Table 4)	2.77%	3.33%	3.89%
Since protein A is 41% of total core protein (Table 3), percentage of charged isomers with respect to core protein	1.14%	1.37%	1.59%

Since total core protein used was 20nmol (Experiment started with 1mg total hnRNP protein of which 80% was core protein, that is 800μg core protein).

	A_4	A_5	A_6
pmol of charge isomers are	228	274	318
and pmol of ADP-ribose (X2, X3 and X4 for A_4, A_5 and A_6 respectively)	456	822	1272

Therefore total amount of ADP-ribose = 2.25nmol. It was possible to load only half of the sample on the TLC plate, therefore approximately 1nmol was present on the plate. Since UV light can detect approximately 20nmol of ADP-ribose, the method could only detect the presence of ADP ribose if core protein A had more ADP ribose groups than assumed or if core protein A as well as B, C and D were poly ADP-ribosylated. To conclusively decide whether core proteins are ADP-ribosylated or not, therefore, the experiment would need to be repeated with labelled protein. However, it was not possible to perform the latter experiment in the time available. Among the publications of previous workers, there are conflicting reports about the possible ADP-ribosylation of core proteins. Kostka and Schweiger (1982) have demonstrated the incorporation of labelled NAD into core proteins of rat liver but Song and Adolph (1983) have failed to show similar incorporation in HeLa cells. However the latter workers did not purify the core proteins but examined the ADP-ribosylation of

total nuclear proteins. Since, the 2-dimensional gels of Song and Adolph (1983) contained poly (ADP-ribose) polymerase, which is known to be heavily poly (ADP)-ribosylated (Ogata et al., 1980), it is possible that core protein ADP-ribosylation (if any), was missed.

It is well known that core proteins have N, N-dimethylated arginine residues (Karn et al., 1977; Beyer et al., 1977; Wilk et al., 1985). Only a proportion of the total arginine residues (between 15-25%) are however dimethylated. Since the methyl groups are attached to the basic amino group in the side chain of arginine, this modification will reduce the basicity of the proteins containing the modified arginine residues. It is not known whether the acidic isomers of any of the core proteins contain more dimethylated arginine residues than the basic ones, but it is possible that the varied dimethylation of the arginine residues could contribute to at least some of the charge heterogeneity of the core proteins.

Whether core proteins merely provide an inert packaging material for hnRNA, or actively participate in its splicing is still to be proven. However, the charged modifications of core proteins may assist in the correct aligning of hnRNA for the excision of introns and the linking of the exons in correct order. Histones which like the core proteins are low molecular weight, basic nuclear proteins, are also modified by charged groups. Some of the charged modifications of histones like acetylation, ADP-ribosylation and ubiquitination have been implicated in the control of gene transcription (Reeves, 1984). Acetylation and hyperacetylation of histones has been correlated with active

chromatin regions (Georgieva et al., 1982). Poly ADP-ribosylation of nucleosomal histones and H1 may facilitate gene transcription by relaxing chromatin structure and preventing the formation of higher order configurations (Aubin et al., 1983) and different levels of chromatin organization has also been correlated with differential pattern of phosphorylation of the various histones (Gurley et al., 1978).

In recent years, in vitro splicing systems have been developed (Padgett et al., 1983; Krainer et al., 1984) and very recently, a "splicing complex" associated with functional splicing has been identified (Brody and Abelson, 1985). hnRNP particles and hence core proteins could be a part of this functional "splicing complex" (which Brody and Abelson, 1985 call a spliceosome). If the core proteins do indeed prove to be a part of the spliceosome, then knowledge of their variants and charge modifications will contribute to an understanding of their function.

REFERENCES

Alt, F. W., Bothwell, A. L. M., Knapp, M., Siden, E., Mather, E., Koshland, M., and Baltimore, D. (1980). Cell 20 293-301.

Aubin, R. J., Frechett, A., DeMurcia, G., Mandel, P., Lord, A., Grondin, G., Poirier, G. G. (1983). EMBO J. 2, 1680-1693.

Aziz, S., and Knowler, J. T. (1980). Biochem. J. 187, 265-267.

Babich, A., Nevins, J. R., Darnell, J. E. (1980). Nature (London) 287, 246-248.

Baird, M., Driscoll, C., Schreiner, H., Sciaratta, G. V., Sansone, G., Niazi, G., Ramirez, F., and Bank, A. (1981). Proc. Natl. Acad. Sci. USA 78, 4218-4221.

Bajszar, G., Szabo, G., Simoncsits, A., and Molnar, J. (1978). Mol. Biol. Rep. 4, 93-96.

Banerjee, A. K. (1980). Microbiol. Reviews 44, 175-205.

Beavo, J. A., Bechtel, P., and Krebs, E. G. (1984). Meth. Enzymol. 38c, 299-308.

Berget, S. M. (1984). Nature (London) 309, 179-182.

Beyer, A. L., Bouton, A. H., Hodge, L. D., and Miller, O. L. (1981a). J. Mol. Biol. 147, 269-295.

Beyer, A. L., Bouton, A. H., and Miller, O. L. (1981b). Cell 26, 155-165.

Beyer A. L. (1983). Mol. Biol. Rep. 9, 49-58.

Beyer, A. L., Christensen, M. E., Walker, B. W., and LeStourgeon, W. M. (1977). Cell 11, 127-138.

Beyer, A. L., Miller, O. L., and McKnight, S. L. (1980). Cell 20, 75-84.

Birchmeier, C., Folk, W., and Birnstiel, M. L. (1983). Cell 35, 433-440.

Birnstiel, M. L., Busslinger, M., and Strub, K. (1985). Cell 41, 349-359.

Black, D. L., Chabot, B., and Stiez, J. A. (1985). Cell 42, 737-750.

Blake, C. (1983). Trends Biochem. Sci. 8, 11-13.

Blanchard, J. M., Brunel, C., and Jeanteur, P. (1977). Eur. J. Biochem. 79, 117-131.

Boardman, M., Basi, G. S., and Storti, R. V. (1985). Nucleic Acids Res. 13, 1763-1776.

Bradford, M. M. (1976). Anal. Biochem. 72, 248-254.

Brawerman, G. (1981). Crit. Rev. Biochem. 10, 1-38.

Bray, D., and Brownlee, S. M. (1973). Anal. Biochem. 55, 213-221.

Breathnach, R., Mandel, J. L., and Chambon, P. (1977). Nature (London) 270, 314-319.

Brody, E., and Abelson, J. (1985). Science 228, 963-967.

Brunel, C., and Lelay, M. N. (1979). Eur. J. Biochem. 99, 273-283.

Busby, D. W. G., House, W., and MacDonald, J. R. (1964). in: Virological Technique, 120-127, Chuch Ltd., London.

Busslinger, M., Portmann, R., and Birnstiel, M. L. (1979). Nucleic Acids Res. 6, 2997-3008.

Busslinger, M., Moschonas, N., and Flavell, R. A. (1981). Cell 27, 289-298.

Bustin, M., Hopkins, R. B., and Isenberg, I. (1978). J. Biol. Chem. 253, 1694-1699.

Caballero, E., Baldoma, L., Ros, J., Boronat, A., and Aquilar, J. (1983). J. Biol. Chem. 258, 7788-7792.

Calvet, J. P., and Pederson, T. (1977). Proc. Natl. Acad. Sci. USA 74, 3705-3709.

Capetanaki, Y. G., Ngai, J., Flytzanis, C. N., and Lazarides, E. (1983). Cell 35, 411-420.

Chauveau, J., Moule, Y., and Rouiller, C. C. (1956). Exp. Cell Res. 11, 317-324.

Choi, Y. D., and Dreyfuss, G. (1984a). Proc. Natl. Acad. Sci. USA 81, 7471-7475.

Choi, Y. D., and Dreyfuss, G. (1984b). J. Cell Biol. 99, 1997-2004.

Christensen, M. E., Beyer, A. L., Walker, B., and LeStourgeon, W. M. (1977). Biochem. Biophys. Res. Commun. 74, 621-629.

Chung, D. W., and Davie E. W. (1984). Biochemistry 23, 4232-4236.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977). J. Biol. Chem. 252, 1102-1106.

Cole, C. N., and Stacy, T. P. (1985). Mol. Cell Biol. 5, 2104-2113.

Comings, D. E., and Peters, K. E. (1981). In "The Cell Nucleus" (H. Busch, ed.), Vol 9. 89-118. Academic Press, New York.

Cruz-Alvarez, M., Szer, W., and Pellicer, A. (1985). Nucleic Acids Res. 13, 3917-3923.

Darnell, J. E., Wall, R., and Tushinski, R. J. (1971). Proc. Natl. Acad. Sci. USA 68, 1321-1325.

Del Grande, R. W., and Traugh, J. A. (1982) Eur. J. Biochem. 123, 421-428.

Derenzini, M., Pession-Brizzi, A., and Novello, F. (1981). J. Ultrastruct. Res. 77, 66-82.

DiMaria, P. R., Kaltwasser, G., and Goldenberg, C. J. (1985). J. Biol. Chem. 260, 1096-1102.

Dreyfuss, G., Choi, Y. D. and Adam, S. A. (1984). Mol. Cell Biol. 4, 1104-1114.

Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R., and Hood, L. (1980). Cel 20, 313-319.

Easley, C. W., Petterson, B. W., and Fisher, W. R (1983). Biochim. Biophys. Acta 751, 145-152.

Economidis, I. V., and Pederson, T. (1983a). Proc. Natl. Acad. Sci. USA 80, 1599-1602.

Economidis, I. V., and Pederson, T. (1983b). Proc. Natl. Acad. Sci. USA 80, 4296-4300.

Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, L. E., and Weber, P. (1981). Anal. Biochem. 118, 131-137.

Eschenbruch, M., and Burk, R. R. (1982). Anal. Biochem. 125, 96-99.

Faiferman, I., Hamilton, M. G., and Pogo, A. O. (1970). Biochim. Biophys. Acta 204, 550-563.

Faiferman, I., and Pogo, A. O. (1975). Biochemistry 14, 3808-3816.

Fakan, S., Leser, G., and Martin, T. E. (1984). J. Cell Biol. 98, 358-363.

Fitzgerald, M., and Shenk, T. (1981). Cell 24, 251-260.

Fracker, P. J., and Speck, J. C. (1978). Biochem. Biophys. Res. Comm. 80, 849-857.

Freytag, S. O., Beaudet, A. L., Bock, H. G. O., and O'Brien, W. E. (1984). Mol. Cell Biol. 4, 1978-1984.

Fuchs, J. P., Judes, C., and Jacob, M. (1980). Biochemistry 19, 1087-1094.

Fuchs, E., and Green, H. (1979). Cell 17, 573-582.

Funakoshi, S., and Deutsch, H. F. (1969). J. Biol. Chem. 244, 3438-3446.

Furuichi, Y., LaFiandra, A., and Shatkin, A. J. (1977). Nature (London) 266. 235-239.

Gall, J. G., and Callan, H. G. (1962). Proc. Natl. Acad. Sci. USA 48, 562-570.

Gallinaro, H., Puvion, E., Kister, L., and Jacob, M. (1983). EMBO J. 2, 953-960.

Gallinaro, H., and Jacob, M. (1979). FEBS Lett. 104, 176-182.

Gallinaro, H., Stevenin, J., and Jacob, M. (1975). Biochemistry 14, 2457-2554.

Gallinaro-Matringe, H., Stevenin, J., and Jacob, M. (1977). Differentiation 9, 147-155.

Garrels, J. I. (1979). J. Biol. Chem. 254, 7961-7977.

Gattoni, R., Stevenin, J., and Jacob, M. (1980). Eur. J. Biochem. 108, 203-211.

Georgiev, O., and Birnstiel, M. L. (1985). EMBO J. 4, 481-489.

Georgieva, E. I., Paschev, I. G., and Tsanev, R. G. (1982). Arch. Biochem. Biophys. 216, 88-92.

Gilbert, W. (1978). Nature (London). 271, 501.

Goodman, P. A., and Karn, R. C (1983). Biochem. Genetics 21, 405-416.

Grabowski, P. J., Padgett, R. A. and Sharp, P. A. (1984). Cell 37, 415-425.

Grebanier, A., and Pogo, A. O. (1979). Cell 18, 1091-1099.

Green, M. R., Maniatis, T., and Melton, D. A. (1983). Cell 32, 681-694.

Greenberg, J. R., and Perry R. P. (1971). J. Cell Biol. 50, 774-787.

Grigera, P. R., and Tisminetzky, S. G. (1984). Virology 136, 10-19.

Gronow, M., and Chapleo, M. R. (1979). FEBS Lett. 103, 352-354.

Gurley, L. R., Walters, R. A., Barham, S. S., Deaven, L. L. (1978). Exp. Cell Res. 111, 373-383.

Gurley, L. R., Valdez, J. G., Prentice, D. A., and Spall W. D (1983). Anal. Biochem. 129, 132-144.

Hames, B. D., and Perry, R. P. (1977). J. Mol. Biol. 109, 437-454.

Hamkalo, B., and Miller, O. L. (1973). Annu. Rev. Biochem. 42, 379-396.

Hardy, S. F., Grabowski, P. J., Padgett, R. A., and Sharp, P. A. (1984). Nature (London) 308, 375-377.

Herbert, M. G., and Wall, R. J. (1979). J. Mol. Biol. 135, 879-890.

Heinrich, P. C., Gross, V., Northemann, W., and Scheurlen, M. (1978). Rev. Physiol. Biochem. Parmacol. 81, 101-132.

Heinrich, P. C., and Northemann, W. (1981). Mol. Biol. Rep. 7, 15-24.

Hernandez, N., and Keller, W. (1983). Cell 35, 89-99.

Higgs, D. R., Goodburn, S. E. Y., Lamb, J., Clegg, J. B., Weatherall, D. J., and Proudfoot, N. J. (1983). Nature (London) 306, 398-400.

Hinterberger, M., Petterson, I., and Steitz, J. A. (1983). J. Biol. Chem. 258, 2604-2613.

Hofer, E., and Darnell, J. E. (1981). Cell 23, 585-593.

Holcomb, E. R., and Friedman, D. L. (1984). J. Biol. Chem. 259, 31-40.

Houmard, J., and Drapeau, G. R. (1972). Proc. Natl. Acad. Sci. USA 69, 3506-3509.

Hugle, B., Guldner, H., Bautz, F. A., and Alonso, A. (1982). Exp. Cell Res. 142, 119-126.

Hunkapillar, M. W., Lujan, E., Ostrander, F., and Hood, L. E. (1983). Methods Enzymol. 91, 227-236.

Isenberg, I. (1979). Ann. Rev. Biochem. 48, 159-191.

Ivanova, E., Pironcheva, G., and Djondjurov, L. (1981). Eur. J. Biochem. 113, 569-573.

Jacob, M., Devilliers, G., Fuchs, J. P., Gallinaro, H., Gattoni, R., Judes, C., and Stevenin, J. (1981). In "The Cell Nucleus" (H. Busch, ed.) Vol. 8, 194-245. Academic Press, New York.

Jaggi, R. B., Felber, B., Maurhofer, S., Weber, R., and Ryffel, G. U. (1980). Eur. J. Biochem. 109, 343-347.

Jeanteur, Ph. (1981). In "The Cell Nucleus" (H. Busch, ed.) Vol. 9, 145-170. Academic Press, New York.

Jeffreys, A. J., and Flavell, R. A. (1977). Cell 12, 1097-1108.

Karn, J., Vidali, G., Boffaa, L. C., and Allfrey, (1977). J. Biol. Chem. 252, 7307-7322.

Karp, D. R., Atkinson, J. P., and Shreffler, D. C. (1982). J. Biol. Chem. 257, 7330-7335.

Katan, M., Stevely, W. S., and Leader, D. P. (1985). Eur. J. Biochem. 152, 57-65.

Keller, E. B., and Noon, W. A. (1984). Proc. Natl. Acad. Sci. USA 81, 7417-7420.

Kidwell, W. R., and Purnell, M. R. (1984). Methods Enzymol. 106a, 505-512.

Kindle, K. L., and Firtel, R. A. (1978). Cell 15, 763-778.

King, C. R., and Piatigorsky, J. (1983). Cell 32, 707-712.

Kish, V. M., and Pederson, T. (1975). J. Mol. Biol. 95, 227-238.

Knowler, J. T. (1976). Eur. J. Biochem. 64, 161-165.

Knowler, J. T. (1983). Int. Revs. Cytol. 84, 103-153.

Konarska, M. M., Padgett, R. A., and Sharp, P. A. (1984). Cell 38, 731-736.

Konarska, M. M., Grabowski, P. J., Padgett, R. A., and Sharp, P. A. (1985). Nature (London) 313, 552-557.

Kornberg, R. D. (1977). Annu. Rev. Biochem. 46, 931-954.

Kostka, G., and Schweiger, A. (1982). Biochim. Biophys. Acta 969, 139-144.

Krainer, A. R., Maniatis, T., Ruskin, B., and Green, M. R. (1984). Cell 36, 993-1005.

Kramer, A., Keller, W., Appel, B., and Luhrmann, R. (1984). Cell 38, 299-307.

Kucherer, C., Marty, L., and Blanchard, J. M. (1982). Biochem. Biophys. Res. Commun. 105, 603-609.

Kulguskin, V. V., Krichevskaya, A. A., Lukanidin, E. M., and Georgiev, G. P. (1980). Biochim. Biophys. Acta 609, 410-424.

Laemmli, U. K. (1970). Nature (London) 227, 680-685.

Lahiri, D. K., and Thomas, J. O. (1985). J. Biol. Chem. 260, 598-603.

Lamb, M. M., and Daneholt, B. (1979). Cell 17, 835-848.

Langford, C. J. and Gallwitz, D. (1983). Cell 33, 519-527.

Langford, C. J. Klinz, F. J., Donath, C., and Gallwitz, D. (1984). Cell 36. 645-653.

Lerner, M. R., and Steitz, J. A. (1979). Proc. Natl. Acad. Sci. USA 76, 5495-5499.

Lerner, E. A., Lerner, M. R., Janeway, C. A., Steitz, J. A. (1981). Proc. Natl. Acad. Sci. USA 78, 2737-2741.

Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. L., and Steitz, J. A. (1980). Nature (London) 283, 220-224.

Leser, G. P., Escara-Wilke, J., and Martin, T. E. (1984). J. Biol. Chem. 259, 1827-1833.

LeStourgeon, W. M., and Beyer, A. (1977). In "Methods in Chromatin and Chromosomal Protein Research, G. Stein and L. Kleinsmith, eds. (New York, Academic Press) 387-406.

LeStourgeon, W. M., Beyer A. L., Christensen, M. E., Walker, B. W., Poupore, S. M., and Daniels, L. P. (1978). Cold Spring Harbor Symp. Quant. Biol. 42, 885-897.

LeStourgeon, W. M., Lothstein, L., Walker, B. W., and Beyer, A. L. (1981). In "The Cell Nucleus" (H. Busch, ed.). Vol. 9, 49-87. Academic Press, New York.

Lewin, B. (1980). In "Gene Expression 2", 728-760, John Wiley and Sons, USA.

Lewin, R. (1985). Science 228, 977.

Lothstein, L., Arenstorf, H. P., Chung, S. Y., Walker, B. W., Wooley, J. C., and LeStourgeon, W. M. (1985). J. Cell Biol. 100, 1570-1581.

Louis, Ch., Zimmerman, H. P., and Sekeris, C. E. (1978). Cytobiologie 16, 171-184.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). J. Biol. Chem. 193, 265-275.

Malcolm, D. B., and Sommerville, J. (1974). Chromosoma 48, 137-158.

Mantieva, V. L., Avakjan, E. R., and Georgiev, G. P. (1969). Mol. Biol. USSR 3, 545-553.

Martin, T. E., Billings, P., Levey, A., Ozarslan, S., Quinlan, T., Swift, H., and Urbas, L. (1974). Cold Spring Harbor Symp. Quant. Biol. 38, 921-932.

Martin, T. E., and Okamura, C. S. (1981). In "The Cell Nucleus" (H. Busch, ed.) Vol. 9, pp119-144. Academic Press, New York.

Martin, T. E., Jones, R., and Billings, P. (1979). Mol. Biol. Rep. 5, 37-42.

Mason, P. J., Jones, M. B., Elkington, J. A., and Williams, J. G. (1985). EMBO J. 4, 205-211.

Mather, E. L., Nelson, K. J., Haimovich, J., and Perry, R. P. (1984). Cell 36, 329-338.

Maundrell, K., Maxwell, E. S., Puvion, E., and Scherrer, K. (1981). Exp. Cell Res. 136, 435-445.

Maundrell, K., and Scherrer, K. (1979). Eur. J. Biochem 99, 225-238.

Maxam, A. M, and Gilbert, W. (1980). Meth. Enz. 65, 499-560.

Maxwell, E. S., and Fischer, M. S. (1979). Biochim. Biophys. Acta 562, 319-330.

Mayrand, S., Setyono, B., Greenberg, J. R., and Pederson, T. (1981). J. Cell. Biol. 90, 380-384.

McDevitt, M. A., Imperiale, M. J., Ali, H., and Nevins, J. R. (1984). Cell 37, 993-999.

McKnight, S. L., and Miller, O. L. (1976). Cell 8, 305-319.

McKnight, S. L., and Miller, O. L. (1979). Cell 17, 551-563.

McKnight, S. L., Sullivan, N. L., and Miller, O. L. (1976). Prog. Nucleic Acid Res. 19, 313-318.

McLauchlan, J., Graffney, D., Whitton, J. L., and Clements, J. B. (1985). Nucleic Acids Res. 13, 1347-1368.

Miller, O. L., and Beatty, B. R. (1969). J. Cell Physiol. 74, 225-232.

Molnar, J., Bajszar, G., Marczinovits, I., and Szabo, G. (1978). Mol. Biol. Rep. 4, 157-161.

Montell, C., Fisher, E. F., Caruthers, M. H., and Berk, A. J. (1983). Nature (London) 305, 600-605.

Moore, C. L. and Sharp, P. A. (1985). Cell 41, 845-855.

Morton, R. K. (1954) Biochem. J. 57, 595.

Mount, S. M., Pettersson, I., Hinterberger, M., Karmer, A., and Steitz, J. A. (1983) Cell 33, 509-518.

Mount, S. M. (1982). Nucleic Acids Res. 10, 459-472.

Nabeshima, Y., Fujii-Kuriyama, Y., Muramatsu, M., and Ogata, K. (1984). Nature 308, 333-338.

Nevins, J. R. (1982). Cell 28, 1-2.

Nevins, J. R., and Darnell, J. E. (1978). Cell 15, 1477-1493.

Nevins, J. R., and Wilson, M. C. (1981). Nature (London) 290, 113.

Niessing, J., and Sekeris, C. E. (1970). Biochim. Biophys. Acta 209, 484-492.

Niessing, J., and Sekeris, C. E. (1972). FEBS Lett. 22, 83-88.

Northermann, W., and Heinrich, P. C. (1979). Biochim. Biophys. Acta 564, 67-78.

Northermann, W., Klump, W., and Heinrich, P. C. (1979) Eur. J. Biochem 99, 447-456.

O'Farrell, P. H. (1975). J. Biol. Chem. 250, 4007-4021.

O'Farrell, P. Z., Goodman, H. M., and O'Farrell, P. H. (1977). Cell 12, 1133-1142.

Ogata, N., Kawaichi, M., Ueda, K., and Hayaishi, O. (1980). Biochemistry Int. 1, 229-236.

Ohshima, Y., Itoh, M., Okada, N. and Miyata, T. (1981). Proc. Natl. Acad. Sci. USA 78, 4471-4474.

Oka, J., Ueda, K., and Hayaishi, O. (1978). Biochem. Biophys. Res. Commun. 80, 841-848.

Padgett, R. A., Hardy, S. F., and Sharp, P. A. (1983). Proc. Natl. Acad. Sci. USA 80, 5230-5234.

Padgett, R. A., Konarska, M. M., Grabowski, P. J., Hardy, S. F., and Sharp, P. A. (1984). Science 225, 898-903.

Pagoulatos, G. N. and Yaniv, M. (1978). Eur. J. Biochem. 91, 1-10.

Parker, P. T., Stabel, S., and Waterfield, M. D. (1984). EMBO J. 3, 953-959.

Pederson, T. (1974a). J. Mol. Biol. 83, 163-183.

Pederson, T. (1974b). Proc. Natl. Acad. Sci. USA 71, 617-621.

Pederson, T. (1983). J. Cell Biol. 97, 1321-1326.

Periasamy, M., Brunel, C., Blanchard, J. M., and Jeanteur, P. (1977). Biochem. Biophys. Res. Commun. 79, 1077-1083.

Perry, R. P., and Kelley, D. E. (1976). Cell 8, 433-442.

Peters, K. E., and Comings, D. E. (1980). J. Cell. Biol. 86, 135-155.

Peterson, G. L. (1979) Anal. Biochem. 100, 201-220.

Peterson, R. F. (1972). Methods Enzymol. 25, 178-182.

Pfleiderer, G., and Sumyk, G. (1961). Biochim. Biophys. Acta 51, 482-493.

Pikielny, C. W., Teem, J. L., and Robash, M. (1983). Cell, 34, 395-403.

Pogo, A. O. (1981). In "The Cell Nucleus" (H. Busch, ed.), Vol. 8, pp331-367. Academic Press, New York.

Proudfoot, N. J., and Brownlee, G. G. (1976). Nature (London) 263, 211-214.

Pullman, J. M. and Martin, T. E. (1983). J. Cell. Biol. 97, 99-111.

Puvion-Dutilleul, F., Puvlion, E., and Bernhard, W. (1978). J. Ultrastruct. Res. 63, 118-131.

Quinlan, T. J., Billings, P. B., and Martin, T. E. (1974). Proc. Natl. Acad. Sci. USA 71, 2632-2636.

Quinlan, T. J., Kinniburgh, A. J. and Martin, T. E. (1977). J. Biol. Chem. 252, 1156-1161.

Raj, N. B., Ro-Choi, T. S., and Busch, H. (1975). Biochemistry 14, 4380-4385.

Razzell, W. E. (1963) Meth. Enzymol. 6, 236-258.

Rech, J., Brunel, C., and Jeanteur, Ph. (1979). Biochem. Biophys. Res. Commun. 88, 422-427.

Reddy, V. B., Thimmappaya, B., Dhar, R., Subramanian, K. N., Zain, B. S., Pan, J., Ghosh, P. K., Celma, M., and Weissman, S. M. (1978). Science 200, 494-502.

Reeves, R., Chang, D., and Chung, S.-C. (1981). Proc. Natl. Acad. Sci. USA 78, 6704-6708.

Reeves, R., and Chang, D. (1983). J. Biol. Chem. 258, 679-687.
Reeves, R. (1984). Biochim. Biophys. Acta 782, 343-393.

Rickwood, D., MacGillivary, A. J., and Whish, W. J. D. (1977). Eur. J. Biochem. 79, 589-598.

Risau, W., Symmons, P., Saumweber, H., Frasch, M. (1983). Cell 33, 529-542.

Robinson, A. B., and Rudd, C. (1974). Curr. Top. Cell. Regul. 8, 247-295.

Robinson, A. B., Scotchler, J. W., McKerrow, J. H. (1973). J. Am. Chem. Soc. 95, 8156-8159.

Rogers, J. and Wall, R. (1980). Proc. Natl. Acad. Sci. USA 77, 1877-1879.

Rosenfeld, M. G., Amara, S. G., and Evans, R. M. (1984). Science 225, 1315-1320.

Ruskin, B., Krainer, A. R., Maniatis, T., and Green, M. R. (1984). Cell 38, 317-331.

Ryffel, G. U., Wyler, T., Muellenes, D., and Weber, R. (1980). Cell 19, 53-61.

Sachs, A. B., and Kornberg, R. D. (1985). Mol. Cell. Biol. 5, 1993-1996.

Sadofsky, M., Connelly, S., Manley, J. L., and Alwine, J. C. (1985). Mol. Cell. Biol. 5, 2713-2719.

Salditt-Georgieff, M., Harpold, M., Chen-Kiang, S., and Darnell, J. E. (1980). Cell 19, 69-78.

Samarina, O. P., Lukanidin, E. M., Molnar, J., and Georgiev, G. P. (1968). J. Mol. Biol. 33, 251-263.

Samarina, O. P., and Krichevskaya, A. A. (1981). In "The Cell Nucleus" (H. Busch, ed.), Vol. 9, pp1-48. Academic Press. New York.

Samarina, O. P., Aitkhozihina, N. A. and Besson, J. (1973). Mol. Biol. Rep. 1, 193-199.

Saumweber, H., Symmons, P., Kabisch, R., Will, H., and Bonhoeffer, F. (1980). Chromosoma 80, 253-275.

Scheer, U., and Sommerville, J. (1982). Exp. Cell Res. 139, 410-416.

Schwarzbauer, J. E., Tamkun, J. W., Lemischka, I. R., and Hynes, R. O. (1983). Cell 35, 421-431.

Sekeris, C. E., and Niessing, J. (1975). Biochem. Biophys. Res. Commun. 62, 642-650.

Setyono, B., and Greenberg, J. R. (1981). Cell 24, 775-783.

Sharp, P. A. (1985). Cell 42., 397-400.

Shatkin, A. J. (1976). Cell. 9, 645-653.

Snow, P. M., Cooligan, J. E., and Terhorst, C. (1985). J. Biol. Chem. 260, 2700-2708.

Sommerville, J. (1981). In "The Cell Nucleus" (H. Busch, ed.), Vol. 8, pp1-57. Academic Press, New York.

Sommerville, J., Chrichton, C., and Malcolm, D. B. (1978). Chromosoma 66, 99-114.

Somasekhar, M. B., and Mertz, J. E. (1985). Nucleic Acids Res. 13, 5591-5609.

Song, M.-K. H. and Adolph, K. W. (1983). Biochem. Biophys. Res. Comm. 115, 938-945.

Steck, G., Leuthard, P., and Burk, R. R. (1980). Anal. Biochem. 107, 21-24.

Stevenin, J., and Jacob, M. (1974). Eur. J. Biochem. 47, 129-137.

Stevenin, J., Mandel, P., and Jacob, M. (1970). Bull. Sco. Chim. Biol. 52, 703-720.

Stevenin, J., and Jacob, M. (1979). Mol. Biol. Rep. 5, 29-35.

Suria, D., and Liew, C. C. (1979). Can. J. Biochem. 57, 32-42.

Takahashi, N., Roach, A., Teplow, D. B., Prusiner, S. B., and Hood, L. (1985). Cell. 42, 139-148.

Tamkun, J. W., Schwarzbauer, J. E. and Hynes, R. O. (1984). Proc. Natl. Acad. Sci. USA 81, 5140-5144.

Thomas, P. S., Shepherd, J. H., Mulvihill, E. R., and Palmiter, R. D. (1981b). J. Mol. Biol. 150, 143-166.

Thomas, J. O., Raziuddin, S. A., Boublik, M., and Szer, A. (1981a). Proc. Natl. Acad. Sci. USA 78, 2888-2892.

Thomas, J. O., Glowacka, S. K., and Szer, W. (1983). J. Mol. Biol. 171, 439-455.

Tilghman, S. M., Tiemeier, D. C., Seidman, J. G., Peterlin, B. M., Sullivan, M., Maizel, J. V., and Leder, P. (1978a). Proc. Natl. Acad. Sci. USA 75, 725-729.

Tilghman, S. M., Curtis, P. J., Tiemeier, D. C., Leder, P. and Weissman, C. (1978b). Proc. Natl. Acad. Sci. USA 75, 1309-1313.

Tolan, D. R., Lambert, J. M., Bolieau, G., Fanning, T. G., Kenny, J. W., Vassos, A., and Traut, R. R. (1980). Anal. Biochem. 103, 101-109.

Towbin, H., Staelin, T., and Gordon, J. (1979). Proc. Natl. Acad. Sci. USA 76, 4350-4354.

Treisman, R., Proudfoot, N. J., Shander, M., and Maniatis, T. (1982). Cell 29, 903-911.

Tsai, M., Ting, A., Nordstrom, J., Zimmer, W., and O'Malley, B. W. (1980). Cell 22, 219-230.

Tsanev, R. G., and Djondjurov, L. P. (1982). J. Cell Biol. 94, 662-666.

Valentini, O., Biamonti, G., Pandolfo, M., Morandi, C., and Riva, S. (1985). Nucleic Acids Res. 13, 337-346.

van Eekelen, C. A., Ohlsson, R., Philipson, L., Mariman, E., van Breck, R., and van Venrooij, W. J. (1982). Nucleic Acids Res. 10, 7115-7131.

van Eekelen, V. A. and van Venrooij, W. J. (1981). J. Cell Biol. 88, 554-563.

van Santen V. L., and Spritz, R. A. (1985). Proc. Natl. Acad. Sci. USA 82, 2885-2889.

van Venrooij, W. J., and Janssen D. B. (1978). Mol. Biol. Rep. 4, 3-8.

Wahli, W., Dawid, I. B., Wyler, T., Jaggi, R. B., Weber, R., and Ryffel, G. U. (1979). Cell 16, 539-549.

Walker, B. W., Lothstein, L., Baker, C. L., and LeStourgeon, W. M. (1980). Nucleic Acids Res. 8, 3639-3657.

Wilk, H.-E., Angeli, G., and Schaefer, K. P. (1983). Biochemistry 22, 4592-4600.

Wilk, H.-E., Werr, H., Friedrich, D., Kiltz, H. H., and Schafer, K. P. (1985). Eur. J. Biochem. 146, 71-81.

Wilks, A. F., and Knowler J. T. (1980). Electrophoresis 1, 155-158.

Wilks, A., Cato, A. C., Cozens, P. J., Mattaj, I. W., and Jost, J.-P. (1981). Gene 16, 249-259.

Wilks, A. F. (1980). Ph.D. Thesis. University of Glasgow.

Wilks, A. F. and Knowler, J. T. (1981a). Bioscience Rep. 1, 407-411.

Wilks, A. F. and Knowler, J. T. (1981b). Biochim. Biophys. Acta 652, 228-233.

Woo, S. L., Beattie, W. G., Catterall, J. F., Dugaiczyk, A., Staden, R., Brownlee, G. G., and O'Malley, B. w. (1981). Biochemistry 20, 6437-6446.

Woo, S. L., Dugaiczyk, A., Tsai, M. T., Lai, E. C., Catterall, J. F., and O'Malley, B. W. (1978). Proc. Natl. Acad. Sci. 75, 3688-3692.

Wray, W., Boulikas, T., Wray, V. P., and Hancock (1981). Anal. Biochem. 118, 197-203.