Nuclear proteins associated with gene expression in mouse plasmacytoma cell lines and in rat liver nuclei treated with the carcinogen acetylaminofluorene

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2) Analysis of Plasmacytoma Nonhistone Chromatin Proteins; John C Lincoln and David I Stott; Cell Biology International Reports 4, (1980),p405.

3) Nuclear Proteins and the Control of Gene Expression in Lymphocytes and Plasma Cells; David I Stott and John C Lincoln; The Immune System 2,pp 77-83. A Festschrift for Neils Kaj Jerner on his Seventieth Birthday S.Karger (Basel) (1981).

4) Nuclear Proteins Associated with Gene Expression in Plasmacytoma Cell Lines; David I Stott and John C Lincoln; Molecular Immunology,20,pp 839-850,(1983).

Summary

The subject matter of this thesis is the role of nuclear non histone proteins in the control of gene expression. To understand how these proteins may be able to regulate gene expression we first look at their molecular environment, viz. the structure of chromatin; some of the evidence linking non histone (chromatin) proteins with gene expression is then examined, together with the background of the experimental work reported here.

In the first experimental chapter, the binding of a labelled carcinogen ([^{14}C]-acetylaminofluorene) to rat liver nuclear protein fractions was examined. Most of the recovered carcinogen (80%) was bound to the non-histone protein fraction. After two weeks the label was more evenly distributed; the highest specific activity being in the protein fraction most tightly bound to DNA. Pre-feeding rats with sodium sulphate and phenobarbitone reduced binding to all nuclear fractions. Labelled proteins were further analysed by isoelectric focusing which revealed a complex binding pattern. In the second experimental chapter the nucleoplasmic and non-histone chromatin proteins from four related and two unrelated mouse plasmacytoma cell lines were analysed by biosynthetic labelling with [^{35}S]-methionine followed by one and two dimensional electrophoresis. In this work in clonal cell lines it was hoped that by looking at

cells that were "frozen" at one stage of differentiation, viz. the plasma cell, that it would be possible to both limit the differences between the cell lines and correlate particular proteins with phenotypic differences. The majority of proteins are common to all of the cell lines studied as would be expected if the majority of nuclear proteins are concerned with functions common to all plasma cells. There are however both qualitative and quantitative differences in the nuclear protein patterns of mutant and parent cell lines which appear to correlate with differences in gene expression. The turnover of nuclear proteins in two of the cell lines, MOPC 315.40 (IgA) producer and MOPC 315.32 (λ_2 chain producer), which exhibited these differences, was studied using pulse-chase techniques and the half lives of both nuclear protein fractions and nuclear proteins which differed between the cell lines were calculated.

Finally, the results of these experiments are discussed in the context of findings by other authors working in similar areas and in the light of recent developments concerning the role of these proteins in gene expression.

1. INTRODUCTION

The aim of the work described in this thesis is to examine the role which non-histone chromatin proteins may play in the control of gene expression. The investigation is in two parts; the first part is an examination of the binding of a carcinogen (2-acetylaminofluorene) to nuclear proteins and DNA, and the larger second part is a study of nuclear proteins which may be associated with gene expression in mouse plasmacytoma cell lines.

In this thesis the abbreviation NHCP will refer specifically to non-histone chromatin proteins; these proteins are functionally defined as remaining bound to DNA after extraction of soluble nuclear proteins, RNP particles and histones; the abbreviation NHP will refer to any subset of nuclear non-histone proteins. To understand how these proteins may regulate gene expression we will first examine the structure and organisation of chromatin which provides their molecular environment. Subsequently we will examine evidence linking these proteins with gene expression and look at mechanisms by which control may be brought about, examining briefly some current lines of investigation into the regulation of DNA transcription. Finally, the aims of the experiments detailed in this thesis will be discussed in the context of previous observations.

1.1 The Structure of Chromatin

THE NUCLEOSOME

The primary repetitive unit of chromatin organisation is a nucleoprotein subunit called the nucleosome. Its existence was originally postulated as a result of nuclease digestion experiments (Noll, 1974) and by visualisation under the electron microscope (Olins et al., 1974). Recently the structure of the nucleosome core particle has been solved to 7Å resolution using X-ray diffraction (Richmond et al., 1984). The nucleosome core particle is composed of a flat disc of proteins (two each of histones H2A, H2B, H3 and H4) round which is wound approximately 146 base pairs of DNA. The DNA is in a right handed double helix configuration folded into a left hand superhelix which is bent fairly sharply at several locations (Richmond et al., 1984).

The next level of chromatin organisation is the mononucleosome, which is produced in the early stages of chromatin digestion with micrococcal nuclease. Particles containing approximately 200 b.p. of DNA are released with the concomitant loss of 1 molecule of histone H1 per nucleosome. Histone H1, although not an integral part of the nucleosome core, is essential for the formation of regular higher order structure in

chromatin (Thoma et al.,1979). The distance between nucleosomes along the DNA fibre can vary widely between species (and between tissues in the same organism) with approximately 165 to 250 b.p. per nucleosome (Kornberg, 1977). A number of authors (Defer et al.,1978,Bakayev et al.,1978,Chan and Liew,1979) have demonstrated the release of non-histone proteins in the early stages of nuclease digestion which co-migrate with nucleosomes in sucrose gradients,including high mobility group proteins (discussed on page 24). However this does not prove that they are associated with nucleosomes in vivo as the observed relationships could be caused by interactions between NHCP and nucleosomes occurring during preparation. Detailed reviews of investigations into nucleosome structure are available (McGhee and Felsenfield, 1980, Kornberg and Klug, 1981, Felsenfield, 1978).

HIGHER ORDER STRUCTURES

Early studies of chromatin using the electron microscope revealed thick fibres with a diameter of between 250 and 300 Å (Ris and Kornberg, 1970). The question as to how nucleosomes are packaged into this fibre has been the subject of a large number of studies. Unfortunately, the wide range of experimental conditions used in these experiments makes meaningful comparisons of results difficult. Two main hypotheses have emerged from these studies. The first suggests that nucleosomes form a superhelix or "solenoid" with a pitch of approximately 100Å and with 6-7 nucleosomes per turn (Thoma et al.,1979). The alternative hypothesis is that between 7 and 20 nucleosomes form clusters termed "superbeads" which are arranged tandemly to give the thick fibre (Hozier et al., 1977). Both of these structures would be stabilised by histone H1 which would be located at the region of the entry and exit points of the DNA on the nucleosome (Thoma et al.,1979).

More recent X-ray diffraction evidence (Widom and Klug, 1985), obtained using partially oriented chromatin fibres prepared by drawing concentrated solutions of chicken erythrocyte chromatin into capillaries,would appear to support the solenoid model. However,the results are by no means definitive and a

number of questions remain (reviewed by McGhee and Felsenfield,1986,Butler, 1983). Nevertheless it now seems clear that the 300 Å fibre is probably a superhelical structure containing a small number of nucleosomes per turn. It is possible that the "superbeads" represent a partial disruption of solenoid segments, which may have functional significance (Cartwright et al., 1982). Above the level of the thick 300 Å fibre not enough is known to give a definitive picture until we look at the structure of entire chromosomes.

Paulson and Laemmli have removed the histones from HeLa metaphase chromosomes and found that the dehistonised chromosome consisted of a central proteinaceous skeleton which they termed the "scaffold", from which projected a large number of DNA loops (Paulson and Laemmli, 1977,Earnshaw and Laemmli,1983). However, the existence of a specific "scaffold" remains controversial (Okada and Comings,1980) and may be the result of protein aggregation , a common problem in the study of NHCP. Wray et al. (1980) analysed metaphase chromosomal proteins and interphase chromatin proteins using polyacrylamide gel electrophoresis and found that proteins of approximately 50,000 Mr and 200,000 Mr were prominent components of metaphase chromosomes.

Regarding interphase chromatin a number of authors (Mullenders et al.,1982,Hancock and Hughes,1982(a), Hancock and Hughes 1982(b)(review)) suggest that interphase DNA is attached to a skeletal structure in the form of loops 20 - 200 kilobases in length,and that the peripheral lamina underlying the nuclear envelope is important in the organisation of DNA. Earnshaw et al.(1985) using a polyclonal antibody that recognised mitotic chromosome "scaffolds", suggested that DNA topoisomerase II was a major component of mitotic chromosome "scaffolds". Type II DNA topoisomerases (see Liu,1983,for review) are defined by their ability to pass DNA double strands via enzyme bridged double stranded breaks. The line of evidence presented was that that antibody to mitotic chromosome "scaffolds" inhibited the strand passing activity of topoisomerase II. In addition the same antibody recognised identical partial proteolysis fragments to an antibody raised against purified bovine topoisomerase II.

In later experiments Earnshaw and Heck (1985) used indirect immunoflourescence and immunoelectron microscopy to investigate the location of topoisomerase II. They found no evidence for the rigid core like "scaffold" (Paulson and Laemmli,1977,Earnshaw and Laemmli,1983) but found that the "scaffold" component was localised in numerous separate "islets".

Topoisomerase II appeared to be localised along the axial region of swollen chromosomes indicating that it occurred at the base of each chromatin loop. Despite the controversial nature of the "scaffold" it is possible that the type of organisation shown may be a useful pointer to the higher order structure and organisation of chromosomes.

DIGESTION OF CHROMATIN BY NUCLEASES

In addition to the work of Paulson and Laemmli other clues to the structure of chromatin have been provided by the study of the sensitivity of different parts of chromatin to digestion by particular nucleases (reviewed by Elgin,1976 and 1984 ,Yaniv and Cereghini,1986).

In 1976 Weintraub and Groudine found that haemoglobin gene sequences in chick red blood cells were rendered acid soluble after DNAse I digestion of only 10% of the genome. This result was only observed with red blood cell nuclei and not with nuclei obtained from fibroblasts, brain cells or a population of blood cell precursors. Further research (Stalder et al.1980) revealed that this sensitivity extended to at least 8kb to the 3' side of the gene cluster and to 6 or 7kb on the 5' side. The authors used low levels of DNAse I to

measure the sensitivities of coding and adjacent noncoding regions ,discovering that coding regions have a very sensitive structure, whilst adjacent noncoding regions have a moderately sensitive structure. In addition, they found that DNAse I introduced specific double stranded cuts into both α and β globin gene clusters. Similar results were obtained by Carmon et al.(1982), who found that the DNAse I sensitivity of genes coding for muscle specific protein expressed during myogenesis were not sensitive to digestion in a number of cell types, including muscle precursor cells. Changes leading to DNAse I sensitivity occurred during the transition to terminally differentiated cells.

Lawson et al. (1980, 1982, 1983) and Anderson (1983) working on the the chick ovalbumin gene, found that this gene and its structurally related genes X and Y existed in a 100 kilobase DNAse I sensitive "domain" in oviduct tissue. In contrast the entire domain was resistant to DNAse I in spleen, liver and erythrocyte nuclei. However,the domain remained DNAse I sensitive when transcription of ovalbumin and its related genes was halted by the withdrawal of hormone from oestrogen stimulated chicks. Thus it appears that DNAse I detects an altered chromatin state broadly associated with the potential for a gene to be expressed.

On the other hand it has been found that the sensitivity of chromatin to micrococcal nuclease is closely associated with the frequency of transcription of sensitive genes (Cartwright et al.,1982).

The available evidence suggests a two tiered pattern of chromatin structure in genes with a potential for transcription with the presence of sites hypersensitive to nuclease cleavage superimposed upon a generally increased sensitivity. Some authors have suggested that the "domain" characterised by sensitivity to DNAse I may represent one of the loops of DNA found attached to the "scaffold" of Paulson and Laemmli.

1.2 Evidence Linking Non-Histone Chromatin Proteins with Gene Expression

The non-histone chromatin proteins are a heterogeneous group characterised by their DNA binding properties. They include structural proteins of the nuclear matrix, enzymes (especially those connected with nucleic acid and nuclear protein metabolism), high mobility group proteins, HnRNP particles,actin, proteins associated with chromosome segregation during cell division, and effectors of transcription.

There is now a large body of indirect evidence that non-histone chromatin proteins are involved in gene expression (reviewed by Yaniv and Cereghini, 1976, Stein, 1978, Smulson, 1979, Bustin, 1979, Cartwright *et al.*, 1982, Elgin, 1982, Karavanov and Afanasjev, 1983, Reeves, 1984). However, the precise mechanisms by which proteins exert control of gene expression have yet to be fully established. Early investigations into the role of these proteins involved systems in which there were many changes in gene expression. Results in the main were correlative in nature leaving the precise mode of action of NHCP involved in gene expression undefined.

IMMUNOSPECIFICITY OF NHCP-DNA COMPLEXES

One early experimental approach was to look at the immunospecificity of NHCP-DNA complexes using a complement fixation assay to assess antigenic differences between chromatins derived from different sources. Chytil and Spelsberg (1971) found tissue specific differences between chick oviduct and other organs using this method; Chiu et al. (1974) found differences between normal rat liver and Novikoff hepatoma cells. Zardi et al. (1973) extended these findings by showing that antibodies reacted not only with chromatin but also with NHCP derived from that chromatin, in a species specific reaction. These experiments were correlative in nature and one would be surprised if there were not differences between chromatins derived from different sources.

As time went on experiments became more sophisticated, using antibodies to NHCP as probes to examine the distribution of NHCP in the nucleus and on individual chromosomes. Silver and Elgin(1977) used immunoflourescence techniques to study the chromosomal distribution of three molecular weight subfractions of Drosophila NHCP , they found the fluorescence patterns of polytene chromosomes distinctive and reproducible. They also examined the distribution of their respective

antigens during "heat shock" in which the larvae of Drosophila were subjected to a temperature of 35°C for twenty minutes. This treatment is known to induce a specific set of transcriptionally active chromosome "puffs" with concomitant reduction in the activity of previously active loci. One antiserum detected puffs or loci known to puff. Loci induced by heat shock treatment which previously stained at low levels stained brightly on "heat shock". This work was extended by the production of antisera against individual NHCP (Silver and Elgin,1978), in two cases the antigen was distributed in a limited,specific, and reproducible way whilst a third protein was generally distributed.

In 1979 Campbell et al. raised an an antiserum to a chromosomal protein fraction specific for HeLa cells , the antigen was apparently located in the "scaffold" region of the chromosomes. Extensive nuclease digestion of the "scaffold" resulted in the loss of antigenic activity ,suggesting that DNA binding was required for antigenic activity. Subsequent experiments by Dunn et al. (1980) showed that the antigenic activity could be restored by the addition of Human or HeLa DNA but not Calf or Rat DNA. However DNA from Green African Monkey kidney cells restored antigenic activity to almost the same degree as Human or HeLa DNA ,perhaps indicating that the DNA sequence bound by this protein is conserved

among primates. However, the antigen was not detectable in Monkey chromatin. The development of monoclonal antibodies has made it possible to obtain antibodies to specific NHCP without the difficult job of purifying the antigen first. Howard et al. (1981) produced monoclonal antibodies against a fraction of proteins released from Drosophila embryo nuclei treated with DNAse I. They were able to demonstrate that a particular NHCP preferentially associated with active loci or loci that will be or have been active. Again heat shock loci were only stained with antibodies after they had been induced to puff. In similar experiments Dangli and Bautz (1983) used monoclonal antibodies directed against chromosomal proteins of Drosophila Melanogaster to demonstrate differences in their chromosomal distribution after heat shock regarding their disappearance from activated loci and their appearance in activated loci.

ELECTROPHORETIC ANALYSIS OF NON-HISTONE PROTEINS

One alternative experimental approach used was to examine the NHP complement of nuclei directly using ion exchange chromatography and polyacrylamide gel electrophoresis. In one of the earliest studies Elgin and Bonner (1970) examined the nonhistone chromosomal proteins from a variety of sources (Pea Bud,Rat Liver,Rat Kidney,Chicken Liver and Chicken Erythrocytes) using SDS polyacrylamide gel electrophoresis. They found a high degree of homology and little heterogeneity between NHCP fractions with only the Pea Bud preparation showing striking differences from the remainder. However the limited resolving power of the electrophoresis system used would make it difficult to distinguish proteins other than those present in large quantities. These proteins, as they explain, are likely to be structural proteins and common enzymes of nucleic acid metabolism.

One problem with many early methods of chromatin fractionation was that the use of denaturing agents made it difficult to ascribe biological functions to the isolated fractions. Later authors (Levy et al.,1972, MacGillivray et al.,1972) used combinations of guanidinium hydrochloride/urea and salt/urea respectively to solubilise chromatin. This chromatin was

then fractionated using ion exchange chromatography and analysed by polyacrylamide gel electrophoresis. In a later paper MacGillivray and Rickwood (1974) analysed the components of ^{32}P-labelled nonhistone protein fractions obtained from Mouse kidney,liver and brain chromatins using the fractionation procedure described above, followed by 2-Dimensional Polyacrylamide Gel Electrophoresis. They found that many of the phosphorylated and non phosphorylated proteins were common to chromatins from each tissue,only a few species being tissue specific.

Tallman et al. (1979) examined the NHCP patterns of liver chromatin isolated from male inbred Rats and the males of a heterotic parental hybrid (showing increased hybrid vigour over the inbred parent). A number of differences were observed in the electrophoresis patterns,however the scans of the gels shown revealed only a limited number of peaks with large variations in absorbance between different preparations. A more meaningful analysis might have been made using 2-dimensional polyacrylamide gel electrophoresis. In addition they omitted an analysis of the liver chromatin from the other strain used in the hybridisation which could have provided valuable additional information.

Barrett and Gould (1973), also using an early form of 2-dimensional polyacrylamide gel electrophoresis, compared NHP from rat liver, chicken liver and chicken reticulocytes, finding greater similarity between the two liver tissues than the two chicken tissues. Bekhor et al. (1974), using SDS gel electrophoresis, showed differences in the NHCP complements of various rabbit organs.

DIRECT MEASUREMENT OF INTERACTIONS BETWEEN NHP AND DNA

More recent studies have moved away from purely correlative experiments and tried to directly examine the interaction between NHCP and DNA.

One method of directly measuring the interaction between NHCP and DNA is by using a nitro-cellulose filter assay; free native DNA is not bound to these filters whereas proteins and their complexes with DNA are retained. Umansky et al. (1975), employed this technique on preparations of NHCP from rat liver and thymus. They showed that although most non-histone protein fractions formed complexes with DNA, the majority bound non-specifically. Only a small proportion of NHP bound specifically to homologous native DNA. Similar findings were reported by Gates and Bekhor (1979) who isolated proteins still bound to DNA after extraction of chicken liver chromatin with 2 molar

sodium chloride They observed preferential binding of NHCP to homologous DNA compared to heterologous (E.Coli) DNA.

Lesser and Comings (1978) used an excess of unlabelled E. Coli DNA to sequester non specific binding proteins. They found that mouse liver NHCP combined with labelled mouse DNA was bound to filters to a three to four fold greater extent than when combined with E.Coli DNA. Bluthmann et al. (1978a,b) used hydroxyapatite chromatography and single stranded DNA agarose affinity chromatography to isolate two NHCP which exhibited stronger binding to single stranded DNA than double stranded DNA. Using similar techniques Dastague and Crepin (1979) showed that the binding of mouse NHCP to mouse DNA was twice as great as the binding to Drosophila DNA. This observation was confirmed by using Drosophila NHCP in the reverse experiment.

Lesser et al. (1982) extended the use of the techniques discussed above by using cloned α fetoprotein sequences to identify specific binding proteins in a sequential nitro-cellulose filter binding assay. They identified two proteins of Mr 53,000 and Mr 51,000 which bound specifically near the centre of the cloned α fetoprotein gene segment, and nonspecifically to the ends of all DNA molecules.

EFFECTS OF NHCP ON TRANSCRIPTION

Other authors have looked at the effects of NHCP fractions on DNA transcription *in vitro*. Stein *et al*. (1975) showed that chromatin reconstituted using S-phase non-histone proteins transcribed histone messenger RNA sequences whereas chromatin reconstituted using G_1 non-histone proteins did not. This suggested that these proteins played a key role in the regulation of histone genes during the cell cycle. However prokaryotic *E.Coli* RNA polymerase was used to transcribe the histone genes making interpretation of the results difficult. Sequences transcribable by *E.Coli* RNA polymerase may not necessarily be those transcribed by endogenous RNA polymerases *in vivo* which will have different initiation and promoter requirements.

Bekhor and Samal, 1977,(see also Samal and Bekhor,1977) extracted NHP from rabbit liver chromatin and found two fractions that enhanced RNA synthesis (using homologous RNA polymerase B) from DNA/histone complexes. They found these effects species specific. Kostraba *et al*. (1977a,b) have isolated a NHCP from Ehrlich ascites tumour chromatin that inhibits transcription by binding to DNA, acting at the initiation step of RNA synthesis. However, this protein did not inhibit transcription from chromatin, leaving its "*in vivo*" function unclear. They later isolated a

similar protein from calf thymus chromatin whose electrophoretic mobility and subunit structure differed from the tumour protein (Kostraba et al . ,1977b). On the other hand, Legraverend and Glazer (1980) have purified a 66,000 Mr protein from rat liver,using preparative thin layer electrofocusing followed by ion exchange chromatography . This protein stimulated wheat germ RNA polymerase II using liver chromatin or calf thymus DNA as a template, transcription with E. coli RNA polymerase was not stimulated.

Further evidence for the biological role of non-histone proteins has been provided by Duprat et al. (1977) who studied the effects of NHCP on primary embryonic induction and cytodifferentiation in Salamanders. Treatment of isolated endoderm and/or Blastoporal lip with liver NHCP for four hours before or after their association does not alter their induction or cytodifferentiation. However continuous treatment with homospecific NHCP inhibited cytodifferentiation. NHCP extracted from the livers of other species had no effect. This work was extended by Mathieu et al. (1982) who extracted a non-histone fraction from amphibian (pleurodele) liver cells which inhibited the morphological differentiation of neuroblast cells. This inhibition was species specific as treatment of axolotl neuroblasts elicited no response.

EFFECTS OF HORMONES ON NHCP AND TRANSCRIPTION

Hormones are agents which are able to alter the gene expression of cells and as such are excellent model systems for investigation (for reviews see Cox,1980,Shapiro,1983). One much used experimental system concerns the effect of steroid hormones on the chick oviduct (Spelsberg et al., 1971, 1976, Parker et al., 1976, Tsai et al., 1976a,b, Palmiter and Lee, 1980). Early studies by Spelsberg et al. (1971) using [^{3}H]-progesterone revealed that it formed a complex with a cytosolic receptor protein which bound extensively to chromatin. On the other hand progesterone in combination with cytosol from liver, spleen or chick serum showed very little binding to oviduct chromatin. In addition, extracted progesterone-receptor complex was bound more extensively to oviduct chromatin than other chromatins. They also used chromatin reconstitution techniques to demonstrate that the acidic proteins of the oviduct chromatin (NHP) were responsible for the association of the progesterone-receptor complex with oviduct chromatin. Later Spelsberg and Cox (1976), used immature chicks treated with oestrogen for 14 days and then withdrawn from the hormone to investigate the effects of oestrogen, progesterone, and oestrogen + progesterone on endogenous RNA polymerase activities and template capacities. The appearance of ovalbumin mRNA

was also measured by hybridisation with labelled complementary DNA. They found that progesterone repressed RNA polymerase I and II activity whilst oestrogen alone and oestrogen plus progesterone enhanced their activities. The effects were detectable almost immediately (15 min. after injections). Changes in template capacity (measured using E.Coli RNA polymerase which may in fact be more of a probe of chromatin structure than of actual capacity) correlated with changes in RNA polymerase II activities. However, levels of ovalbumin mRNA increased within 2-3 hours of oestrogen, progesterone or oestrogen + progesterone treatment. Accumulation of ovalbumin mRNA was therefore not always accompanied by an increase in RNA polymerase II activity or chromatin template capacity.

In other experiments using this system Parker et al. (1976) fractionated NHCP from hen oviduct chromatin using a variety of methods and characterised these fractions using polyacrylamide gel electrophoresis.

Parker, O'Malley et al., 1976,Tsai, O'Malley et al., 1976a,b, using chromatin reconstitution techniques, indicated that isolated NHP from the oviducts of oestrogen stimulated chicks were capable of activating the in vitro transcription of the ovalbumin gene. However these experiments were performed using E.Coli

RNA polymerase which can use single strand "nicks" in DNA as initiation sites. The reported results could be attributed to changes in the accessibility of the ovalbumin gene to E.Coli RNA polymerase. In addition these results were also called into question by Palmiter and Lee (1980) who used a direct assay for specific gene transcription, to demonstrate that oestrogen and progesterone had no effect on transcription of the ovalbumin gene using E. coli polymerase. They found that transcription by endogenous RNA polymerase was raised by 5 and 30 fold respectively. They suggest that the specific transcription found by O'Malley et al. using E. coli RNA polymerase was an artefact caused by the polymerase copying contaminating ovalbumin mRNA. In addition there are many problems associated with the use of prokaryotic RNA polymerases in eukaryotic systems which makes it difficult to obtain meaningful results from chromatin reconstitution experiments.

More recent work on the effect of steroid hormones on the ovalbumin and related genes has examined the sensitivity of the genes to nuclease digestion. Ruh and Spelsberg (1983) have partially purified the chromatin acceptor proteins for oestrogen-receptor complexes. They found that the binding of the receptor complex to purified chromatin could be saturated whilst

the binding to DNA remained linear. This nuclear binding was tissue specific and binding sites appeared to be associated with a NHCP fraction extracted from chromatin with 5 molar guanidine hydrochloride. Similar experiments with progesterone-acceptor complexes have shown the acceptor protein in this case to be a low molecular weight protein tightly bound to DNA (Thrall and Spelsberg, 1980, Spelsberg, 1982).

Research has also been carried out on other steroid hormones. Matuo et al. ,1982,(a,b) have isolated a NHCP of Mr 20,000 from the dorsolateral prostate of rats. The NHCP was found to be androgen dependent (it is reduced after castration but restored by administration of androgen). Hamana and Iwai,1978, studied the interaction of the glucocorticoid receptor with chromatin extracted from a variety of rat tissues. Binding of the receptor was highest to liver chromatin and was related to NHCP content but not histone content,binding to DNA was the same in all tissues studied (liver,thymus,prostate,uterus).

CHANGES IN CHROMATIN RELATED TO GENE EXPRESSION

Evidence for the involvement of non-histone proteins in the control of gene expression has been presented. The role of particular known NHP is now discussed, together with the role of post synthetic modification of these and other proteins.

HIGH MOBILITY GROUP (HMG) PROTEINS

The high mobility group proteins, first described by Goodwin, Sanders and Johns (1973), represent one of the most extensively studied groups of NHP. When HMG proteins were originally prepared 16 bands were enumerated on polyacrylamide gels ,later a seventeenth was found (Goodwin et al. ,1975). Subsequent studies have shown that many bands first observed were degradation products, the exceptions being HMG1,HMG2,HMG14 and HMG17. These proteins are characterised by being lysine rich and having a high content of acidic and basic amino acids. Indeed HMGI includes a continuous sequence of 41 aspartic and glutamic acid residues (Walker et al., 1978), and HMG2 contains a similar fragment. These proteins are best considered as two pairs of closely related proteins HMG1 and 2 forming one pair and HMG14 and 17 the other. Although they appear to be too abundant to be

responsible for the activation of specific genes, nuclease digestion experiments suggest they may be associated with active genes (Georgieva et al. ,1981) and much work has been carried out to establish their precise role.

The four HMG proteins have been found to be associated with monomer nucleosomes (Goodwin et al. 1977);in later experiments (Goodwin et al ,1979) it was found that monomer nucleosomes obtained by brief micrococcal nuclease digestion of rabbit thymus were enriched 2 - 4 fold in HMG14 and 17, suggesting that these proteins are associated with transcribed sequences. In addition polyacrylamide gel electrophoresis of these nucleosomes showed that HMG14 and 17 were bound to nucleosomes lacking histone H1 but having 160 bp of DNA. They suggested that HMG14 and 17 bound to the 15 bp of DNA immediately contiguous to the 145 bp core particle.

Weisbrod et al. (1980), using chromatin reconstitution techniques, found that most actively transcribed sequences are rendered sensitive to the action of DNAse I by HMG 14 and 17 and that these proteins can restore DNAse I sensitivity to nucleosome core particles depleted of HMG group proteins. They subsequently used HMG-proteins bound to agarose

(Weisbrod and Weintraub, 1981) to purify "active" nucleosomes.

Stoute and Marzluff (1982) have proposed that HMG proteins 1 and 2 are required for transcription of chromatin by endogenous RNA polymerase. Their evidence is that the addition of HMG1 and 2 to chromatin depleted of NHCP (which dramatically reduces transcription) stimulates RNA synthesis 3 to 5 fold. However, the level of transcription reported was only half that of non-extracted chromatin, suggesting that other factors are at least as important. In addition HMG1 has an amino acid composition so highly charged that the interaction of such a peptide would be expected to have a significant, but not necessarily specific, effect on chromatin structure which may explain its effects on transcription.

It has been demonstrated that the binding of HMG14 and 17 to 160 bp nucleosome cores produces two additional bands when these cores are separated on non-denaturing polyacrylamide gels (Mardian et al.,1980),indicating that nucleosome cores have two specific binding sites for these proteins. Schroeter and Bode (1982) extended these findings showing that HMG14 binding to the nucleosome core is co-operative at physiological ionic strength. They concluded that HMG1

and 2 bind to the 40 bp of linker DNA with little binding to the nucleosome cores. On the other hand McGhee et al.(1982) found that HMG14 and 17 did not prevent the transition of chromatin higher order structure (ie the transition from 10 nm to 30 nm fibre).

The precise role of HMG proteins remains controversial. Goodwin and Johns (1978) and Seale et al. (1983) both using limited nuclease digestion to degrade transcribed sequences (Weintraub and Groudine, 1976) found very little release of HMG proteins and concluded that they were not specifically associated with that part of the genome. On the other hand, Levy-Wilson and Dixon (1978) have reported that HMG proteins are associated with transcriptionally active nucleosomes.

The problem of reconciling such apparently contradictory data may be attributed to the widely different sources of chromatin used in these experiments [Ehrlich ascites cells (Goodwin et al., 1978), calf thymus (Georgieva et al., 1981), mouse myeloma cells (Stoute and Marzluff, 1982), chicken erythrocytes (Schroeter and Bode, 1982), trout testis (Weintraub and Groudine, 1976, Levy-Wilson and Dixon, 1978,Goodwin and Johns, 1978), He La cells (Seale et al., 1983),) and chick embryo erythrocytes (Weisbrod et al., 1980, Weisbrod and Weintraub, 1981)] and that the reported

nuclease digestion and chromatin reconstitution experiments been presented with little quantitation.

Bustin et al. (1981) have demonstrated that the mRNA for HMG proteins HMG1 and HMG2 are polyadenylated and as such resemble those coding for the bulk of cellular proteins and therefore differ from those for histones which are not polyadenylated.

High mobility group proteins are subject to post-synthetic modification. Saffer and Glazer (1982) showed that HMG14 and HMG17 (but not HMG1 and HMG2) were ADP-ribosylated in cultured cells in addition to histone H1.

A number of authors have examined the cellular distribution of HMG group proteins using immunochemical means. Smith et al. (1978), using antisera to HMG1 and HMG2, found that they were localised on chromosomes during mitosis,mouse chromosomes appeared to show a banding pattern. Kurth and Bustin (1982) looked at the distribution of antigenic sites of antibodies to HMG1 in Chironimus Thummi polytene chromosomes and found a distinct banding pattern which changed during the development of the organism. They also detected HMG1 in the cytoplasm. Einck et al. (1984) have studied the localisation of HMG proteins by microinjection of antibody fragments into living fibroblasts. Their

results indicated that the majority of HMG1 and HMG2 is found in the cytoplasm whilst HMG17 is found predominantly in the nuclear fraction. Microinjection of anti HMG17 and anti histone inhibited transcription whereas anti HMG1 and HMG2 did not.

1.3 Post-synthetic modification of nuclear proteins

PHOSPHORYLATION

The phosphorylation and dephosphorylation of proteins catalysed by protein kinases and phosphoprotein phosphatases respectively are important in the regulation of many cellular activities; for example,in their mediation of the effects of cyclic AMP in the control of various metabolic pathways. The phosphorylation of nuclear proteins has,not surprisingly, been the subject of extensive study,so much so that only a brief description of some of the work to date is possible.

Both NHP and histones are phosphorylated by protein kinases which transfer the terminal phosphate group from a variety of nucleoside (and deoxynucleoside) triphosphates to serine and threonine residues; these phosphate groups can be subsequently cleaved by phosphatase enzymes (Kleinsmith, 1975). Early work by Kish and Kleinsmith (1974) revealed an extraordinary degree of heterogeneity among chromatin associated protein kinases and their substrates. They also observed that cyclic AMP could be stimulatory or inhibitory depending on the substrate and kinase fractions used. These findings were extended by Dokas et al. (1978) who fractionated a soluble rat liver

nuclear extract containing total RNA polymerase activity using DEAE-Sephadex chromatography. The unbound fraction contained protein kinase activity which was stimulated by cyclic AMP. The unbound fraction contained protein kinases associated with, but not part of, RNA polymerases I and II which were dramatically inhibited by cyclic AMP.

Thompson et al. (1975),in a study of in vivo and in vitro phosphorylation of nuclear proteins in rat liver found a group of low molecular weight NHP with a very high incorporation of labelled phosphate. These proteins were found at the interface of the phenol and aqueous phases of solutions used to separate proteins and nucleic acids.

Stahl and Knippers (1980) extracted protein kinases from the chromatin of various mammalian cell types finding various partially overlapping substrate specificities. Kitzis et al. (1980) examined the distribution of labelled phosphoproteins released from the chromatin of hepatoma tissue culture cells by limited micrococcal nuclease digestion. The smallest released fragments were enriched in phosphoproteins,in addition protein kinase activity was firmly bound to the released particles suggesting that these proteins are associated with actively transcribing chromatin.

If the phosphorylation of nuclear proteins is related to gene expression one would expect changes occurring during the cell cycle to be reflected in changes in protein kinase activity and degree of phosphorylation of NHP. Karn et al. (1974) showed that the uptake of [^{32}P]-orthophosphate into nuclear proteins in synchronised HeLa Cells varies through the cell cycle, being increased in early G_1 and S phases and minimal during the late G_2 to M period. Stein et al. (1975) used single stranded RNA complementary to histone mRNA to detect histone mRNA in HeLa cells . They found that RNA from S phase cells contained mRNA sequences whereas G1 phase cells did not. In chromatin reconstitution experiments they found that chromatin reconstituted with S phase NHCP transcribed histone genes whereas chromatin from G1 cells does not. However they used E.Coli RNA polymerase in the transcription assay which will have different initiation and promoter requirements to eukaryotic RNA polymerase molecules.

Thompson et al., (1976) using HeLa S3 chromatin showed that a chromosomal phosphoprotein activated transcription of histone m-RNA sequences with chromatin from a phase in the cell cycle when histone genes are normally silent. In an extension of this work Thompson et al. (1979) fractionated nuclear non histone

phosphoproteins and nuclear protein kinases using phosphocellulose chromatography. Each of the five kinase fractions produced a distinctive phosphorylation pattern on non histone phosphoproteins. They found no significant sensitivity to cAMP or cGMP in any of the fractions. On the other hand Limas and Chan-Stier (1979) found that cAMP stimulated nuclear protein kinase fractions isolated from rat myocardial tissue. In more recent experiments Iynedjian and Arslan(1984) have shown that phosphorylation of a subset of nuclear proteins is increased in rat liver cells stimulated with glucagon, and that the effect of glucagon was mimicked by cAMP.

Chintaman et al. (1984) have examined the phosphorylation of NHP during the cell cycle in HeLa cells. They found that the phosphorylation rate increased 8-10 fold during G2,reached a peak during mitosis, and decreased in a similar fashion as the cells divided and entered G1. A number of NHP and histone H1 were phosphorylated suggesting that the phosphorylation of NHP may play a part in mitosis. Philips et al. (1979) extended this work by looking at the variation in nuclear protein kinase activity during the cell cycle, using unfractionated chromosomal proteins as a substrate. Using HeLa S3 cells they showed that the activity of nuclear protein kinases increased during S

and G_2 phase, reduced during mitosis but increased again in G_1 phase. They also demonstrated cell cycle related substrate specificity of kinases fractionated by cellulose phosphate chromatography. In addition they found evidence for NHCP protein kinase activity being associated with nucleosomes. Saffer and Glazer (1982), examined the distribution of phosphorylated HMG proteins in a number of cell types using nuclease digestion techniques. They suggested that phosphorylated HMG14 and 17 may be associated with intranucleosomal regions of active genes. Phosphorylation of NHCP is also associated with lymphocyte activation; this is discussed in detail later.

ACETYLATION

The acetylation of histones has long been thought to be associated with gene expression (reviewed by Delange and Smith, 1971); although many questions remain unanswered as to the precise relationship with RNA synthesis. The acetylation of NHCP has been observed on the *in vitro* treatment of rat liver nuclei with sodium butyrate (Jiakuntorn and Mathias , 1981, 1982) and in human liver cells stimulated to proliferate by epidermal growth factor (Kaneko, 1983) but has not attracted the same attention as histone acetylation. In 1978 Nelson *et al*. (1978) reported that DNAse I

preferentially digested chromatin containing hyperacetylated histones. Their conclusions agreed with those of Jiakuntorn and Mathias (1981, 1982) who studied the incorporation of [^{3}H]-acetate into rat liver slices using various modulators of gene expression. However, Perry and Chalkley (1982), found that although histone acetylation increased the solubility of chromatin (and hence its susceptibility to nuclease attack) they suggested that essentially all DNA sequences in an unsynchronised population were associated with rapidly acetylated histones, a view supported by Yukioka et al. (1983).

POLY ADP-RIBOSYLATION

ADP-ribosylation of proteins (for extensive reviews, see Pekala and Moss, 1983, and Ueda and Hayaishi ,1985) is catalysed by the chromosomal enzyme ADP-ribosyl transferase. This enzyme was first reported in 1963 by Chambon et al. who later demonstrated that its activity was dependent on DNA (Chambon et al., 1966). Miller et al (1975) found that endonucleases stimulated the incorporation of labelled ADP-ribose from NAD into poly (ADP-ribose) suggesting that poly(ADP-ribose) had a role in DNA repair. However, its significance was not fully appreciated until 1976 when

Rechsteiner et al. using a human cell line derived from HeLa cells showed that ADP-ribosyl transferase accounts for the vast majority of cellular NAD breakdown. Rickwood et al. (1977) used purified mouse nuclei to study the incorporation of [^{32}P]-NAD into poly(ADP-ribose). Analysis of the labelled products revealed that a considerable amount of free poly(ADP-ribose) was produced as well as that bound to proteins,the free polymer having the longer chain length. These results were questioned by Minaga et al. (1979) who found that in rat liver over 99% of poly(ADP-ribose) of chain length greater than 4 was covalently bound to non histone protein. They suggested that the free poly(ADP-ribose) found by Rickwood et al. was an artefact of the isolation procedure as protein-poly(ADP-ribose) adducts are unstable at alkaline pH).

Stone et al. (1977) demonstrated that two molecules of histone H_1 could be joined by a single chain of poly-ADP-ribose. Smulson and his associates (Giri et al., 1978 a,b, Jump et al., 1979) using chromatin digested by micrococcal nuclease, found that modified histones associated preferentially with mononucleosomes and dimers and that the higher oligomers contained modified NHP. In addition, they showed that ADP-ribosyl transferase was associated with intranucleosomal "linker" regions and with extended

regions of chromatin (DNA replication fork). Other authors have shown that ADP-ribosyl transferase is dependent on DNA containing "nicks" and that it is involved in cytodifferentiation (Miller, 1975, Farzin et al., 1982). Johnstone and Williams (1982) have demonstrated that ADP ribosylation is obligatory early in the mitogen induced activation of human peripheral blood lymphocytes. It has also been demonstrated that ADP-ribosyl transferase activity is required for DNA repair (Miller, 1975, Durkacz et al., 1980) and for accurate transcription initiation (Ohtsuki et al., 1984).

Proteins can also be modified post-synthetically by de-amidation and by methylation. Less attention has been paid to the role of these modifications in gene expression; however Quick et al. (1981) demonstrated that methylated proteins were tissue specific and associated with proteins tightly bound to DNA.

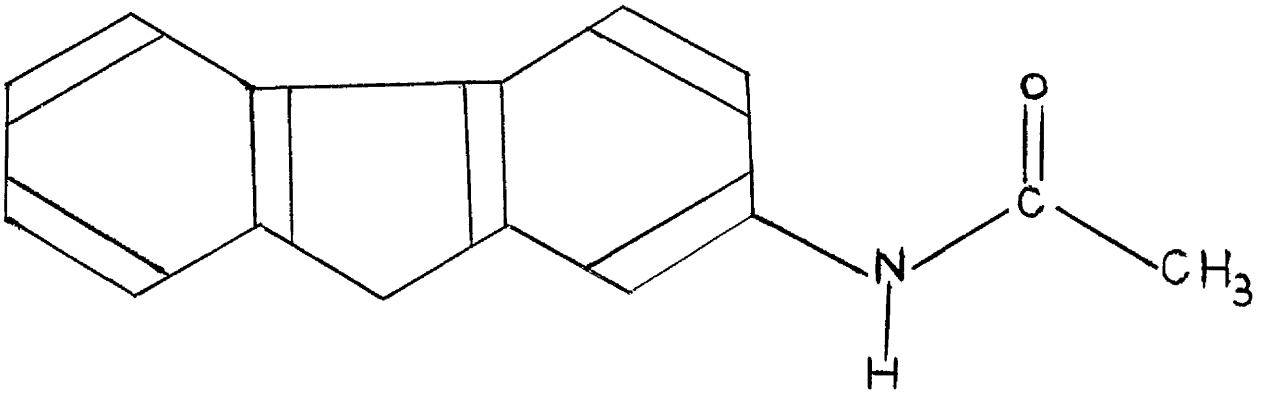
Smulson et al. (1984) used antibody to poly-ADP-ribose coupled to sepharose to fractionate oligonucleosomes and histones. They found that endogenously acetylated histones had a high affinity for the polymer coupled antibody. They suggested that a correlation may exist between these post-translational modifications and that identical histone molecules may

be accessible to both modifications.

DNA METHYLATION

There is now much evidence that methylation of DNA is involved in gene expression (reviewed by Adams and Burdon, 1982, Doerfler, 1983 and Razin and Szyf, 1984). It has been shown by the use of restriction enzymes and paper chromatography that the major modified base is that of cytosine in the dinucleotide CG. There is a correlation between undermethylation and euchromatin and that undermethylation is associated with active genes (Adams and Burdon (1982). Methylation patterns can be maintained since hemi-methylated DNA produced in replication appears to be the natural substrate for DNA methylases (Adams and Burdon, 1982).

1.4 The binding of acetylaminofluorene to nuclear proteins and DNA



2-Acetylaminofluorene

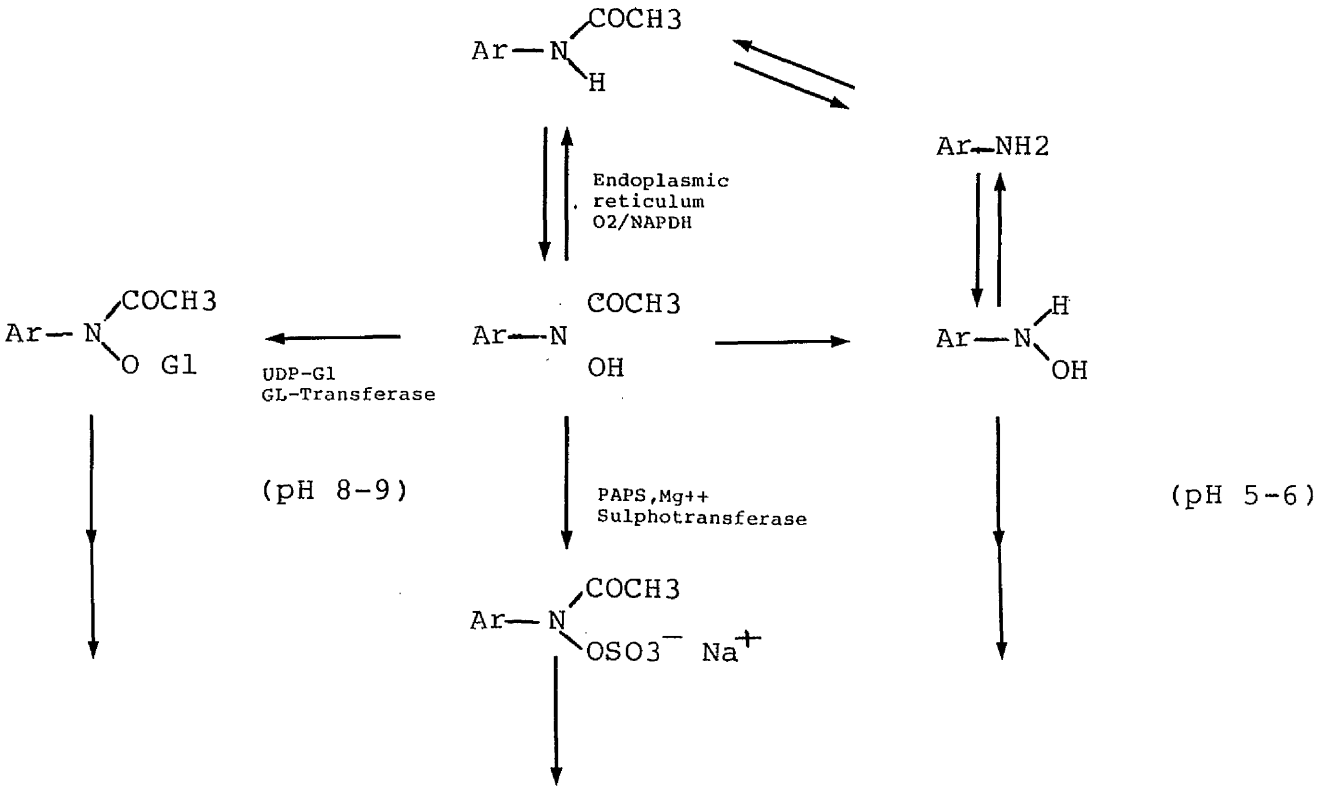
Chemical carcinogens comprise a very diverse group of organic and inorganic compounds with various tissue and species specificities. Carcinogenic compounds induce neoplasia through interactions with cellular components which lead to seemingly irreversible changes in gene expression. If we study and elucidate the mechanism of action of the carcinogen on cellular macromolecules we may gain some insight into the ways in which gene expression is controlled.

Chemical carcinogenesis in humans was first documented by Pott (1775) a surgeon at St Bartholemews Hospital,London, who called attention to the high incidence of scrotal cancer in the chimney sweeps of London. He correctly attributed this to their constant contact with soot and tar.

The aromatic amines are a group of compounds formerly used in the manufacture of aniline based dyes. Indeed, the first description of the relationship between aniline dyes and bladder cancer in humans as an occupationally related malignant disease was made as long ago as 1895 (Rehn,1895). The carcinogen 2-acetylaminofluorene belongs in this group of compounds, it was intended for use as an insecticide. However, despite its lack of acute toxicity,it was found to be carcinogenic in the rat (Wilson et al.,1941). The compound 2-acetylaminofluorene is not itself carcinogenic but is a pre-carcinogen in that it has to be converted metabolically into its reactive form. Because of this and because it causes tumours far away from its site of administration it has been the object of a great deal of study (reviewed by Kriek, 1974). Methods of activation of AAF are shown in Fig. 1.

Figure 1.

Metabolic activation of Acetylaminofluorene in rat liver



Reaction with guanine in RNA, DNA; Met, tyr, trp and cys in proteins.

The chemically reactive metabolites (underlined) have been shown to react non-enzymatically <u>in vitro</u> under physiological conditions with nucleic acids and proteins.

<u>Abbreviations</u>: Ar, 2 Fluorenyl; Gl, glucuronyl; PAPS, 3 phosphoadenosine 5' phosphosulphate.

Early studies appeared to indicate that AAF underwent a two step activation with the sulphate ester as the ultimate carcinogen. Evidence for this included the following observations (reviewed by Kriek, 1974). Weisburger et al., (1972) investigated the activation steps required for carcinogenesis by AAF in rat liver. Acetanilide inhibited the hepatocarcinogenicity of AAF and its activated metabolite N-hydroxy-AAF. Hepatocarcinogenicity could be restored to N-hydroxy-AAF but not AAF by the addition of sulphate to the diet. Thus sulphate ester formation was considered to be a required second activation step. However the required sulphotransferase enzyme system was found in cytoplasmic but not nuclear fractions (Gutmann et al., 1972). More recent evidence obtained by examining the metabolism of AAF in isolated nuclei suggests that binding of AAF to nuclear macromolecules is a consequence of de-acetylation rather than sulphate esterification (Schut et al., 1978, Sakai et al., 1978, Stout et al., 1980). These authors found that nuclei could mediate the binding of N-OH-AAF to DNA and that the addition of 3-phosphoadenosine 5-phosphosulphate generating components did not increase binding to DNA,which would be expected if sulphate esterification was an important step in carcinogen activation. In addition the dominant adduct in nuclear DNA following AAF or N-OH-AAF treatment is

the de-acetylated form (AF). However the susceptibility of rats and mice to hepatocarcinogenesis by N-OH-AAF correlates well with the activity of sulphotransferase (Weisburger et al.,1972) suggesting a role for this method of activation. Presumably its cytotoxic effects stimulate cell proliferation allowing the expression and fixation of mutagen altered genes,thus acting as a promoter of carcinogenesis. Therefore the sulphate ester is probably the ultimate entity in the alkylation of cytoplasmic components.

Schmidt et al.(1984) have studied the antigenic changes taking place in nonhistone proteins during azo-dye hepatocarcinogenesis (using N,N-dimethyl-p(m-tolyazo)-aniline) in the rat. Groups of animals were fed a carcinogen containing diet and animals were sacrificed every three weeks to give a time series of chromatin fractions prepared during carcinogenesis. Antisera to the dehistonised chromatin from azo-dye induced tumours were used to measure the progressive appearance of tumour specific antigens. Normal chromatin had little immunoreactivity. Changes in reactivity were evident after three weeks and the antigenic profiles gradually assumed the characteristics of hepatoma. However there are a number of problems with this type of approach: The feeding of azo dyes will produce

considerable cytotoxicity causing cell division, the progressive increase in the amount of tumour specific antigens may merely reflect the increasing proportion of tumour cells in what is already a heterogeneous cell population,and in addition the antigen source could be from a mixed tumour type.

In similar experiments to the ones described above Burkhardt et al. (1984) used antisera to Morris Hepatoma dehistonised chromatin to investigate the appearance of tumour specific NHCP antigens in rats fed 3-methyl 4-dimethyl-azobenzene. Again immunoreactivity of liver NHCP to the antisera increased over time (with the same reservations as expressed above). The antisera did not react with either foetal liver or regenerating liver suggesting that the NHCP shown are associated with the neoplastic state.

Although evidence is accumulating that DNA is the critical target in the initiation of carcinogenesis by chemicals the role of nuclear proteins may also be important. For example, seemingly irreversible changes in gene expression occur without apparent change in DNA content during differentiation. It is therefore important to study the effects of carcinogen binding to NHP. To this end the binding of 9-[^{14}C]-labelled AAF to rat liver nuclear protein fractions was measured. Rats

were pre-fed with phenobarbitone (which stimulates the microsomal mixed function oxygenases that catalyse the activation of AAF to N-OH-AAF) and sodium sulphate(to stimulate the sulphotransferase activation) in an attempt to modify carcinogen activation. Experiments were carried out with lower specific activity carcinogen (to assess whether proteins become saturated with AAF) and the long term binding of carcinogens to nuclear fractions was measured. Finally, the NHP fractions were further analysed by isoelectric focusing and the histones by polyacrylamide gel electrophoresis.

1.5 Nuclear proteins associated with gene expression in lymphocytes and plasma cells

The immune system of higher animals is a very elaborate system of defence mechanisms which is able to recognise and respond to a virtually unlimited number of non-self determinants (antigens). The immune response mechanism can be divided into two parts:- A humoral response,mainly used to combat free floating antigens,including bacteria and free viruses and a cellular response,dealing with the intracellular phase of viral infections,tumour cells and parasites,responding to infected cells and their cell surface antigens. The humoral immune response is mediated by B-lymphocytes that recognise unique antigens and divide and differentiate into antibody secreting plasma cells. The cellular immune response is largely mediated by T-lymphocytes (processed by the thymus) which have various functions (T-helper cells,T-suppressor cells T killer cells etc.). The receptor cells which mediate these responses are all believed to to be related (reviewed by Hood et al.,1985).

Immunoglobulin molecules contain pairs of light and heavy chains,in most immunoglobulins an identical pair of light chains and a different pair of identical heavy chains form a tetramer. Both light and heavy chains have variable (v) regions that recognise antigens and constant regions which mediate the different effector functions e.g. complement fixation,mast cell degranulation etc. In mice immunoglobulin light chains are encoded by two unlinked gene families, k and λ.

Using recombinant DNA technology mechanisms for the generation of antibody diversity, once such puzzling phenomena, have been elucidated. Immunoglobulin gene rearrangement during B-lymphocyte differentiation leads to the expression of a particular immunoglobulin gene (reviewed by Joho et al., 1984). Briefly,the light chain variable regions are encoded by separate V_L and J_L (joining) segments while the heavy chain variable regions are encoded by V_H D_H (diversity) and J_H gene segments. These gene segments join together to generate complete light and heavy chain genes. This process is antigen independent. The next phase of lymphocyte differentiation is antigen dependent and leads to the formation of IgM-secreting plasma cells in the primary immune response. Further antigenic stimulation may cause further differentiation into plasma cells secreting antibodies of different isotypes. This "heavy

chain switch" is accomplished by translocation of the V_H gene from a position next to one C_H gene to a new C_H gene with concomitant deletion of the old C_H gene and intervening DNA (Joho et al., 1984). Although the genetics of lymphocyte differentiation are now fairly well understood the mechanism by which changes in gene expression occur still need to be established.

One investigative approach has made use of mitogens; these are polyclonal activators of lymphocyte proliferation and differentiation and, as such, provide excellent model systems.

Levy et al.(1973) using a double isotope labelling approach, observed stimulation of NHCP synthesis (including selective stimulation of certain NHCP) as early as 1-3 hours after phytohaemagglutinin (a T cell mitogen) stimulation of lymphocytes isolated from the mesenteric lymph nodes of guinea pigs. In these experiments NHCP were found to account for little more than 15% of the total chromatin proteins but over 70% of the [^{3}H]-leucine label. The low ratio of NHCP to histones may be due protein degradation as protease inhibitors were not used in the preparations of nuclei or in the chromatin fractionation procedures. A number of NHCP were found to be selectively stimulated. Johnson et al. (1974) used the T cell mitogen

concanavalin A (conA) to stimulate equine lymphocytes. They found that conA induced cellular non-histone proteins to bind to chromatin within 15 minutes of its addition. Phosphorylation of certain nuclear non histone proteins was also stimulated, reaching a maximum 8 hours after conA addition. Further work (Johnstone and Hadden, 1975) has shown that cGMP and cholinergic agents (which may mediate the effect of added mitogen) stimulated incorporation of phosphate into specific lymphocyte NHP, whilst cAMP had an inhibitory effect. Decker and Marchalonis (1977),using a double labelling technique, also observed selective increase in the synthesis of NHCP following anti-immunoglobulin or concanavalin-A stimulation of rabbit peripheral blood lymphocytes. These results have been confirmed and extended by other authors (Facchini et al., 1979,Mednieks et al.,1979,Milner and McCormick,1980, Ohtsuki et al.,1980).

Stott et al. (1978a,b, 1979, 1980 a,b, 1981, 1982, 1983) have examined the synthesis and phosphorylation of the NHCP of lymphocytes stimulated by the B cell mitogen lipopolysaccharide. They have demonstrated both stimulation of synthesis and de-novo synthesis of proteins (Stott et al., 1978, 1980a, 1982), the degree of stimulation being characteristic for each

individual protein. In addition, several NHCPs showed linear increases in synthesis which extrapolated back to zero time, indicating that the changes were very early events in lymphocyte triggering occurring well before any detectable commencement of lymphocyte differentiation (Stott,1982). Selective stimulation of phosphorylation of proteins was first observed after four hours in culture. It was suggested that the first nuclear event occurring after lipopolysaccharide stimulation was the appearance of certain NHCPs in the nucleus, the result of increased synthesis, followed by selective phosphorylation of NHCPs and further protein synthesis, including de-novo synthesis.

The work described in this thesis was carried out using cloned myeloma cell lines (for details of their derivation see Mosmann et al., 1979 and Materials and Methods) which are effectively 'frozen' at one stage of differentiation, viz. the plasma cell. By looking at such closely related cell lines it was hoped to restrict the number of differences between their nuclear protein patterns. One of the problems in interpreting previous analyses of nuclear protein patterns was that they employed systems in which there were heterogeneous cell populations and numerous differences in gene expression (Chytil and Spelsberg, 1971, Chiu et al., 1974, Zardi et al., 1973, Barrett and Gould, 1973, Bekhor et al., 1974,

Stein et al., 1975). This makes it very difficult to correlate the observed nuclear protein differences with phenotypic differences. In this work it was hoped to facilitate this correlation by comparing the nucleoplasmic and NHCP patterns of mutant plasmacytoma cell lines and the parent cell line from which they were derived.

Because nuclear proteins are extremely heterogeneous (MacGillivray and Rickwood, 1974, Peterson and McConkey, 1976), high resolution one and 2-D polyacrylamide gel electrophoresis (O'Farrell, 1975, O'Farrell and O'Farrell, 1977) combined with fluorography (Bonner and Laskey, 1974, Laskey and Mills, 1975) was used for their analysis.

Peterson and McConkey (1976) analysed HeLa cell NHCP biosynthetically labelled with ^{35}S-methionine using 2-dimensional polyacrylamide gel electrophoresis. They were able to resolve 470 different species,the majority of which were not detectable in the cytoplasm. The majority of these proteins were represented by 10^3 to 10^4 copies per haploid genome. The predominant protein found in NHCP,nucleoplasmic and cytoplasmic fractions was found to be actin. Although they considered the limit of detection in their system to be proteins occurring at 500 copies per haploid genome they hoped

to increase the sensitivity to 10 molecules per haploid genome in certain regions of the NHCP pattern. Using protein fractions that are biosynthetically labelled with high specific activity [^{35}S]-methionine it is theoretically possible to detect proteins in concentrations as low as 1-10 molecules per cell (Stott, 1980(b)) using this system, although the practical detection limits will probably be an order of magnitude higher. Even in these circumstances it would be necessary for the putative regulatory molecules to be present in an area of the gel relatively free from other proteins.

The objectives of the experiments described in this thesis are as follows:

1) To examine the relative binding of the carcinogen acetylaminofluorene to rat liver nuclear fractions.

2) To examine how this binding is affected by pre-feeding animals with phenobarbitone and sodium sulphate which will affect carcinogen metabolism.

3) To examine the nuclear protein patterns of related and unrelated plasmacytoma cell lines and to establish whether differences in nuclear protein patterns reflect differences in gene expression.

4) To examine the turnover of nuclear protein fractions and individual polypeptides in plasmacytoma cell lines.

Pulse-chase experiments were carried out on selected cell lines to determine the turnover of protein fractions and of selected proteins.

2 MATERIALS AND METHODS

General laboratory chemicals were purchased from BDH (UK) Ltd., enzymes and other proteins from Sigma (UK) Ltd. Photographic materials were bought from Kodak (UK) Ltd. and ion exchange resins from Bio-rad laboratories.

RADIOCHEMICALS

L-[^{35}S]-methionine (636-1,200 Ci/mol) was purchased from Amersham International.

[9-^{14}C]-2-acetylaminofluorene was synthesised according to the method of Nguyen-Hoang-Han et al. (1970) and tested for purity by thin layer chromatography.

CELL CULTURE MATERIALS

The basic medium used for cell culture was RPMI 1640 (powdered) medium which was purchased from Gibco-Bio-cult (UK) Ltd. This was supplemented by foetal calf serum (heat inactivated), benzylpenicillin and streptomycin which were obtained from Flow Laboratories (UK) Ltd.

CULTURE MEDIUM

The standard culture medium consisted of the following:

	RPMI 1640 medium	
plus	10% foetal calf serum	
	Benzylpenicillin	50 units/ml
	Streptomycin	50 μg/ml
	Sodium bicarbonate	2 g/litre

OTHER MEDIA

For freezing cell lines the concentration of foetal calf serum was increased to 20% and 10% dimethylsulphoxide was added to prevent the formation of ice crystals which damage cells. For regrowing frozen cells, a medium containing 50% standard medium and 50% medium "preconditioned" by growing cells (filter sterilised before use) was used.

CELL CULTURE

Plasmacytoma cell lines were grown in 50 ml tissue culture flasks purchased from Nunclon (Denmark). Cells were grown in suspension culture at a concentration of between 2 and 8 x 10^5 cells per ml. The medium was replenished every 2-3 days by removing half of the suspension medium and replacing it with fresh pre-warmed culture medium.

Cell counts were carried out on a routine basis using a Hawksley-Neubauer haemocytometer, cell viability was assessed using trypan blue (.05%) exclusion. There were very few problems of contamination by bacteria, mycoplasma etc using these techniques.

PLASMACYTOMA CELL LINES

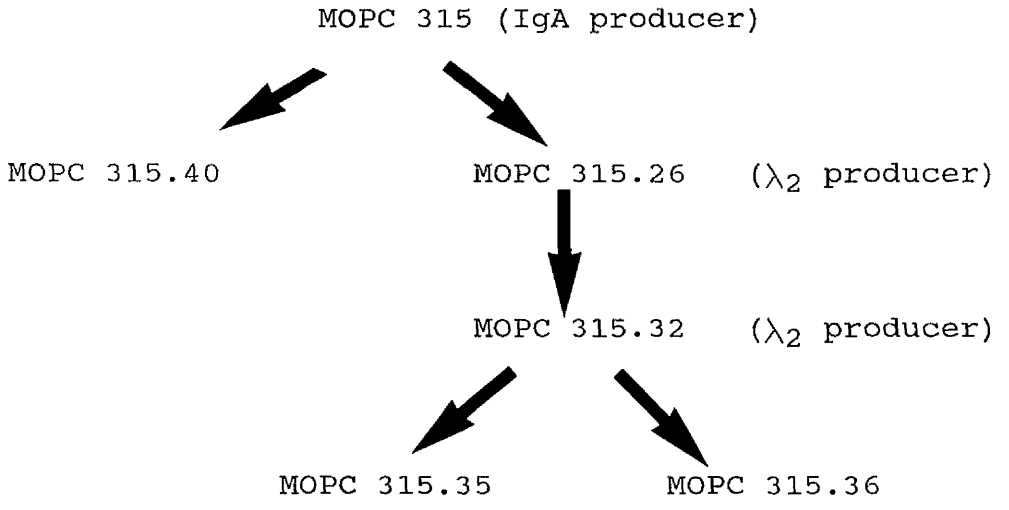
The plasmacytoma cell lines used in this series of experiments were kindly donated by Dr T Mossman and Professor A R Williamson. Their products and sensitivities are listed in the table below.

Cell line	Products	Sensitivities
MOPC 315.40	IgA (α,λ_2, j)	-
MOPC 315.32	λ_2	TG^{R*}, HAT^{S*}
MOPC 315.35	-	TG^{R}, HAT^{S}
MOPC 315.36	-	TG^{R}, HAT^{S}
Y 5781.4	μ, k, j	-
5563T	γ_3 K	-

* TG^{R} : Cells are resistant to thioguanine

* HAT^{S} : Cells will not grow in medium containing hypoxanthine, aminopterin and thymidine.

DERIVATION OF CELL LINES (Mossmann, Baumal and Williamson, 1979).



BACKGROUND NOTES:

The MOPC 315 cell line was produced in a BALB/C mouse by three intraperitoneal injections of 0.5 mls of mineral oil given at 2,4 and six months of age (Eisen et al.,1968). The cell line secretes an IgA (λ_2) antibody specific for 2,4 dinitrophenol and 2,4,6 trinitrophenol. It has been used extensively in experiments on B-Cell regulation and differentiation (reviewed by Lynch et al.,1978 and Abbas,1979)

The derivation of each of the clones is summarised above,each arrow represents one cloning. MOPC 315.40 is a a subclone of MOPC 315, MOPC 315.26 was

derived by testing a number of clones derived from MOPC 315 for loss of heavy chain production without resort to mutagenesis. MOPC 315.26 was then grown in 6-thioguanine (1 ng/ml) to select a resistant mutant,again no mutagenesis was used. The selected mutant MOPC 315.32 could grow in 6-thioguanine (10 ng/ml) but not in medium containing hypoxanthine, aminopterin and thymidine. MOPC 315.35 and MOPC 315.36 were derived by ultra-violet irradiation of MOPC 315.32 to a 0.1% survival rate. Three non-secreting clones were found out of 10,000 tested.

Y5781.4 cells represent native ,IgM secreting plasma cells ,equivalent to 80 - 110 hr. stimulated B-cells and have been extensively characterised by Andersson et al.(1974). 5563T is a tissue culture adapted IgG(k) secreting cell line derived from the 5563 mouse plasmacytoma. It has also been extensively characterised (Williamson,A.R. and Askonas,B.A.,1967).

FREEZING CELL LINES

Aliquots of the plasmacytoma cell lines were preserved in liquid nitrogen for reasons enumerated below:

1. For renewal of cultures in the event of contamination or loss of viability;

2. Cell lines tend to change after many generations in culture; for example, plasmacytoma cell lines can lose the ability to produce immunoglobulin heavy chains. This makes it desirable to return to the original cell lines at the beginning of a series of experiments to ensure reproducibility;

3. Cell culture is both costly and time-consuming making it preferable to discontinue culture of cell lines that are not immediately required.

The preservation of cell lines was accomplished as follows: cells growing in log phase were cooled slowly to approximately $4^{o}C$, harvested and resuspended in ice cold "freezing medium" (described previously) at a concentration of 5×10^{6} cells/ml. Small (1-2 ml) aliquots in ampoules were placed in an expanded polystyrene box which was cooled overnight in a -70^{o} deep freeze (this ensured that the cells cooled down

very slowly). After 24 hours the ampoules were transferred to a liquid nitrogen freezer.

In contrast to the freezing procedure it is necessary to thaw out the cells very quickly. To this end the ampoules were placed in a 37^{o}C water bath until the pellets could be dislodged into 20 ml of pre-warmed tissue culture medium. This both warms the cells and dilutes the dimethylsulphoxide which is toxic. Subsequently the cells were harvested and resuspended in 5 ml of medium "preconditioned" by growing cells (described on page 56) followed by incubation at 37^{o}C. After 24 hours the cell viability was estimated using trypan blue exclusion and the medium replenished.

RADIOLABELLING CELL LINES

MATERIALS

Phosphate buffered saline

NaCl	0.15 M
NaN_3	0.01%
0.01 M sodium phosphate buffer	pH 7.2

Hanks basal salt solution (concentrations in mg/litre)

$CaCl_2.2H_2O$	185.5
KCl	400
KH_2PO_4	60
$MgSO_4.7H_2O$	200
NaCl	8,000
$NaHCO_3$	350
Na_2HPO_4	47.5
Glucose	1,000
Sodium phenol red	17

Labelling Medium

Plasmacytoma cells were labelled in cell culture medium (RPMI 1640 + 10% foetal calf serum) from which methionine was omitted and replaced with [^{35}S]-methionine (636-1,200 Ci/mol) such that the medium contained 200 μ Ci/ml (giving 200 μ Ci per 5 x 10^6 cells).

PROCEDURE FOR RADIOLABELLING CELL LINES

The plasmacytoma cell lines to be labelled were growing in log phase for several days prior to labelling. Between 2.5 and 5 x 10^7 cells were used in each experiment and the initial cell viability was between 90% and 98% as measured by trypan blue exclusion. Plasmacytoma cells were washed twice in Hanks basal salt solution (containing 5% foetal calf serum) and suspended in labelling medium at a concentration of 5 x 10^6 cells/ml. The cells were incubated for four hours at 37^{o}C in a 25 ml disposable tissue culture flask (Nunclon, Denmark). After four hours' incubation the cells were spun down (400 g for 5 minutes) and washed in ice cold phosphate buffered saline containing 1 mM unlabelled methionine to halt further incorporation of label. The labelling medium was retained, stored at -20^{o}C and later tested for the presence of labelled immunoglobulin. The cell pellets were quick frozen in a bath of solid CO_2/ethanol and stored at -70^{o}C until required.

PULSE-CHASE EXPERIMENTS

Initially the cell lines to be tested were labelled in the same manner as in other experiments

(described above). At the end of the labelling period cells were washed twice in pre-warmed Hanks basal salt solution containing 5% foetal calf serum and 1 mM unlabelled methionine (to displace and free L-[^{35}S]-methionine). The cells were then resuspended in fresh pre-warmed culture medium at a concentration of 5 x 10^5 cells/ml and incubated at 37^{o}C. Samples were taken at intervals after the beginning of incubation (0, 1, 2, 4 and 20 hours). The samples were subsequently treated in the same way as other radiolabelled cell samples. Cell viability dropped slightly during the course of the incubation from an initial level of around 90% to 80-85%.

THE PREPARATION OF NUCLEAR PROTEIN FRACTIONS FROM RADIOLABELLED CELLS

CELL LYSING SOLUTION (HK)

	Sucrose	0.25 M	pH 8.0
	$CaCl_2$	3 mM	
	Tris/HCl buffer	10 mM	
	Na_2MoO_4	0.5 %	
Solution A	NaCl	0.08 M	pH 6.3
	E.D.T.A.	0.02 M	
	Na_2MoO_4	1 mM	
Solution B	NaCl	0.35 M	
	Na_2MoO_4	1 mM	
Solution C	Tris/HCl	2 mM	pH 7.5
	E.D.T.A.	0.1 mM	
	Na_2MoO_4	1 mM	
Solution D	Tris/HCl	0.1 M	pH 7.5
	$MgCl_2$	2 mM	
	$CaCl_2$	2 mM	
	Na_2MoO_4	1 mM	
Solution E	Deionised Urea	8 M	pH 8.0
	E.D.T.A.	0.02 M	
	Tris/HCl	0.25 M	

Solution F	Deionised Urea	9 M	}	
			}	
	L-Lysine	1 mM	}	
			}	pH 8.0
	Na_2MoO_4	1 mM	}	
			}	
	Tris/HCl	0.01 M	}	

Trichloroacetic acid 5% and 100%.

Bovine pancreatic deoxyribonuclease (Sigma, 200 Kunitz units/mg) 1 mg/ml in solution D.

Bovine pancreatic ribonuclease (Sigma, 100 Kunitz units/mg) 1 mg/ml in solution D.

The DNAse and RNAse solutions were prepared and then quick frozen in a bath of solid CO_2/ethanol in 100 μl aliquots and stored at -70^{o}C (DNAse) or -20^{o}C (RNAse).

To prevent the degradation of nuclear protein fractions by chromatin associated proteases extraction procedures were carried out in the presence of 1 μM pepstatin and 1 mM phenylmethylsulphonylfluoride (PMSF) which completely inhibits chromatin associated protease activity (Stott and Williamson, 1978a). 1 μl of PMSF (dissolved in dimethyl-formamide) and 1 μl of pepstatin (dissolved in 50% ethanol) were added per ml of solution to HK and A-F, with rapid stirring, on the day of use.

Sodium Molybdate (1 mM) was added to the solutions to inhibit nuclear protein phosphatases.

PREPARATION OF NUCLEAR PROTEIN FRACTIONS FROM LABELLED CELLS

The preparative procedure used is a modification of the method of Wilson and Spelsberg (1973) described by Stott and Williamson (1978b). The procedure has been further modified and scaled down so that manipulations can be carried out in 1.5 ml Eppendorf centrifuge tubes. This reduction in scale allows the preparation of samples of a specific activity higher then hitherto possible, this in turn increases the resolution of the electrophoresis system. In addition, centrifugation times (using a fixed speed micro-haematocrit centrifuge at 10,000g) are greatly reduced. Using this method it is possible to reduce the number of cells required for preparative purposes from 5×10^8 to 5×10^7, any shortfall below 5×10^7 cells was made up by the addition of unlabelled "carrier" mouse spleen cells up to the requisite number. Extractions were carried out using a Potter-Elvehjem teflon/glass homogeniser (0.11-0.15 mm clearance). The homogeniser speeds quoted refer to the free running speed of the pestle in air. All procedures were carried out at 0-4^{o}C except where otherwise stated.

PREPARATION OF NUCLEI

1) Washed plasmacytoma cells were suspended in 1 ml of lysing solution (HK.) and disrupted by homogenisation at 2,400 r.p.m. using 10 strokes of the pestle. The homogenate was centrifuged at 900 g for 5 minutes, the supernatant (cytoplasmic fraction) was retained to check for antibody synthesis by the cell lines. The pelleted nuclei were then washed twice in 5 ml of the same solution, a sample of nuclei from the last wash was examined using phase contrast microscopy to check for cytoplasmic contamination and whether any lysis of nuclei had occurred. Only preparations containing clean nuclei were used in experiments. If there was any evidence of cytoplasmic contamination homogenisation and washes were repeated until a "clean" sample was obtained.

PREPARATION OF CHROMATIN

2) The nuclear pellet was suspended in 1 ml of solution A and the nuclei ruptured by homogenisation at 8,400 r.p.m. using 20 strokes of the pestle. This homogenate was centrifuged at 10,000 g for 2 minutes (using the fixed speed micro-haematocrit centrifuge housed in a cold cabinet). The supernatant (nucleoplasmic proteins) was retained in an Eppendorf

tube, the pellet was re-homogenised and spun down twice more. A sample of the homogenate was examined under a phase contrast microscope to verify that all the cell nuclei were broken.

3) The pellet from step (2) was resuspended in 1 ml of solution B, homogenised at 6,000 r.p.m. using 5 strokes of the pestle and centrifuged as in step (2). The supernatant (containing proteins loosely bound to chromatin (fraction B)) was retained in an Eppendorf tube.

4) The pellet from step (3) was resuspended in 1 ml of solution B and centrifuged as in step (2). The supernatant was discarded as it contained only negligible quantities of protein, the pellet (chromatin) was retained for further analysis.

It should be pointed out that steps (3) and (4) were only carried out in initial experiments being omitted when it was established that fraction B contained only a small proportion of labelled proteins (which were of limited heterogeneity).

REMOVAL OF HISTONES

5) The pellet remaining after steps (2) or (4) was homogenised gently in 1 ml of ice cold 0.2 M H_2SO_4 and

left to stand for 15 minutes at 0°C. After centrifugation (as in (2)) the supernatant (histones) was saved in an Eppendorf tube. The pellet (dehistonised chromatin) was washed in 1 ml of 0.2 M H_2SO_4 and centrifuged as in step (2). The supernatant was discarded and the sides of the Eppendorf tube carefully dried with tissue.

6) 50 μg of haemoglobin was added to A and B (to aid precipitation by acting as a "carrier protein") followed by 50 μl of 100% trichloroacetic acid, 100 μl of tricholoracetic acid was added to the histone solution if required. The protein fractions were again centrifuged as in step (2), the supernatants were discarded and the sides of the tubes dried carefully with tissue.

REMOVAL OF RNA AND DNA

7) The pellets from step (5) (NHCP) and step (6) (A, B and histones) were homogenised in 1 ml of solution D. The pH of the resulting homogenate was checked using narrow range indicator paper and adjusted to pH 7.0 to 7.5 by the addition of 1 M tris pH8 if necessary. 25 μl of DNAse (1 mg/ml) and 5 μl of RNAse (1 mg/ml) were added to the samples which were then incubated at 30°C for 30minutes.

8) 50 μl of 100% trichloroacetic acid was added to fractions A, B and the NHCP (100 μl to histones), the solutions were vortimixed and left to stand on ice for 10 minutes. After centrifugation (as in step (2)), the supernatants were discarded, the pellets were drained and resuspended in 200 μl of distilled water. Proteins were precipitated by the addition of 1 ml of acetone and the tubes left to stand on ice for 10 minutes. Following centrifugation (as in step (2)), the supernatants were discarded and the protein pellets drained.

PREPARATION OF SAMPLES FOR RUNNING ON GELS

All protein samples were reduced and alkylated to disrupt inter and intra chain disulphide bonds and prevent their reformation. This was carried out to prevent protein aggregation through disulphide "bridges" between different polypeptide chains ensuring that proteins will run as single polypeptide chains on SDS gels.

9) The protein precipitates from step (8) were dissolved in 90 μl of solution E, 10 μl of the reducing agent dithiothreitol (0.5 M, dissolved in solution E) was added to each of the samples which were then incubated at 37^{o}C for 30 minutes. 20 μl of the alkylating agent iodoacetamide (1 M, dissolved in solution E) was then added and the samples incubated for a further thirty minutes at room temperature.

10) The reduced and alkylated proteins were precipitated by 0.6 ml of acetone, left for 10 minutes at room temperature and spun down. The supernatants were discarded and the pellets taken up in 200 μl of distilled water, 1 ml of acetone was added and the samples left to precipitate for 10 minutes at 0^{o}C. The samples were centrifuged as before and the supernatants discarded.

11) Finally, the protein samples were dissolved in 100 μl of solution F, the proteins were incubated at room temperature (with regular vortimixing) to ensure that the samples were completely dissolved. Duplicate 2 μl samples were added to 2 ml of Triton-toluene scintillation fluid for estimation of radioactivity. The protein samples were stored at -20^{o}C until required for further analysis.

PREPARATION OF RAT LIVER NUCLEI

The method employed was a modification of the Chauveau technique (Higashinokagawa et al., 1972). All procedures were carried out at 0-4°C unless otherwise stated. Lightly anaesthetised S.P.F. grade male Wistar rats were killed by cervical dislocation, their livers were immediately removed and washed in ice cold sucrose (0.25 M sucrose, 10 mM Mg^{++}, 0.1 M P.M.S.F.). The livers were minced with scissors and washed with ice cold sucrose until all traces of blood were removed. The minced liver was homogenised in ice cold sucrose (7 up and down strokes with a teflon/glass motor homogeniser, 100 ml of sucrose solution per rat liver) and then filtered through four layers of muslin to remove debris (connective tissue, blood vessels, etc.). This homogenate was centrifuged at 1,000 g for 15 min. and the nuclear pellet re-homogenised in 2.3 M sucrose, 10 mM Mg^{++}, 0.1M P.M.S.F. (100 ml/rat liver). The nuclei were then centrifuged at 50,000 g for 75 min. The nuclear pellet was removed using a small spatula and washed in 0.32 M sucrose, 1 mM Mg^{++}, 0.3% v/v Triton N101 detergent to remove any remaining cytoplasm and the remaining nuclear membrane. The nuclear preparation was finally examined using phase-contrast microscopy to assess the degree of cytoplasmic contamination and the integrity of the nuclei.

PREPARATION OF NUCLEAR FRACTIONS

Proteins were extracted from the rat liver nuclei using a modification of the method of Gronow and Griffiths (1971); all procedures were carried out at 0-4^{o}C except where stated. The nuclear pellet was resuspended in 5 ml of 8M urea, 50 mM phosphate pH 7.6, using a small spatula. This contained 30 μ moles of N-ethyl maleimide to alkylate sulphydryl groups to prevent protein aggregation. The ruptured nuclei were spun down at 3,000 g for 10 minutes and the extraction procedure repeated twice more. The extracts were pooled, dialysed against 8 M urea and stored at -20^{o}C until required for further analysis.

Histones were extracted from the pellet by homogenisation in 5 ml of 0.25 M H_2SO_4 followed by sedimentation at 3,000 g for 10 min. This procedure was repeated twice more and the histones precipitated from the combined extracts using 5 volumes of acetone. The residual material was dissolved in 1% S.D.S. 100 mM Tris, 0.25 mM E.D.T.A., pH 8, and extracted twice with an equal volume of phenol saturated in the same buffer. The phenol layer (containing proteins) was dialysed first against distilled water (to remove phenol) and then against 8 M urea 50 mM phosphate, pH 7.6 (to

solubilise proteins), which were then stored at -20°C. The DNA was precipitated from the aqueous phase by the addition of 2.5 volumes of ethanol followed by incubation overnight at -15°C. Protein concentrations were measured using the method of Lowry (1951) using a solution of bovine serum albumin as a standard. DNA concentrations were measured using the Burton (1976) modification of the diphenylamine estimation using calf thymus DNA as a standard. Radioactivity was measured by taking duplicate 50 μl samples at appropriate points in the preparation of fractions and adding them to a scintillator cocktail containing Triton x 100. These samples were counted in an Intertechnique Multi-8 scintillation counter which computed dpm from an external standard.

POLYACRYLAMIDE GEL ELECTROPHORESIS

S.D.S. GEL ELECTROPHORESIS

The method employed was that of Laemmli (1970) and King and Laemmli (1971) which was modified for the electrophoresis of nuclear proteins by the incorporation of urea (4 M) into the stacking and separating gels. The gels were cast between clean glass plates using silicone plastic tubing as a spacer (2.5 mm bore x 0.8 mm wall thickness) giving a gel thickness of approximately 1.6 mm. Sample wells were cast by inserting a teflon "comb" into the top of the stacking gel. Samples were underlayered below buffer into the wells using Portex tubing attached to the tip of an Eppendorf micro-pipette.

The electrophoresis apparatus used was built in the workshops of the University of Glasgow Department of Virology. It consisted of a large lower buffer reservoir and a smaller upper buffer reservoir, this arrangement allowing the glass plates to be cooled by immersion in the lower electrophoresis buffer. A filter paper wick (Whatman 3 mm) was used to form a bridge between the upper electrophoresis buffer and the top of the gel. Samples were electrophoresed at a constant current of 20-25 mA until the bromophenol blue marker dye had just reached the bottom of the gel (14-16 hours

at room temperature). The length of the separating gel (10% acrylamide) was 20 cm. After electrophoresis the gels were fixed in 5% trichloroacetic acid and processed for fluorography.

POLYACRYLAMIDE GEL ELECTROPHORESIS SOLUTIONS

The following solutions were used for making up one dimensional S.D.S. gel electrophoresis gels and for the second dimension of 2-D gel electrophoresis.

Acrylamide Stock Solution

Acrylamide	333 g
Bisacrylamide	9 g
H_2O	to one litre

The acrylamide solution was deionised by passage through mixed bead ion exchange resin AG 501-X8 (D) 20-50 mesh (Bio-Rad laboratories).

Urea S.D.S. Stock Solution

Urea 9 M (deionised)	90 ml
Sodium dodecyl sulphate 10% w/v	2 ml
H_2O	to 100 ml

This solution was made up freshly on the day of use.

Stacking Gel Buffer (8 x stock solution)

Tris	36.6	g
1N HCl	48	ml
T.E.M.E.D.	0.23	ml
1N HCl to pH 8.8		
H_2O to	100	ml

Riboflavin Solution (8 x stock)

Riboflavin	10	μg
H_2O to	250	ml

The separating gel was polymerised using ammonium persulphate (50 ml per 100 ml of solution G), the stacking gel was polymerised using riboflavin (1.25 mg/ml in stacking gel) and light.

Reservoir Buffer (10 x stock solution)

Tris	150	g
Glycine	720	g
Sodium dodecyl sulphate	50	g
H_2O to	5	litres

Sample Buffer (5 x stock solution)

Tris	7.56 g
S.D.S.	20 g
Bromophenol blue	0.01 g
Adjust to pH 6.8 with HCl	
H_2O	100 ml

Sample Buffer (made up freshly as required)

Urea	5.4 g (giving a final concentration of 9 M)
Sample buffer stock	2.0 ml
H_2O	4.0 ml

Equilibration Buffer (for 2-D gel electrophoresis)

Urea	54 g (giving a final concentration of 9 M)
Sample buffer stock	20 ml
Glycerol	10 g
Lysine (0.1 M)	1 ml
H_2O	31 ml

The stock solutions could be used to give a variety of acrylamide concentrations in the separating gel, the volumes are shown below. The acrylamide concentration in the stocking gel was 4%.

SEPARATING GEL (100 ML)

Final acrylamide concentration (%)

Solution vol. (ml)	5	7.5	10	12.5
Separating gel buffer	12.50	12.50	12.50	12.50
Acrylamide	15.00	22.50	30.00	37.50
Urea/S.D.S. solution	50.00	50.00	50.00	50.00
H_2O	22.50	15.00	7.50	-

The solutions were mixed and degassed, 0.25 ml of 10% ammonium persulphate was then added to catalyse the polymerisation of acrylamide.

Stacking Gel (4% acrylamide)

Stacking gel buffer	4.0 ml
Acrylamide stock solution	3.8 ml
Urea/S.D.S. solution	16.0 ml
H_2O	4.2 ml

The solutions were mixed and degassed, 4.0 ml of riboflavin was then added, the acrylamide was polymerised by exposure to a fluorescent lamp.

TWO DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

This was carried out using a modification of the method of O'Farrell (1975) and O'Farrell and O'Farrell (1977).

FIRST DIMENSION STOCK SOLUTIONS

Solution A

	Amount	Final concn.
Urea	5.5 g	9 M
Solution E (Noniodet P40)	2.0 ml	2 %
Ampholine pH 3.5-10	0.5 ml	2 %
Lysine solution (0.1 M)	0.1 ml	1 mM
H_2O	3.3 ml	

After being made up this solution was divided into 200 μl aliquots and stored at -20^{o}C.

Solution D

Acrylamide		33.3 g
Bisacrylamide		1.9 g
H_2O	to	100 ml

This was deionised by passage through a mixed bed ion exchange resin AG 501-X8 (D) 20-50 mesh (Bio-Rad laboratories).

Solution E

10% w/v Noniodet P40 in H_2O

Solution H

Urea	8 M (deionised)
Lysine	1 mM

This solution was stored frozen at -20^{o}C.

Solution K (stored at -20°C in 200 µl aliquots)

	Amount	Final concn.
Urea	4.8 g	8 M
Ampholine pH 3.5-10	0.25 g	1 % w/v
Lysine (0.1 M)	0.10 ml	1 mM
H_2O	6.10 ml	

Solution L

Urea	9 M (deionised)
Tris	0.01 M pH 8
P.M.S.F.	0.001 M
Pepstatin	0.7 µg

The isoelectric focusing gel was made up as follows:

Urea	5.5 g
Solution D	1.13 ml
Solution E	2.0 ml
H_2O	2.0 ml
Ampholine	0.5 ml (0.2 ml pH 4-6, 0.2 ml pH 6-8, 0.1 ml pH 3.5-10)
Lysine (0.1m)	0.1 ml

The solution was degassed, 2.5 ml of TEMED and 0.1 ml of riboflavin stock solution were added. Glass isoelectric focusing tubes (130 mm x 2.5 mm I.D. cleaned in chromic acid, washed in tap water, washed 3 x in distilled water, siliconised and re-washed) were filled

up to a 10 cm mark with gel solution, overlayered with solution H and polymerised under a fluorescent lamp for 1-2 hours. The lower electrophoresis buffer (anode) was 0.01 M H_3PO_4 and the upper electrophoresis buffer (cathode) was 0.02 M NaOH (degassed).

TWO DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

Two dimensional polyacrylamide gel electrophoresis separates proteins according to their net charge by isoelectric focusing in the first dimension and by S.D.S. gel electrophoresis in the second dimension which separates according to molecular weight. Hence this system separates proteins by two unrelated parameters giving an excellent resolution. The technique has a theoretical upper limit of resolution in the region of 5,000 different proteins (Peterson and McConkey (1976). Isoelectric focusing was carried out in siliconised glass tubes (130 mm x 2.5 mm internal diameter) with a gel length of 10 cm. Focusing was achieved at a constant current of 0.1 milliamps per gel until 400 V was reached, at this point the power pack was pre set to cross over to a constant voltage of 400 V. At the end of the run the voltage was increased to 500 V for 1 hour to "sharpen" the protein bands, the total run length was 5,000-7,000 volt/hours. Sample gels were equilibrated in sample gel buffer including

10% glycerol (see 1-dimension S.D.S. gel solutions) for 2 hours (with one change of buffer), quick frozen in sealed tubes immersed in solid CO_2/ethanol and stored at -70°C.

The pH gradient was measured by slicing a blank gel into 5 mm sections, eluting the ampholines into 200 µl of degassed 0.01 M KCl and measuring the pH using a flat membrane electrode (Pye Unicam, Cambridge).

The second dimension gel was basically the same system as used in 1-dimension S.D.S. electrophoresis. One of the glass plates had a notch allowing the isoelectric focusing gel to sit on top of the stacking gel where it was immobilised by the addition and polymerisation of a little more stacking gel. Two first dimension gels could be accommodated on top of the same second dimensional gel, they were positioned with their acidic ends towards the centre of the gel separated by a small piece of gel containing labelled "marker" proteins of known molecular weight. This marker gel was made by incorporating [^{14}C]-labelled proteins into an I.E.F. gel. After polymerisation this was equilibrated in sample gel buffer and stored at -20°C until required.

The second dimension gels were subsequently run and processed in the same way as S.D.S. gels. When a large number of protein fractions were being compared

(as in the pulse-chase experiments) an additional electrophoresis tank (built in the workshop of the Department of Virology, University of Glasgow) having two upper buffer reservoirs were used. This allowed up to six samples to be run simultaneously (when it was used in parallel with the original apparatus) allowing a series of samples (such as those from pulse chase experiments) to be run under almost identical electrophoretic conditions.

FLUOROGRAPHY

Materials

Dimethylsulphoxide (D.M.S.O.)		
Diphenyloxazole (P.P.O.)	24% in D.M.S.O.	
Glycerol	2.4% w/v in water	
Kodak X-0 mat-H	X-ray film	Kodak plc, Wythenshawe, Manchester
Kodak DX 80 developer		
Kodafix solution		
Acetic acid	2 % w/v in water	
Electronic flash gun	Sunpak CX 14	

Fluorography was used to increase the sensitivity of autoradiography by incorporating the scintillant diphenyloxazole (P.P.O.) into the gel, the method is described by Bonner and Laskey (1974) and Laskey and Mills (1975). Because P.P.O. is insoluble in water the water in the gel was replaced by dimethylsulphoxide. This was accomplished by rocking the gel in dimethylsulphoxide (3 x 500 ml, 20 min between changes) followed by 200 ml of 24% w/v P.P.O. in dimethylsulphoxide for 45 min. This gives a final concentration of 16% P.P.O. in the dried gel. The gel was washed under cold running water for 45 min. to remove dimethylsulphoxide and precipitate the P.P.O. in

the gel. Before drying the gel was rocked for 20 min. in 500 ml of 1.2% glycerol (this helps to prevent cracking). The gel was then dried overnight onto blotting paper supported by sintered glass. After drying the gel was placed in contact with a sheet of Kodak X-o-mat-H film between two sheets of glass and exposed at $-70^{o}C$.

To improve film sensitivity and provide a linear response to radioactivity, the film was pre-exposed with one flash from an electronic flash gun (Sunpak CX 14 at 25 cm) covered with two sheets of orange paper (from X-ray film packing). This gives a background density of 0.15 O.D. units (measured at 540 nm), the exposed side of the film being placed next to the gel.

PREPARATION OF MOUSE SPLENIC LYMPHOCYTES

In some radiolabelling experiments the number of plasmacytoma cells available for the preparation of nuclear protein fractions was insufficient to ensure a reasonable yield of proteins. When this occurred mouse splenic lymphocytes were added to the plasmacytoma cell suspension to act as "carrier cells". Their preparation is described below.

Mice were killed by cervical dislocation, their spleens removed by dissection and rinsed with phosphate buffered saline. The spleens were then transferred to a nylon tea strainer and pressed through into a petri dish containing 10 ml of PBS using the rubber tip of a syringe plunger. The spleen cells were dispersed by gently sucking them up and down in a 5 or 10 ml pipette and transferred to a conical plastic tube. This cells were left to stand for 2-3 minutes to allow large clumps to settle. The supernatant was carefully transferred to a clean conical tube and the cells spun down at 400g for five minutes. The lymphocytes were then resuspended in 10 ml of PBS and a cell count taken. Finally the cells were spun down as previously described and resuspended in 10 mls of PBS, the required number of cells were removed, spun down at 400 g and taken up in cell lysing solution (HK.).

3. PRELIMINARY EXPERIMENTS

Before embarking on a series of experiments, it is advisable to carry out preliminary studies to establish the suitability of the available experimental methods and find the optimal conditions under which experiments should be carried out.

The experimental procedures used to prepare and analyse nuclear protein fractions can be conveniently divided into four stages:

1. Radiolabelling cultured cells with [^{35}S]-methionine.

2. Preparation of cell nuclei.

3. Preparation of nuclear protein fractions from cell nuclei.

4. The analysis of protein fractions by 1 and 2 dimensional polyacrylamide gel electrophoresis followed by fluorography.

The suitability of techniques already employed in the laboratory were compared to other methods in preliminary experiments. The purpose was to optimise the incorporation of [^{35}S]-methionine into plasmacytoma cells, to improve the yield of clean nuclei from these cells and to find a quick, efficient method of

fractionating nuclear proteins. In addition, experiments were carried out to improve the resolution of these proteins on 2-D polyacrylamide gels and reduce the streaking of NHCP spots on gels caused by the tendency of NHCP to aggregate.

3.1 Radiolabelling of Mouse Plasmacytoma Cell Proteins

The amount of [^{35}S]-methionine incorporated into proteins will depend on a number of factors; the important ones being the viability and metabolic activity of the plasmacytoma cells, the amount and specific activity of label used, the culture conditions and the length of labelling time.

First,labelled [^{35}S] - methionine is transported across the cell membrane by an active transport mechanism (for a review see Christenson,1984). The major metabolic functions of methionine are for utilisation in protein synthesis and for conversion to S-adenosylmethionine which is the predominant biological methyl group donor. Initially the intracellular pool will consist of unlabelled methionine and it will take time for this pool to become saturated with labelled methionine. The pool of tRNA will similarly be charged with unlabelled amino acids. Therefore,initially at least, there will be an underestimation of the true rate

of protein synthesis.

Thereafter the kinetics of the incorporation of amino acids into protein becomes extremely complex and will depend on a number of factors including:

1. The relative rates of synthesis and degradation of cellular proteins and secretion of immunoglobulin
2. The rate of re-utilisation of label from degraded proteins
3. The rate at which the [^{35}S] - methionine pool becomes exhausted
4. The proportion of cells at different stages in the cell cycle
5. The viability of the cells during the incubation period

In addition altered methionine metabolism has been found to be associated with neoplastic cells,a number of which have been found to be methionine dependent (for extensive review see Hoffmann, 1983),although no information is available on the cell lines used in these experiments.

To ensure optimal metabolic activity the cells used for labelling experiments were carefully grown in log phase for at least four days prior to an experiment. The cell viability was usually above 95% and always above 90%. It was decided to use [^{35}S]-methionine to label the proteins as it is available at a higher specific activity than [^{14}C]-labelled amino acids, the only disadvantage being that it will only label those proteins containing methionine, fortunately the majority of proteins contain methionine. However, it is important to point out that the intensity of a spot or band on the gel will depend on the relative abundance of methionine in the protein , the amount of protein present and the biological half life of the protein.

Radiolabelling of cell lines was carried out in methionine free RPMI 1640 culture medium containing 10% foetal calf serum. This medium was made up in the laboratory. Therefore the only methionine available for protein synthesis ,apart from the small amount present in foetal calf serum, is that which is labelled. The time course of [^{35}S]-methionine incorporation into the proteins of plasmacytoma cell line MOPC 315.36 was followed to determine the optimal incubation time.

TIME COURSE OF INCORPORATION OF [^{35}S]-METHIONINE INTO PLASMACYTOMA PROTEINS

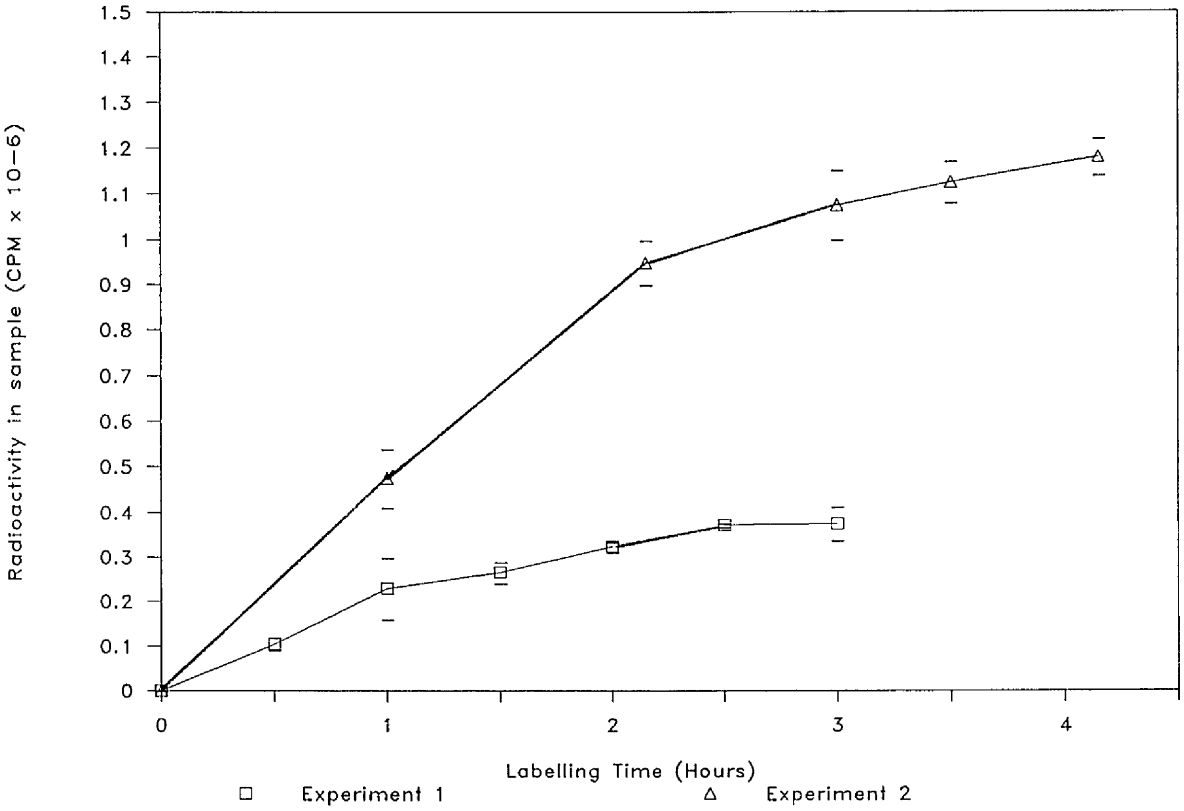
Plasmacytoma cells were washed twice in Hanks basal salt solution + 10% foetal calf serum and resuspended at a concentration of 5 x 10^6 cells/ml in 5 mls of RPMI 1640 medium (made up without methionine) plus 10% foetal calf serum, 1 mCi of [^{35}S]-methionine (636-1,200 Ci/mmol, Amersham International) was added and the cell culture incubated at 37^{o}C for 250 mins. Aliquots of the cell culture (2 x 20 μl) were taken at intervals and diluted in 1 ml of 0.1M NaOH containing 1 mM methionine (to remove any methionine bound nonspecifically to proteins). Bovine serum albumin (200 μg) followed by 150 μl of 100% trichloroacetic acid were added to the samples which were then left for two hours at 0^{o}C. The samples were filtered through glass fibre filter papers which were rinsed with 5% trichloroacetic acid followed by ethanol and then left to dry overnight. The dried filter papers were placed in 4 mls of toluene/PPO/POPOP scintillation fluid for counting.

RESULTS

The time course of [^{35}S]-methionine incorporation into plasmacytoma cells is shown in figure 2. Experiment 1 was performed using the plasmacytoma MOPC 315.32 labelled with 500μCi of [^{35}S]-methionine,this came from an older batch with lower but unknown specific activity than that used in experiment 2. In experiment 2 the cell line MOPC315.36 was used with similar results. It can be seen that the incorporation of label continues for at least four hours (although it is beginning to tail off by this time). Because of the need to maximise the incorporation of [^{35}S]-methionine it was decided to use a labelling period of four hours in subsequent experiments.

Figure 2.

Incorporation of [^{35}S]-Methionine into Proteins



Plasmacytoma cell lines MOPC 315.32 and 315.36 were labelled using 1mCi(Experiment 1) or 500μCi (Experiment 2) of [^{35}S]-Methionine respectively in labelling medium. Samples were taken at intervals and diluted in 0.1N NaOH /1mM Methionine. 200 μg of Bovine Serum Albumin and 150 μl of 100% TCA were added to the sample which was then kept on ice for 2 hours. The suspension was then filtered through a glass fibre filter,rinsed with 5% TCA and dried. The glass fibre filters were then counted in Toluene/PPO/POPOP.

UPTAKE OF LABEL INTO CELL LINES

In a series of experiments of this nature we would wish to ensure that there is consistent uptake of label into cell lines. The level of uptake of [^{35}S]-Methionine into the plasmacytoma cell lines is shown in table 1 and in figures 3 and 4. These figures show the average uptake (and standard deviation) in eight labelling experiments. Proteins loosely bound to chromatin were extracted in five of these experiments. No statistically significant differences in uptake were observed between the cell lines,however this is to be expected because of the small sample size. The average amount of label incorporated was 22.6 x 10^6 cpm with a standard deviation of 6.2 x 10^6 cpm. As would be expected the majority of the label (75%) was incorporated into the cytoplasmic fraction. The most likely cause of apparent differences in uptake between the cell lines is variation in the specific activity of the [^{35}S]-Methionine. Because of the high cost of labelled methionine researchers organised bulk purchases to take advantages of discounts. Although every effort was made to ensure consistent total amounts of label and specific activity the short half life of ^{35}S led to some variability in specific activity of label. However,despite these problems a reasonably consistent level of uptake was maintained.

Table 1 Uptake of Label into Cell Lines

Protein Fraction	Counts Per Minute ($X10_{-6}$)	Standard Deviation	Percent Of Total Counts	Standard Deviation
Cytoplasmic	16.5	3.5	74.5	8.4
Nucleoplasmic (A)	1.0	0.5	4.4	1.6
Loosely Bound (B)	0.8	0.4	4.2	1.9
Histones	1.9	1.7	7.7	6.2
NHCP	2.7	1.9	10.8	5.5
NHCP+B	3.2	1.6	13.4	3.6
Total	22.6	6.2	100	

Figure 3.

Uptake of label into Cell Lines

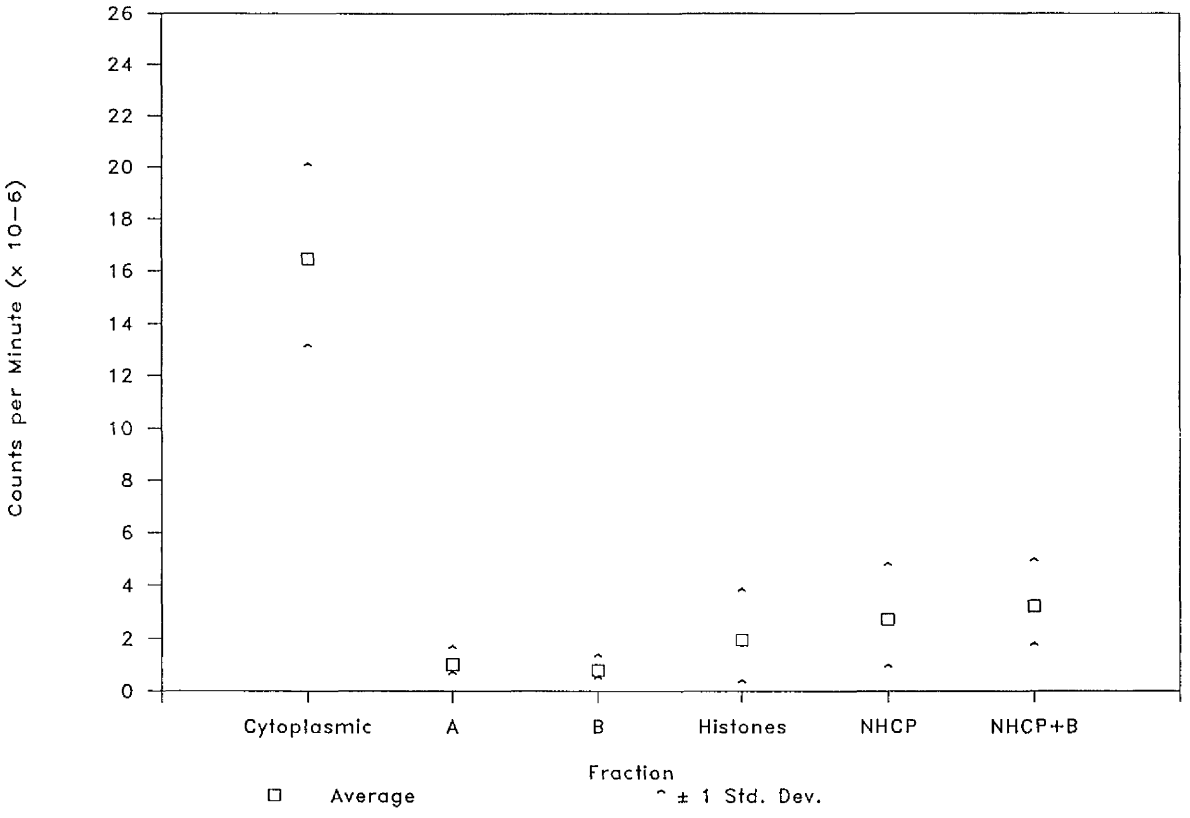


Figure 3. Total label incorporated into cytoplasmic proteins and nuclear protein fractions of Plasmacytoma cell lines. Cells were incubated with [^{35}S]-Methionine for 4 hours at 37 C and the nuclear proteins fractionated and counted. A=Nucleoplasmic proteins, B=Proteins bound loosely to chromatin, NHCP=Non-Histone Chromatin Proteins.

Figure 4.

Relative Uptake of Label Into Cell Fractions

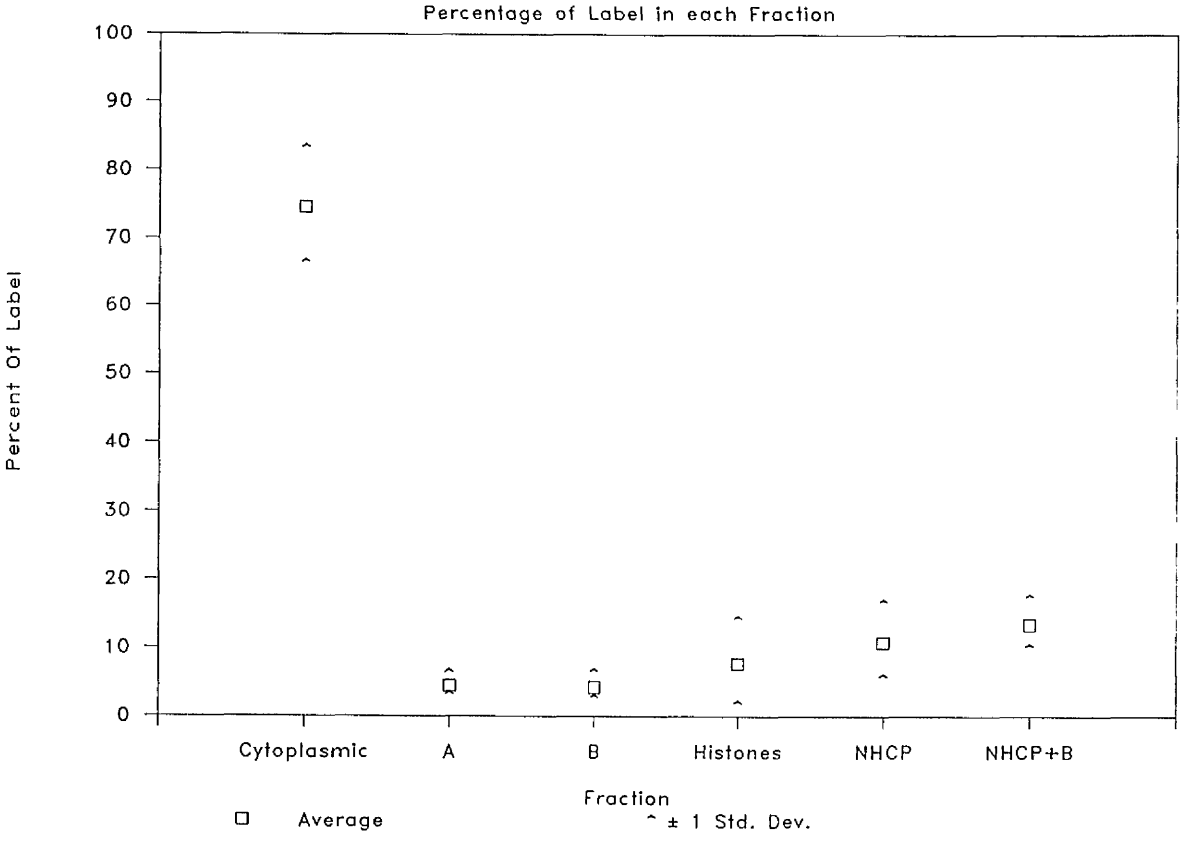


Figure 4. Relative incorporation of label into cytoplasmic proteins and nuclear protein fractions of Plasmacytoma cell lines. Cells were incubated with [^{35}S]-Methionine for 4 hours at 37 C and the nuclear proteins fractionated and counted. A=Nucleoplasmic proteins, B=Proteins bound loosely to chromatin, NHCP=Non-Histone Chromatin Proteins.

3.2 Comparison of Different Methods of Nuclei Preparation

Three methods of preparing nuclei were compared with a view to improving the presently used method of preparing nuclei (adapted from Wilson & Spelsberg, 1973). It was thought that this might be accomplished by sedimenting the nuclei through 2.3M sucrose. In this way it was hoped to strip cytoplasmic contaminants, which have a lower density than 2.3M sucrose, from the nuclei (Higashinokagawa et al.,1972). The parameters used to compare the nuclei were the yield of the nuclei and their condition as assessed by phase contrast microscopy.

METHODS

Mouse spleen cells were prepared and processed according to the following three protocols:

A According to the modified method of Wilson and Spelsberg (1973). Briefly, spleen cells were suspended in 0.25M sucrose, 0.5% Triton X100, 3 mM $CaCl_2$, 0.01M Tris pH 8.0 (lysing solution) homogenised with ten strokes of the pestle at 2,400 r.p.m. (air speed) teflon/glass homogeniser and spun down at 900g for 5 mins. The nuclei were washed twice in the same solution, counted then examined using phase contrast microscopy.

B Initially the same as protocol 'A'. After the final wash in lysing solution the nuclei were resuspended in the lysing solution and overlayered onto 9 ml of 2.3M sucrose, 10 mM Mg^{++}, 0.01M Tris pH 8.0 and centrifuged for 75 min at 50,000g. They were then resuspended in 1 ml of lysing solution, counted and examined.

C The cells were homogenised and spun down as in 'A', taken up in 10 ml of 2.3M sucrose, 10 mM Mg^{++}, 0.01M Tris pH 8 and centrifuged for 75 min at 50,000g. The nuclei were resuspended and counted as in 'B'.

RESULTS

Method	Yield	Condition of Nuclei
A	90%	Little evidence of cytoplasmic contamination. Very little lysis.
B	Approximately 10%	Extensive lysis and aggregation making counting difficult. The whole nuclei that were present appeared clean.
C	Approximately 20%	Some aggregation and lysis although not to the same extent as in 'B'. The nuclei appeared free of cytoplasmic contamination.

It was decided to continue with the original method of preparing nuclei, it is faster, more convenient and appears to give a higher yield of intact nuclei (which appear to be free of cytoplasmic contamination under phase contrast microscopy).

Electron micrographs of representative samples of mouse lymphocyte nuclei prepared by this method in the same laboratory are shown in figure 5. The micrographs were kindly donated by Dr D.I.Stott. A,B, and C show the nuclei of mouse lymphocytes stimulated

for 3 days with the mitogen lipopolysaccharide,many of these nuclei will be from plasma cells similar to the cells used in the experiments described here, D and E show nuclei from unstimulated mouse lymphocytes.

These figures are representative of the nuclei used in the labelling experiments. The nuclei appear to be free of cytoplasmic tags.

A further check of the purity of the nuclei would be to conduct an assay for various non nuclear enzymes for example glucose 6-phosphate dehydrogenase for cytoplasmic contamination,succinate dehydrogenase for mitochondrial contamination and 5' nucleotidase for plasma membrane contamination.

Figure 5.

Electron Micrographs of Lymphocyte Nuclei.

Nuclei were prepared by suspending cells in lysing solution (H.K.) and disrupting them using 10 strokes of a Potter-Elvehjem teflon/glass homogeniser (0.11-0.15mm clearance and centrifuging for 5 minutes at 900g.

The nuclear pellet was then washed twice in the same solution and prepared for electron microscopy. The electron micrographs were donated by Dr D. I. Stott and the cells were prepared using the same method as that employed in the experiments described here.

A,B, and C Nuclei from lymphocytes stimulated using Lipopolysaccharide.

D and E Nuclei from unstimulated mouse lymphocyte nuclei.

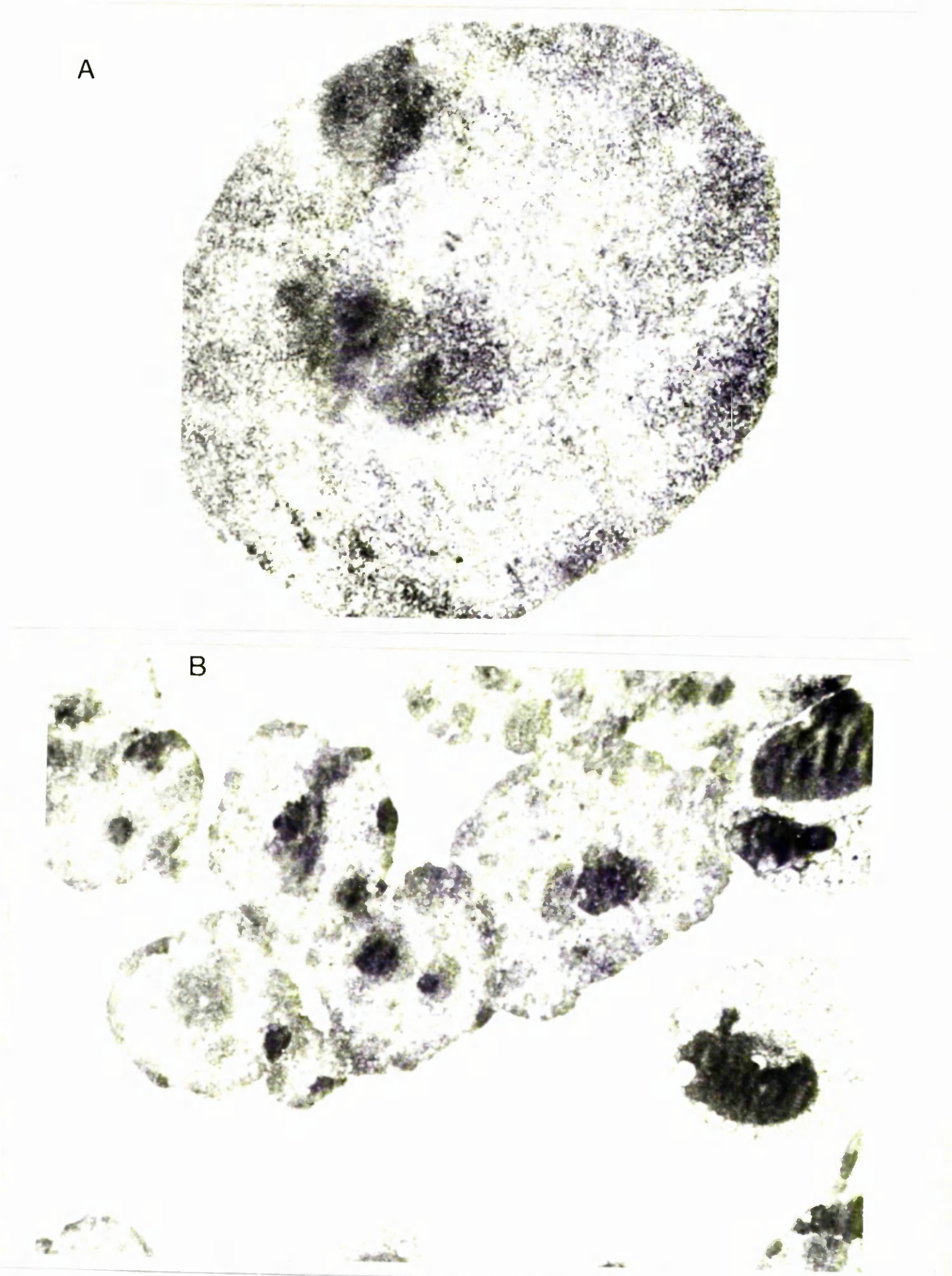
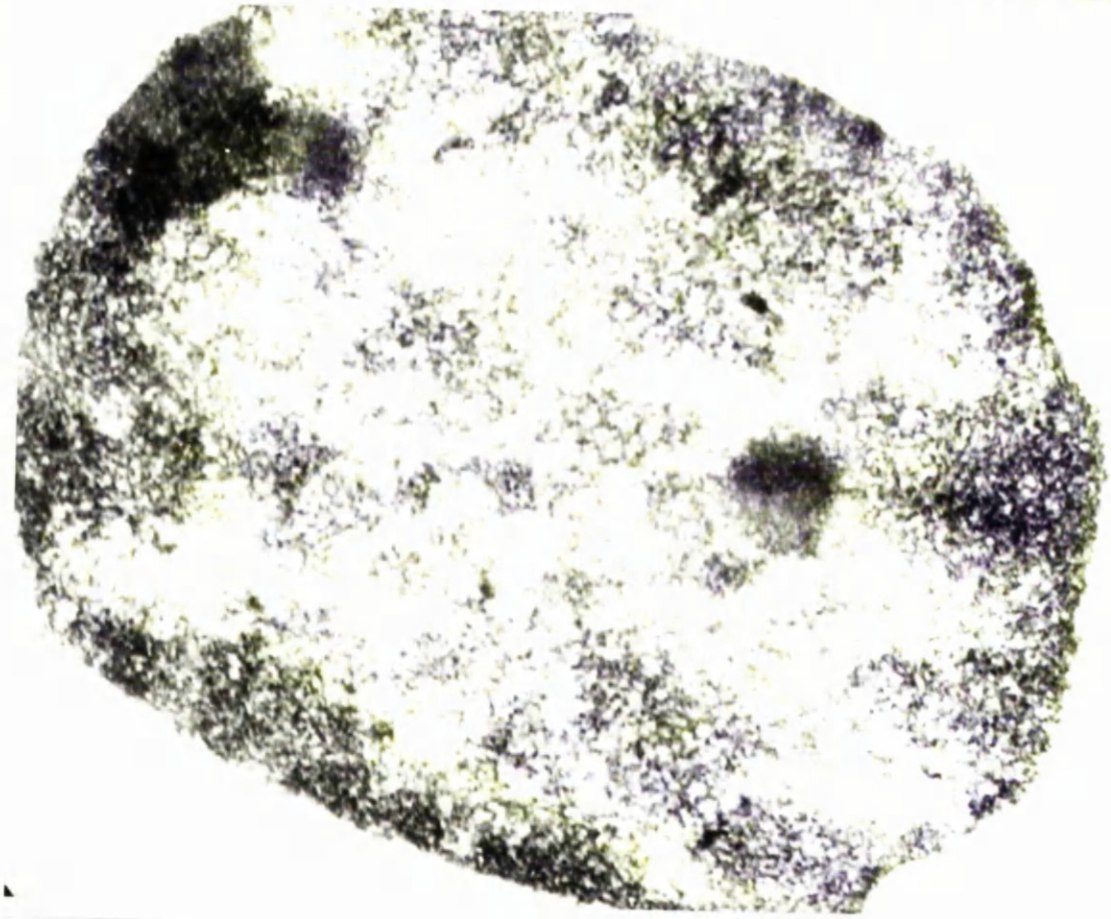

A
B

Figure 3.

C



D

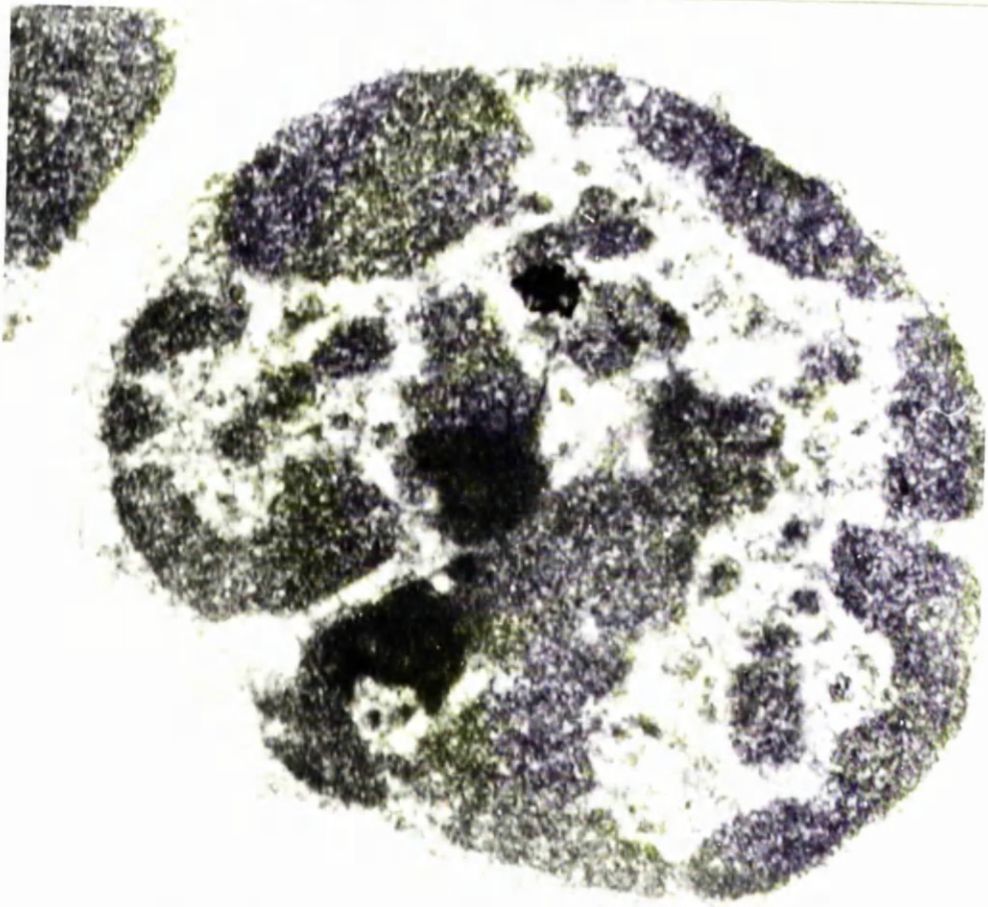
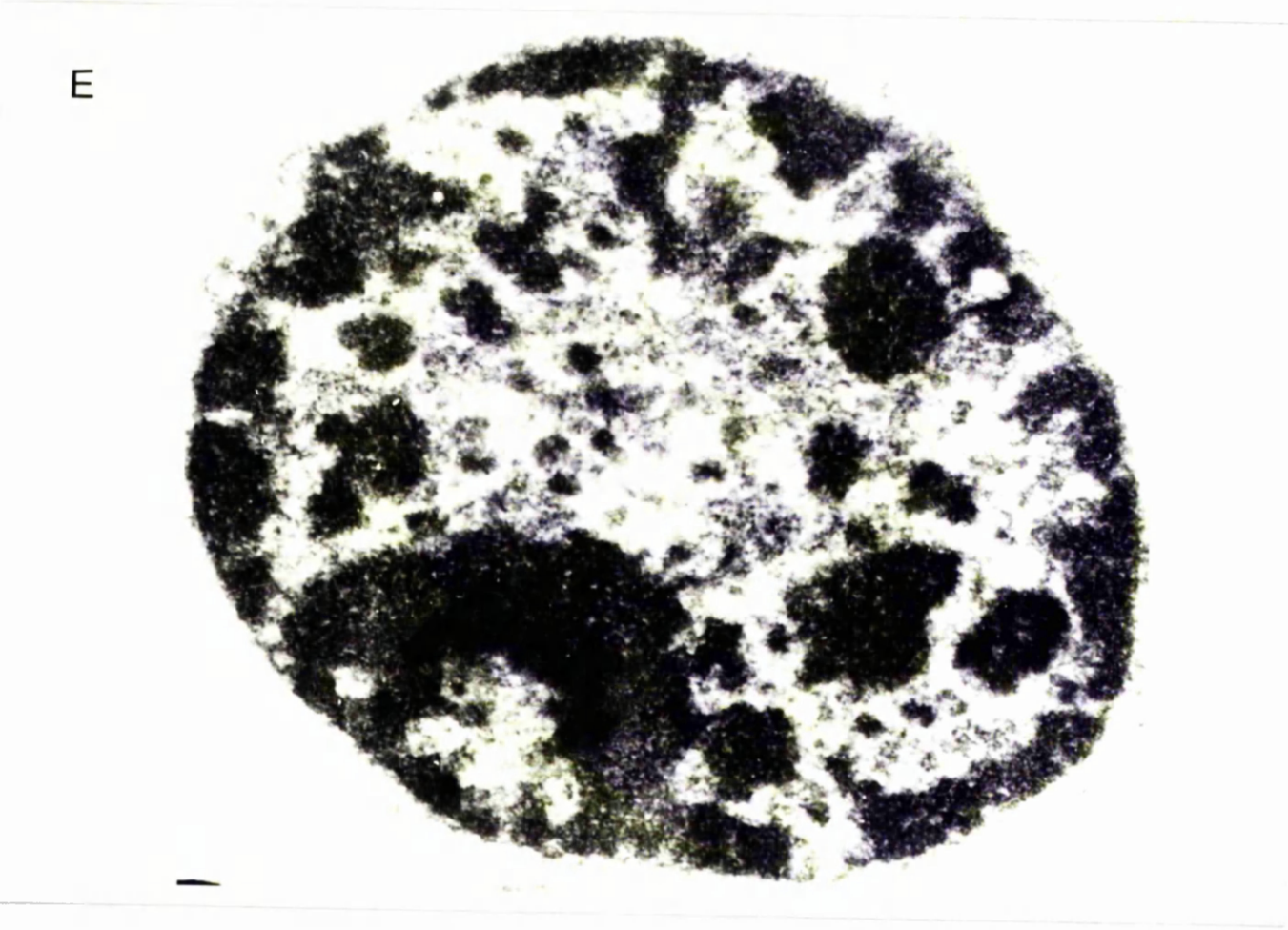


Figure 5.



3.3 Preparation of Nuclear Protein Fractions

A number of changes were made to the method of preparing nuclear protein fractions then used in the laboratory (modified from Wilson and Spelsberg (1973) by Stott and Williamson (1978a)). The most important was the use of a fixed speed microhaematocrit centrifuge (10,000g); this enabled the extraction of nuclear proteins to be carried out on a micro-scale. This eliminated the need for "carrier cells" to provide enough material for extraction procedures which in turn allowed the preparation of protein fractions of a very high specific activity. This improved the resolution of 2-D polyacrylamide gel electrophoresis and reduced the streaking of spots caused by protein aggregation. In addition, the extraction of proteins with 0.35 M NaCl, 1mM $NaMoO_4$ followed by 2mM Tris/HCl 0.1 mM EDTA, 1 mM $NaMoO_4$ was omitted; the reason being that these fractions were found to contain only a small amount of protein of limited heterogeneity.

3.4 Two Dimensional Polyacrylamide Gel Electrophoresis

A major problem encountered in the analysis of NHCP by 2-D polyacrylamide gel electrophoresis is the tendency of these proteins to aggregate when nucleic acids are removed. This causes streaking of spots in the S.D.S. dimension, especially at the basic end. A number of approaches were tried in attempts to minimise this problem; these are detailed below.

The samples of NHCP were spun in the micro-haematocrit centrifuge prior to isoelectric focusing to remove large protein aggregates. Samples of NHCP in SDS sample buffer were boiled before analysis. However this was demonstrated to cause partial degradation of some proteins and was thus discontinued. Protein samples were sonicated but this had no demonstrable effect. Small amounts of DNA may have been 'protected' from digestion during the preparation by binding proteins thus DNA may cause aggregation. It was attempted to reduce streaking by the removal of nucleic acids by chromatography using hydroxyapatite.

3.5 The Chromatography of Nonhistone Chromatin Proteins on Hydroxyapatite

MATERIALS

The hydroxyapatite used was provided by Dr A J MacGillivray.

A Pasteur pipette was used as a column as only 200 μg of protein was being separated.

Loading and sample buffer

NaCl	2.0M
Urea	5.0M
Sodium phosphate buffer	1mM pH 6.8

1st Elution buffer

Guanidinium chloride	2.0M
Urea	5.0M
Sodium phosphate buffer	200 mM pH 6.8

2nd Elution buffer

Guanidinium chloride	2.0M
Urea	5.0M
Sodium phosphate buffer	500 mM pH 6.8

CHROMATOGRAPHY OF NONHISTONE CHROMATIN PROTEINS ON HYDROXYAPATITE

METHOD

Chromatin proteins were fractionated using a modified version of the method of Rickwood and MacGillivray (1975).

A column of hydroxyapatite was formed in a Pasteur pipette, a glass bead was used to retain the hydroxyapatite. The column was equilibrated by allowing running buffer to pass through it for 2 hrs. The sample containing approximately 200 μg of protein was applied to the column as a solution in 0.5 ml of sample buffer. After loading the sample the column was washed through with 2.5 ml of the same buffer, fractions were collected throughout in 0.5 ml aliquots. The elution buffers (3ml of each) were run consecutively through the column, NHCP are eluted by the first buffer, some very tightly bound proteins and DNA in the second. Small (2μl) duplicate samples were taken from each fraction for scintillation counting. The two fractions with the highest counts were pooled, concentrated by precipitation with trichloroacetic acid and analysed by 2-D polyacrylamide gel electrophoresis alongside an equivalent amount of the original sample for comparison.

RESULTS

Fraction	Counts	
1	23,000	
2	1,250	
3	0	Excluded proteins
4	0	
5	0	
6	0	
7	77,000	
8	17,000	
9	7,500	NHCP
10	6,500	Single stranded nucleic acid
11	4,000	
12	3,000	
13	3,000	
14	500	
15	200	DNA fractions
16	0	
17	0	
18	0	

Total counts loaded	=	232,000 cpm	=	100%
Excluded		24,250 cpm	=	10.45%
Eluted in 7-12		115,000 cpm	=	49.57%
Eluted with DNA		3,700 cpm	=	1.59%
Total recovery		142,950		61.61%

Fractions 7 and 8 were combined and analysed by 2-dimensional polyacrylamide gel electrophoresis. However chromatography using hydroxyapatite did not alter the streaking of proteins at the basic end of the gel, so it was decided not to employ this technique.

3.6 Maintaining Antibody Production in Cell Lines

Cell lines are known to adapt to culture conditions when they are maintained for long periods in continuous culture. In particular, plasmacytoma cell lines may lose the ability to produce immunoglobulin heavy chains. Two methods are used to ensure the reproducibility of experiments using plasmacytoma cell lines; the first is to maintain frozen stocks of the original culture which can be returned to at the beginning of each series of experiments, the other is to continually re-clone cultures. The first of these options was taken, primarily because there were sometimes long intervals between cell labelling experiments which made maintaining continuous cultures wasteful in both time and effort as well as in expensive cell culture materials.

In addition to the above the production (or absence) of antibody proteins was tested by specific immunoprecipitation followed by S.D.S. polyacrylamide gel electrophoresis of the precipitated material (described overleaf).

TESTING IMMUNOGLOBULIN PRODUCTION OF CELL LINES

Medium and cell lysates from labelling experiments were tested for the presence of immunoglobulin molecules by immune precipitation followed by polyacrylamide gel electrophoresis of the precipitated material. The relative amount of labelled antibody was calculated by measuring the total amount of label incorporated into proteins (by precipitation with trichloroacetic acid) and comparing this with the amount incorporated into antibody (by immunoprecipitation). Because immune complexes formed during immunoprecipitation can trap other proteins besides antibody molecules a rabbit IgG fraction was used as a control in order to measure this passive precipitation. This can be summarised as follows:

$$\% \text{ specific precipitation} = \frac{I - C}{T} \times 100$$

where I = immunoprecipitate counts,

C = control counts, and

T = total counts (TCA precipitate).

Contamination of immune and control precipitates was minimised by the presence of detergents throughout the procedures and by "cleaning up" the precipitates by centrifugation through a discontinuous sucrose density gradient.

MATERIALS

Antisera

Rabbit anti mouse light chain

Rabbit anti mouse IgA

Rabbit anti mouse IgM

Pig anti rabbit IgG - Provided by Dr F. Franek, Czecheslovak Academy of Sciences,Prague,Czechoslovakia. plus a rabbit IgG fraction (control).

Detergent mixture (10 X stock)

5% S.D.S., 10% Triton X100, 10% deoxycholate.

Density Gradient Solutions

Top layer - 0.5 M sucrose, 0.5% S.D.S., 1% Triton X 100,1% deoxycholate

Bottom layer - 1.0 M sucrose, 0.5% S.D.S., 1% Triton X 100,1% deoxycholate

Materials for polyacrylamide gel electrophoresis.

METHOD

Samples of the cell lysates and labelling medium were made up to 0.5% S.D.S., 1% Triton X 100, 1.0% deoxycholate using the stock detergent mixture. Any precipitate present was removed by centrifugation. 0.5 ml samples were taken and 5 μl of the appropriate antibody solution added (5 μl of a rabbit IgG fraction of the same concentration to the controls) and the

samples incubated at 37°C for 15 mins. An equivalent amount of anti-rabbit antibody (Pig IgG, 150 μl) was added, the samples were mixed and then incubated for a further 45 minutes at 37°C. The samples were then left overnight for precipitates to form. These precipitates were overlayered onto a discontinuous sucrose density gradient. This was formed in a microhaematocrit tube using the density gradient solutions described previously. The tubes were carefully loaded into a bench centrifuge and spun for 15 mins at top speed. The top layer was aspirated and replaced by a 1% S.D.S. solution containing bromophenol blue (to enable the layers to be more easily visualised) and centrifuged as before.

This step was repeated twice more after which all of the liquid was carefully aspirated and the sides of the tubes carefully dried with tissue. The immunoprecipitates and a trichloroacetic acid precipitate of the same volume of sample were prepared for S.D.S. polyacrylamide gel electrophoresis. Before electrophoresis triplicate samples were taken for scintillation counting and appropriate volumes were run on a polyacrylamide slab gel. The counts are shown in table 2.

Table 2 Immunoprecipitation of Secreted and Cytoplasmic Immunoglobulin

Sample	Counts	Specificity of Antibody	% Specific Precipitation
MOPC 315.32	C 0.043 x 10^5	Rabbit anti mouse	0.54%
Cytoplasmic Lysate	I 1.220 x 10^5	light chain	
	T 218 x 10^5		
MOPC 315.35	C 0.034 x 10^5	Rabbit anti mouse	-
Cytoplasmic Lysate	I 0.032 x 10^5	light chain	
	T 11 x 10^5		
MOPC 315.36	C 0.043 x 10^5	Rabbit anti mouse	-
Cytoplasmic Lysate	I 0.030 x 10^5	light chain	
	T 24 x 10^5		
MOPC 315.40	C 0.07 x 10^5	Rabbit anti mouse	9.8%
Incubation medium	I 0.51 x 10^5	IgA	
	T 4.5 x 10^5		
Y5781.4	C 0.06 x 10^5	Rabbit anti mouse	8.5%
Incubation medium	I 0.29 x 10^5	IgM	
	T 2.7 x 10^5		

C = Control; I = Immunoprecipitate;

T = Trichloroacetic acid precipitate.

The polyacrylamide gel clearly showed the presence of light chains in the immune precipitate of MOPC 315.32 but not MOPC 315.35 and MOPC 315.36. Both heavy and light chains were present in the immune precipitate of MOPC 315.40; there was some distortion of the gel in the Y5781.4 track but both heavy and light chains appeared to be present.

4. THE BINDING OF ACETYLAMINOFLUORENE TO RAT LIVER NUCLEAR PROTEINS AND DNA

In these experiments we have studied the binding of the carcinogen acetylaminofluorene to rat liver nuclear protein fractions (in particular the non-histone proteins) and DNA, trying to relate this binding to long term changes in gene expression. The experiments were performed as set out below:

1) Initially experiments were carried out to compare different methods of administering the carcinogen to the rats,the method which yielded the highest labelling of the nuclear protein fractions was then used in subsequent experiments.

2) The possible contamination of urea soluble proteins with [^{14}C]-AAF bound to RNA was examined by digestion of the 8M urea extracted proteins by ribonuclease and by their partitioning between phenol and aqueous phases of a phenol mixture.

3) The specific activity of the carcinogen administered was lowered to assess whether the nuclear fractions were fully saturated with carcinogen.

4) The long term binding of the carcinogen was evaluated by analysing nuclear protein and DNA fractions two weeks after carcinogen administration.

5) Attempts were made to modify the metabolism of acetylaminofluorene by prefeeding the animals with sulphate or phenobarbitone.

6) The binding of carcinogen to nuclear proteins was further examined by isoelectric focusing of non-histone proteins and by polyacrylamide gel electrophoresis of histones under acidic conditions.

4.1 Initial Experiments

In these experiments the administration of acetylaminofluorene to rats by stomach tube and by intraperitoneal injection were compared, two and three μCi doses dissolved in corn oil were used for each procedure. The doses were administered at 24 hour intervals at 10 am. each day to minimise the effects of diurnal variation in liver metabolism, animals were killed 24 hours after the final injection by cervical dislocation. The livers were surgically removed and nuclei prepared and fractionated.

The results of these experiments are given in Tables 3 and 4; they reveal that two doses of carcinogen gave higher specific activities in the NHP and that administration of these doses by intraperitoneal injection gave the highest specific activity of all. In subsequent experiments this regimen was used to administer carcinogen.

Table 3. Specific Activities of Nuclear Proteins Labelled by Two Doses of Carcinogen.

a) **By stomach tube**

Fraction	Recovery (mg protein)	Total Counts (DPM x 10^{-3})	Specific Activity (DPM/mg protein x 10^{-3})	% DPM Recovered
8M urea extract	14.0	86	6.1	50
Histones	10.2	33	3.6	19
Residual proteins	7.5	51	6.8	31

b) **By intra-peritoneal injection**

Fraction	Recovery (mg protein or DNA)	Total Counts (DPM x 10^{-3})	Specific Activity (DPM/mg protein x 10^{-3})	% DPM Recovered
8M urea extract	9.6	96	10.0	57
Histones	4.8	40	8.3	23
Residual proteins	2.6	32	12.3	20
DNA	.044	0.2	4.5	-

Table 4. Specific Activities of Nuclear Proteins Labelled Three doses of Carcinogen.

a) **By stomach tube**

Fraction	Recovery (mg protein or DNA)	Total Counts (DPM x 10^{-3})	Specific Activity (DPM/mg protein x 10^{-3})	% DPM Recovered
8M urea extract	4.4	25	5.6	45
Histones	5.2	13	2.5	24
Residual proteins	5.6	16	2.9	30
DNA	0.24	0.73	3.0	1

b) **By intra-peritoneal injection**

Fraction	Recovery (mg protein or DNA)	Total Counts (DPM x 10^{-3})	Specific Activity (DPM/mg protein x 10^{-3})	% DPM Recovered
8M urea extract	6.2	31	5.0	45
Histones	9.6	18	1.9	26
Residual proteins	4.5	17	3.7	24
DNA	0.39	3	7.2	4

4.2 Contamination of urea soluble proteins with [^{14}C]-Acetylamino-fluorene bound to RNA

The 8M urea extract of rat liver nuclei may contain [^{14}C]-AAF bound covalently to RNA. It is important to quantify the contribution of RNA bound AAF to the total counts in this extract. Two experiments were performed to measure the level of RNA bound acetylaminofluorene. The first was to digest any RNA present with ribonuclease, the second to measure the partitioning of radioactivity between aqueous and phenol phases of a saturated phenol solution (proteins being found predominantly in the phenol phase, RNA in the aqueous phase).

DIGESTION OF 8M UREA EXTRACT WITH RIBONUCLEASE

A sample of urea soluble proteins in 2M urea was incubated with ribonuclease (Sigma UK Ltd., 10 μg/ml) for 5 hr. at room temperature. Two 50 μl samples were taken for scintillation counting, the remaining solution was dialysed overnight against 4M urea. The volume of the sample was measured after dialysis and two 50 μl samples were taken for counting.

RESULTS

Total DPM in sample before dialysis = 3113 dpm

Total DPM in sample after dialysis = 3255 dpm

The small apparent increase in the numbers of counts after dialysis is probably due to minor inaccuracies in the measurement of volumes. As there was no loss of radioactivity during dialysis we may conclude that the acetylaminofluorene in the 8M urea extract is not bound to RNA.

EXTRACTION WITH PHENOL

Proteins are either soluble in phenol or are denatured and precipitated at the phenol phase/aqueous phase interface, on the other hand nucleic acids are partitioned into the aqueous phase. This partitioning is a commonly used method for the separation of nucleic acids and proteins. A sample of 8M urea soluble proteins were extracted twice with phenol, duplicate samples from the phenol and aqueous phase were taken for scintillation counting.

	DPM	% of Total
Total counts in phenol phase =	4,670	87
Total counts in aqueous phase =	680	13

The combined results of both experiments indicate that the [^{14}C]-acetylaminofluorene bound to macromolecules in the 8M urea extract is bound predominantly to proteins.

4.3 Effect of reducing the specific activity of acetylaminofluorene

In this experiment the specific activity of the acetylaminofluorene administered to the rats was lowered from 50 mCi/mM to 10 mCi/mM by adding an appropriate amount of unlabelled AAF. If the nuclear macromolecules were saturated with AAF in earlier experiments then the five fold reduction in specific activity should result in a corresponding reduction in the specific activity of nuclear macromolecules. The results of this experiment are shown in Tables 5 and 6; the specific activities of the 8M urea extract, histones, residual proteins and DNA were reduced by 40, 33, 62 and 85% respectively. From this we may conclude that the nuclear proteins were not saturated with carcinogen in earlier experiments. However, the specific activity of the DNA is reduced

considerably which may indicate that the DNA was saturated with AAF in our experiments. A note of caution must be sounded, however, as the yield of DNA in these experiments is low and rather variable due to the large amount of manipulation of fractions before the DNA is isolated.

Table 5. Specific activities of nuclear macromolecules labelled with AAF.

Experiment	8M Urea Extract (NHP)	Histones	Residual Protein	DNA
Control (average of 2 experiments)	6.02	1.80	4.70	2.16
Animals given phenobarbitone (1mg/ml) in drinking water for 10 days	2.42	0.53	0.63	0.31
Animals given sulphate (10 mg/ml) in drinking water for 10 days	4.33	1.20	1.76	0.76
As control but animals killed 14 days after 2nd dose	0.20	0.29	0.38	0.23
As in control but specific activity of AAF reduced to 10 mCi/ml	6.33	1.2	1.78	0.32

All specific activities are measured in DPM/mg x 10^{-3}.

Animals were given 2 doses of [^{14}C]-AAF (2 x 100 μCi) by intraperitoneal injection at 24 hour intervals; animals were killed 24 hours after the final dose (except in 4).

The specific activity of the AAF was 50 mCi/mM (except in 5).

Table 6. Percentage of total bound radiolabelled carcinogen recovered in nuclear fractions.

Experiment	8M Urea Extract (NHP)	Histones	Residual Protein	DNA
Control	80.9	9.9	5.9	3.3
Animals given phenobarbitone (1mg/ml) in drinking water for 10 days	81.7	10.8	4.3	3.2
Animals given SO_4^{2-} (10 mg/ml) in drinking water for 10 days	67.3	13.2	12.7	6.7
As in control but animals killed 14 days after 2nd dose	39.4	36.3	21.8	2.4
As in control but specific activity of AAF reduced to 10 mCi/mM	75	11	9.2	4.9

Animals were given 2 doses of [^{14}C]-AAF, (2 x 100 µCi) by intraperitoneal injection at 24 hour intervals; animals were killed 24 hours after the final dose (except in 4). The specific activity of the [^{14}C]-AAF was 50 mCi/mM (except in 5).

4.4 The long term binding of acetylaminofluorene to nuclear macro-molecules

The purpose of this experiment was to find out how much protein bound AAF was lost over time due to the elimination of modified proteins. Administration of acetylaminofluorene was performed as in other experiments but the time between administration of the final dose of carcinogen and sacrifice of the rats was extended from 24 hours to 14 days.

The results of this experiments are shown in tables 5 and 6; the histones and DNA have maintained 15% and 11% of their initial specific activity whereas the 8M urea soluble proteins and residual proteins have retained 3% and 8% respectively. These figures support the view that the 8M urea soluble proteins are more metabolically active and subject to a higher turnover than other fractions. However, it is noteworthy that the residual proteins have retained a comparable number of counts to DNA. It is possible that this fraction contains proteins which may mediate gene expression; this could make long term changes in gene expression by protein modification a possibility. However,it is much more likely that these proteins will be performing a structural role than a regulatory one.

4.5 Modification of carcinogen binding by prefeeding with phenobarbitone and sulphate

At the time these experiments were performed it was believed that the metabolic activation of the acetylaminofluorene was a two stage process with the sulphate ester as the ultimate carcinogen (Kriek, 1974, Weisburger et al., 1972, Gutmann et al., 1972).

The first step in the activation of AAF is via N-oxidation in the smooth endoplasmic reticulum. This is accomplished by cytochrome p450 mixed function mono-oxygenase system which is one of the major enzyme systems involved in the detoxification of foreign compounds,including carcinogens. However some carcinogens can be activated by cytochrome p450 to metabolites which bind covalently to macromolecules resulting in cytotoxicity and neoplasia. Mammals possess a multiplicity of cytochromes p450 which differ in molecular weight and substrate specificity. The cytochromes p450 are inducible by wide variety of compounds ,including phenobarbitone, the specific variant being dependent on the compound. It was therefore decided to attempt to stimulate AAF activation by feeding phenobarbitone (Shephard et al.,1984,Dubois et al .,1979).

The administration of phenobarbitone to rats results in rapid and marked enlargement of the liver. This is accompanied by reversible nuclear and whole cell DNA polyploidisation. However phenobarbitone does not alter the number of nuclei per liver cell. In addition there is augmentation of the smooth endoplasmic reticulum and an an increase in protein and RNA synthesis (Bohm and Noltemeter,1981).

This includes an increase in the the level of glutathione S-transferase (ligandin) by the induction of glutathione S-transferase mRNA. This protein catalyses the conjugation of a variety of electrophilic compounds with glutathione,including azo-dye carcinogens (Pickett et al.,1981).

It has been proposed that the second step in the activation of AAF involves the formation of an active sulphate ester. In mammals many drugs (and carcinogens) are metabolised to sulphate conjugates which are rapidly eliminated from the body. The incorporation of sulphate into macromolecules first requires the formation of the co - substrate Adenosine 3' phosphate 5' sulphatophosphate - "active sulphate" from ATP and organic sulphate. Data obtained by Mulder and Schlotens(1978), who injected rats with $Na_2{}^{35}SO_4$ indicate that inorganic sulphate in blood is immediately

available for sulphation in the liver and that the pool size of Adenosine 3' phosphate 5' sulphatophosphate is very small. This is probably because Adenosine 3' phosphate 5' sulphatophosphate is both chemically labile and easily degraded by numerous enzymes including 3' and 5' nucleases and deamidases ,therefore, it may only be synthesised when it is required. Alternatively,other evidence suggests that binding of AAF to nuclear macromolecules is a consequence of de-acetylation rather than sulphate esterification (Schut et al., 1978, Sakai et al., 1978, Stout et al., 1980), the sulphate ester being responsible for the alkylation of cytoplasmic components, which may act as a promoter of carcinogenesis. It was therefore decided to determine the role of sulphation in the activation of AAF by prefeeding the rats with sodium sulphate. The experimental protocols were as follows:

a) **Phenobarbitone**

The rats received phenobarbitone (1 mg/ml) in their drinking water for 10 days prior to administration of carcinogen.

b) **Sodium sulphate**

Sodium sulphate (10 mg/ml) was administered to rats in their drinking water in order to increase the availability of sulphate and stimulate the formation of

the active sulphate ester. In both experiments administration of carcinogen and preparation of nuclear fractions proceeded as before.

RESULTS

The results are shown in Tables 5 and 6; they show a general decrease in the specific activity of the nuclear fractions, especially in the rats fed phenobarbitone. The experimental rats were about 2 weeks older and 30g heavier than the control (230 as compared to 200g) as animals were purchased in batches and it was not possible to carry out all experiments simultaneously. However, this is not enough to account for the observed discrepancies. In the case of phenobarbitone, the enzymes induced are part of the mechanism by which the liver de-toxifies poisons; it is possible that as well as altering the level of enzymes, phenobarbitone may have altered the balance between detoxification and carcinogenic activation, ie. the rate of removal of carcinogenic intermediates may have been stimulated to a greater extent than their rate of production.

The drop of the specific activities of nuclear macromolecules as the result of sulphate feeding was, at the time, more difficult to explain. However, it has

since been shown (Schut et al., 1978, Sakai et al., 1978, Stout et al., 1980), that sulphate esterification is more likely to result in an increase in alkylation of cytoplasmic macromolecules,reducing the proportion of activated AAF entering the nucleus.

REPRODUCIBILITY OF RESULTS

Only a limited number of experiments could be performed due to the high cost of labelled acetylaminofluorene. In the initial experiments two rats were used for each experiment and the material extracted from their livers pooled. Similar protocols were used for later experiments. The control,low specific activity,and long term binding experiments were repeated with very similar results to the ones detailed here. It was not possible to repeat the experiments using phenobarbitone and sulphate treated rats because the supply of acetylaminofluorene became exhausted and the cost of further purchases prohibitive. However these experiments were the last in the series of experiments and by this time the experimental techniques were well practised allowing a greater degree of confidence to be placed in the results.

4.6 Analysis of histones

The histones isolated in a number of experiments were further analysed by polyacrylamide gel electrophoresis on 15% gels using the method of Panyim and Chalkley (1969). The histones were visualised by staining with amido black, bands and interband regions were cut out, dissolved in hydrogen peroxide and the amount of radioactivity present measured by scintillation counting.

RESULTS

The counts present in the histone bands and interband regions are shown in Table 7. In all of the experiments the radioactivity recovered in the histone bands was fairly low, the counts were evenly distributed between the histone components. The histone bands were clearly visible in the gel and an excellent separation was obtained making the number of counts present in the interband region difficult to explain. It may be possible that the carcinogen alters the mobility of histones or that it binds to modified histone molecules leading to the recovery of radioactivity in the interband regions. This electrophoretic analysis of labelled histones was performed three times with similar results.

Table 7. Analysis of histones modified by AAF.

Source of Histones	Applied DPM	DPM Recovered in histone bands	DPM Recovered in interband regions	% applied DPM in histone bands	% recovered DPM in histone bands
Control	2418	625	630	26	50
2 doses AAF administered by stomach tube	4700	1104	620	23	64
3 doses AAF administered intraperitoneally	1800	607	*	34	*

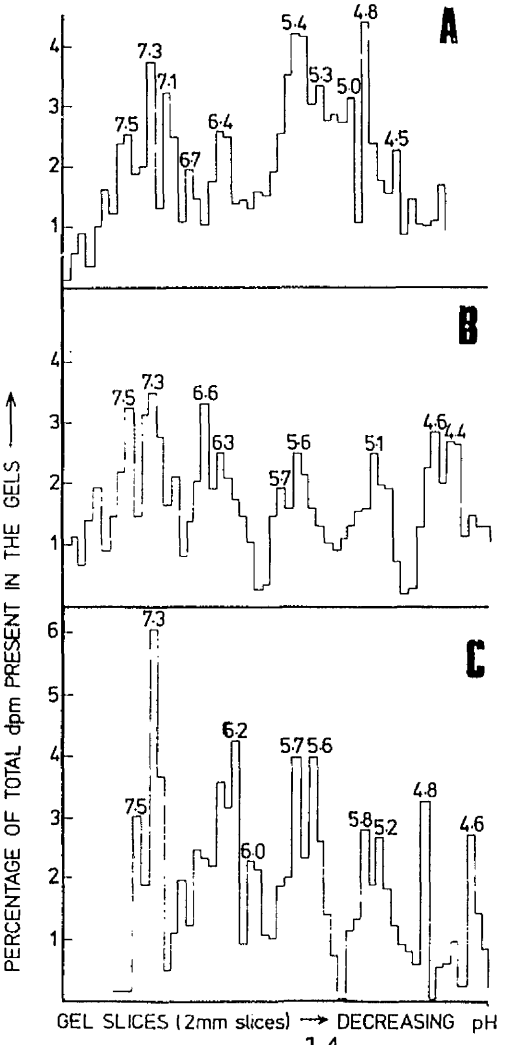
Histones were separated on 15% polyacrylamide slab gels using the Panyim and Chalkley (pH3) technique (1969); the samples were dissolved in 15% sucrose 1 mM HCl. The gels were run at 30 mA/gel for 10 hrs, stained in 0.1% amido black in 7% acetic acid and destained in 7% acetic acid. The histone bands and interband regions were cut out, dissolved in 30% hydrogen peroxide and counted.

4.7 Analysis of non-histone proteins

The distribution of [^{14}C]-acetylaminofluorene in the 8M urea soluble proteins was examined using isoelectric focusing in cylindrical polyacrylamide gels (Gronow and Griffiths, 1971). Approximately 200 μg of protein was loaded onto each gel. After focusing the gels were quick frozen in CO_2/ethanol and sliced into 2 mm sections, dissolved in hydrogen peroxide and counted. The results are shown in Fig. 6, counts are plotted as percentages of total DPM recovered to assist in the comparison of gels. It can be seen that the label is fairly evenly distributed through the gel; the carcinogen being bound to a large number of proteins. It can be seen that prefeeding with phenobarbitone and sulphate appears to alter the distribution of label among the 8M urea soluble protein, a protein of PI 7.3 having a higher peak in the phenobarbitone treated rats. All isoelectric focusing gels were performed at least twice with only minor variations in the results. There was some variation in the relative heights of the peaks,due possibly to the slicing of the peaks at different places. The pH gradients were reasonably consistent varying between 4.2 and 4.6 at the acidic end and 8.5 and 8.9 at the basic end.

Figure 6.

Analysis of AAF Labelled Non-Histone Proteins



Histograms of [^{14}C]-acetylaminofluorene distribution in polyacrylamide gels after isoelectric focusing (2% ampholines pH 3.5-10) of rat liver non-histone proteins extracted with 8M urea, 50 mM phosphate pH 7.6. The figures above the peaks are the approximately pI values. The gels are :- A: control; B: sodium sulphate-treated animals, C: phenobarbitone-treated animals.

5. COMPARISONS OF THE NUCLEAR PROTEIN PATTERNS OF PLASMACYTOMA CELL LINES

In this chapter we will be looking at the nuclear protein patterns of plasmacytoma cell lines. The majority of the work was carried out on subclones of the IgA (λ_2) producing cell line MOPC 315. These cells differ in their ability to express known genes, their lineage is detailed in the materials and methods chapter together with that of the other plasmacytoma cell lines used (5563T,IgG(k) and Y5781.4 ,IgM).

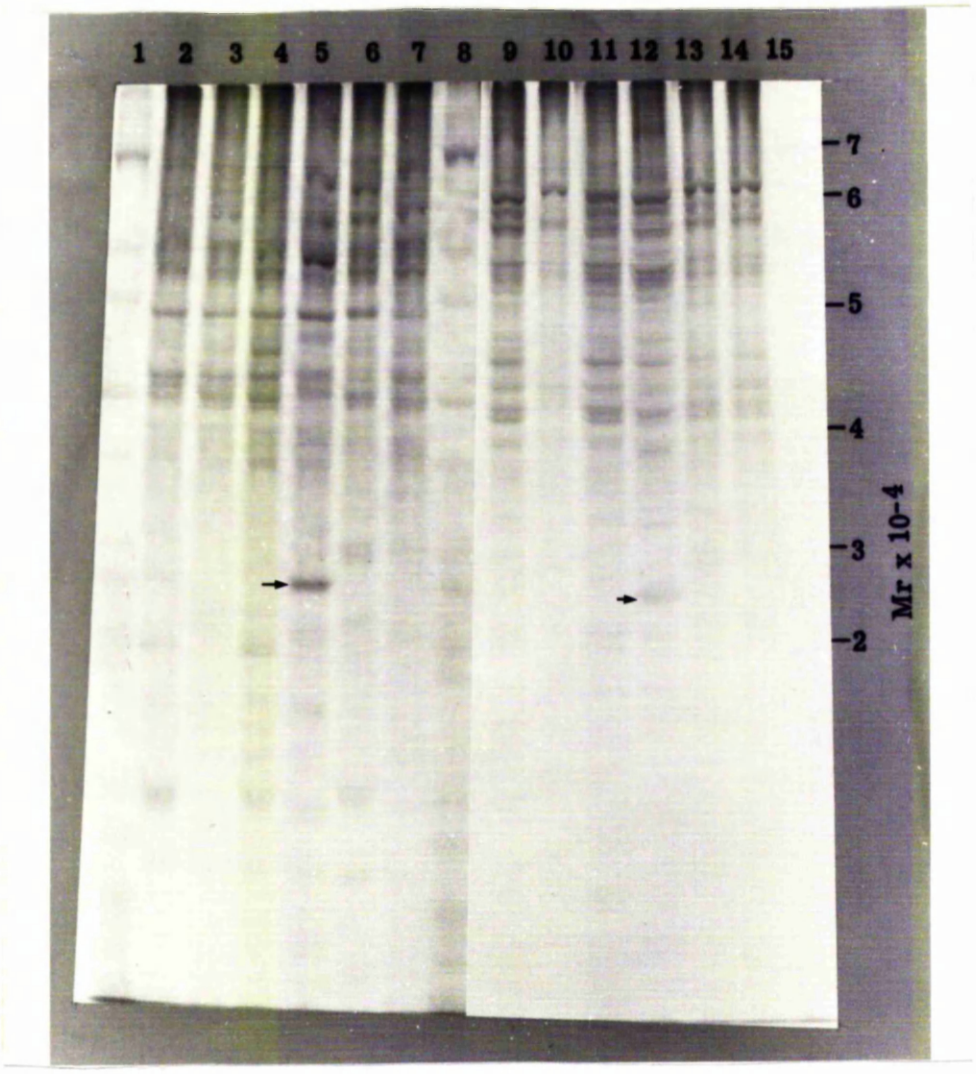
By looking at clonal cell lines in which the number of genotypic differences are limited, it was hoped to correlate particular proteins with the observed phenotypic differences. In addition, the nuclear protein patterns of plasmacytoma Y5781.4 are compared with those of MOPC315.40.

Initially all of the MOPC315 derived cell lines plus plasmacytomas Y5781.4 and 5563T were compared using 1-dimensional slab gel electrophoresis followed by fluorography; the results are shown in Figure 7.

5.1 One dimensional S.D.S. gel electrophoresis

The nuclear proteins of all of the cell lines studied were compared by S.D.S. gel electrophoresis using the method of Laemmli. The fluorograph (Figure 7)

Figure 7.



Nuclear proteins of plasmacytoma cell lines.

Plasmacytoma cells were incubated with L-[^{35}S]-methionine for 4 hours at 37^{o}C and the nuclear proteins fractionated and analysed by one-dimensional polyacrylamide gel electrophoresis. Tracks 1, 8 and 15 : marker proteins of known Mr; 2 - 7 : nucleoplasmic proteins; 9 - 14 : NHCP; 2 and 9 : MOPC 315.32; 3 and 10 : MOPC 315.35; 4 and 11 : MOPC 315.36; 5 and 12 : MOPC 315.40; 6 and 13 : 5563T; 7 and 14 : Y5781.4

reveals that most of the proteins visualised are common to all of the cell lines. However, a protein of Mr 27,000 (arrowed) was observed in both the nucleoplasmic and NHCP fractions of MOPC315.40 (tracks 5 and 12) which was not present in plasmacytomas MOPC315.32,35 or 36, Y5781.4 or 5563T. This protein did not run in the same position as immunoglobulin light chain. It can be seen that there are a number of quantitative differences between the cell lines. Moreover some proteins are observed mainly in the nucleoplasmic fraction; for example, protein of Mr 50,000, whilst others, for example, of Mr 60,000, occur predominantly in the NHCP fraction. However, the close proximity of many bands and slight irregularities in the running of the gel make it necessary to analyse the nuclear proteins in more detail. This may be achieved by using 2-D polyacrylamide gel electrophoresis.

5.2 Comparison of cell lines derived from MOPC 315.40

Figures 8 and 9 show the two dimensional electrophoretic profiles of the NHCP and nucleoplasmic proteins of MOPC 315.40 (IgA producer) the parent cell line and MOPC 315.32 (λ_2 producer). The integrated densities of selected spots were derived by scanning fluorographs with a Joyce-Loebel densitometer. The scan of each spot was traced on to tracing paper of uniform thickness, the peaks were cut out and weighed,the weight

being proportional to the area under the peak. Spots are designated by their co-ordinates (pI/Mr x 10^{-3}). The integrated densities of selected spots from plasmacytomas MOPC 315.32 and MOPC 315.40 are given in Table 8. Detailed scrutiny of the fluorographs reveals a number of qualitative and quantitative differences between the nuclear protein patterns of the cell lines. Proteins 6.4/27, 5.5/44 and 5.3/54 (indicated by arrows) can be detected in both the nucleoplasmic and NHCP fractions of MOPC 315.40 (Figures 8a and 9b respectively) but not in plasmacytomas MOPC315.32,35 or 36, Y5781.4. Protein 6.4/27 is presumably the 27,000 Mr protein observed in the 1 dimensional gel of MOPC 315.40 (Figure 7, tracks 5 and 12).

There are also significant quantitative differences to be found between the cell lines: in the NHCP fractions proteins 7.0/24 and 5.4/48 (arrowed) are found in considerable quantities in MOPC 315.40 but barely detectable in MOPC 315.32.

In the nucleoplasmic fraction protein 4.6/51 is found in large quantities in MOPC 315.40 but barely detectable in MOPC 315.32. On the other hand protein 5.3/38 is intensely labelled in both nucleoplasmic and NHCP fractions of MOPC 315.32(Figures 8b and 9a arrowed), but hardly discernible in MOPC 315.40(Figures 8a and 9b).

There are also a number of differences in distribution of proteins between nucleoplasmic and NHCP fractions; for example, proteins 5.2/50 and 5.4/50 are found predominantly in the NHCP fraction of both cell lines (Figures 9a and 9b).

All of the observed differences in the electrophoretic profiles of the cell lines were reproducible. Electrophoretic analysis was carried out on at least two (and usually more) separate preparations of all of the cell lines. For the cell lines MOPC 315.40 and MOPC 315.32 the entire procedure from labelling cell lines to electrophoretic analysis was performed three times. If the pulse chase experiments are included the analysis was performed 8 times from the preparation of cell lysates and nuclear fractions.

Although there were occasional slight variations in the absolute positions of the proteins,particularly in the IEF dimension (this was probably due to running the isoelectric focusing for too long,this causes the pH gradient and some proteins to be lost from the top of the gel), the positions of proteins could be identified relative to one another and to common abundant proteins did not vary significantly. Thus each spot could be identified reproducibly by its relative position in the total pattern. In addition the ability to run both the

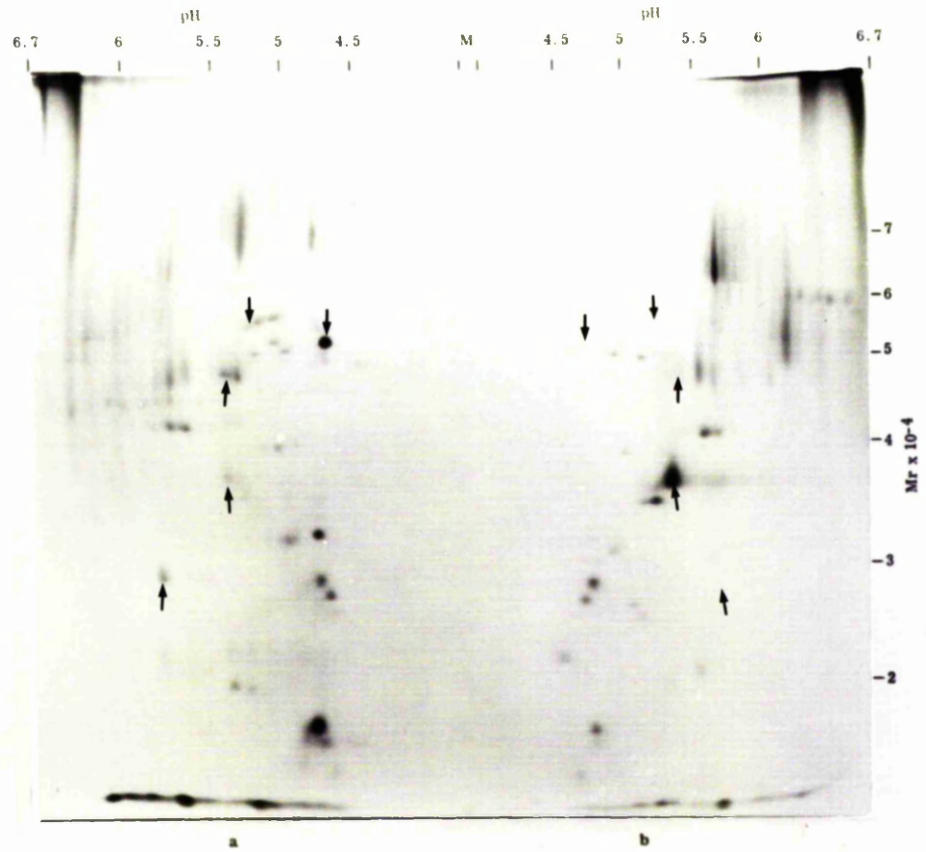
IEF and SDS gel electrophoresis separations on six samples simultaneously gave reproducible nuclear protein patterns.

Charge heterogeneity is observed in a number of proteins,e.g. proteins 5.6-5.9/42 (Figure 8), presumably due to post synthetic modification such as phosphorylation, deamidation acetylation or ADP ribosylation.

It is interesting to note that superimposition of the NHCP and nucleoplasmic fluorographs of MOPC 315.40 reveals that NHCP 5.3/54 corresponds to an addition to the basic end of the chain of spots 5.0/56-5.3/54 in the nucleoplasmic fraction.

The two dimensional electrophoretic profiles of MOPC 315.35 and MOPC 315.36 are shown in Figures 10 and 11. Close scrutiny of the original fluorographs reveals that all detectable proteins are shared by these cell lines. However, the low molecular weight acidic proteins show differences in partitioning between the nucleoplasmic and NHCP fractions. It is not clear whether this represents a genuine difference between the cell lines or merely reflects the sensitivity of binding to chromatin to small changes in extraction conditions. The protein patterns of these cell lines show a marked

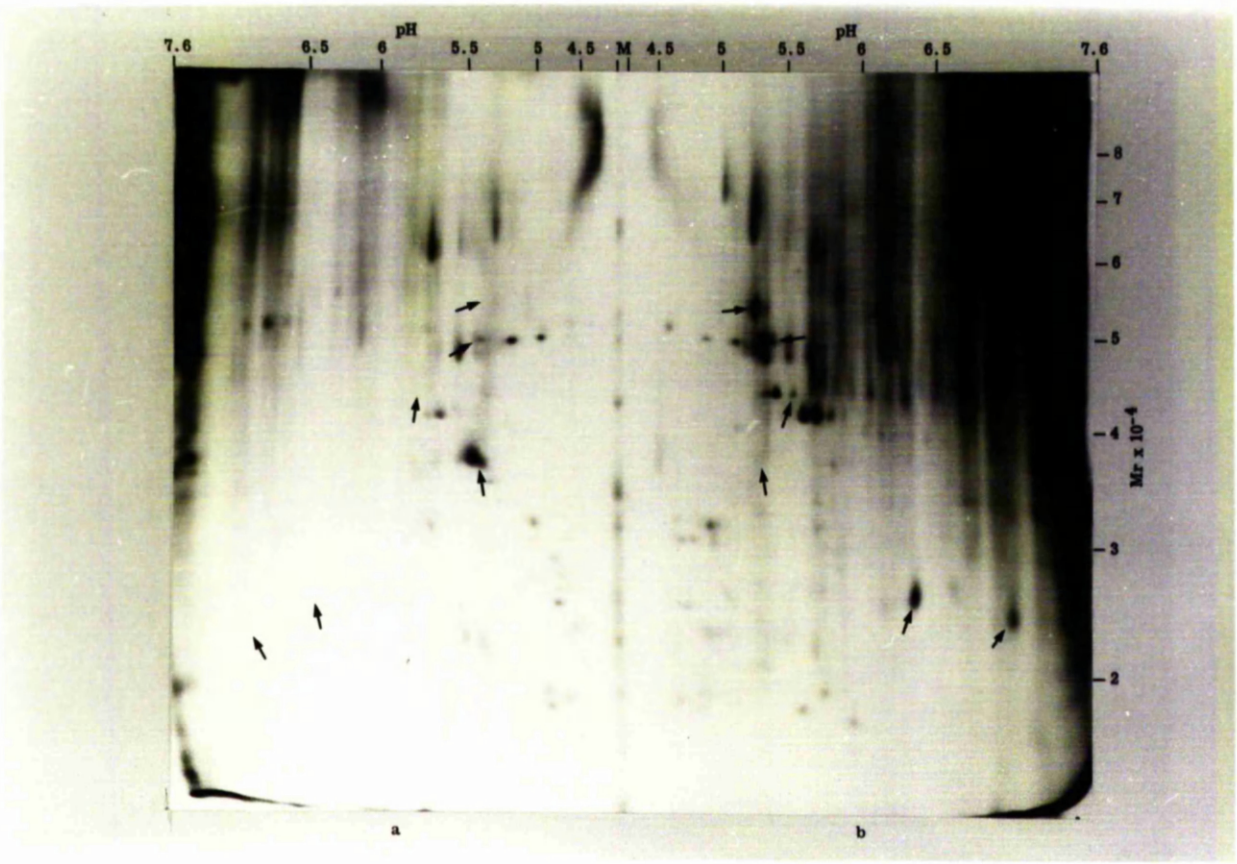
Figure 8.



Nucleoplasmic proteins of plasmacytoma cell lines MOPC 315.32 and MOPC 315.40.

Plasmacytoma cells were incubated with L-[^{35}S]-methionine for 4 hrs at 37^{o}C and the nuclear proteins were fractionated and analysed by 2-D polyacrylamide gel electrophoresis: a) MOPC 315.40; b) MOPC 315.32. The central track (M) contains marker proteins of known Mr. The positions of spots referred to in the text are indicated by arrows.

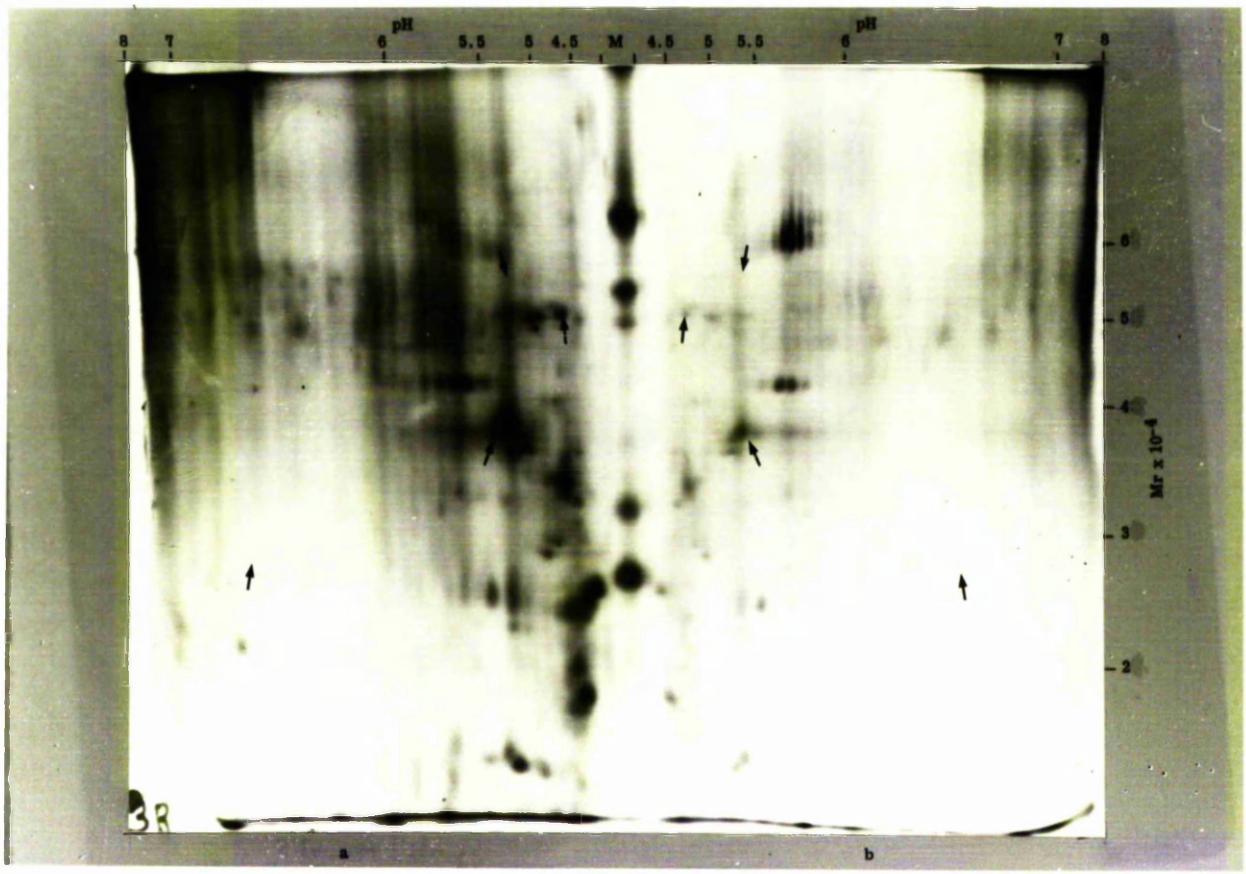
Figure 9.



Non-histone chromatin proteins of plasmacytoma cell lines MOPC 315.32 and MOPC 315.40.

Plasmacytoma cells were incubated with L[$^{-35}$S]-methionine for 4 hrs. at 37^{o}C and the nuclear proteins were fractionated and analysed by 2-D polyacrylamide gel electrophoresis. a) MOPC 315.32; b) MOPC 315.40. The central track (M) contains marker proteins of known Mr. The positions of spots referred to in the text are indicated by arrows.

Figure 10.



Nucleoplasmic proteins of MOPC 315.35 and MOPC 315.36. Plasmacytoma cells were incubated with L[$^{-35}$S]-methionine for 4 hrs. at 37^{o}C and the nuclear proteins fractionated and analysed by 2-D polyacrylamide gel electrophoresis : a) MOPC 315.36; b) MOPC 315.35. The central track (M) contains marker proteins of known Mr. The positions of the spots referred to in the text are indicated by arrows.

Table 8. Integrated densities of selected spots from Figures 8 and 9.

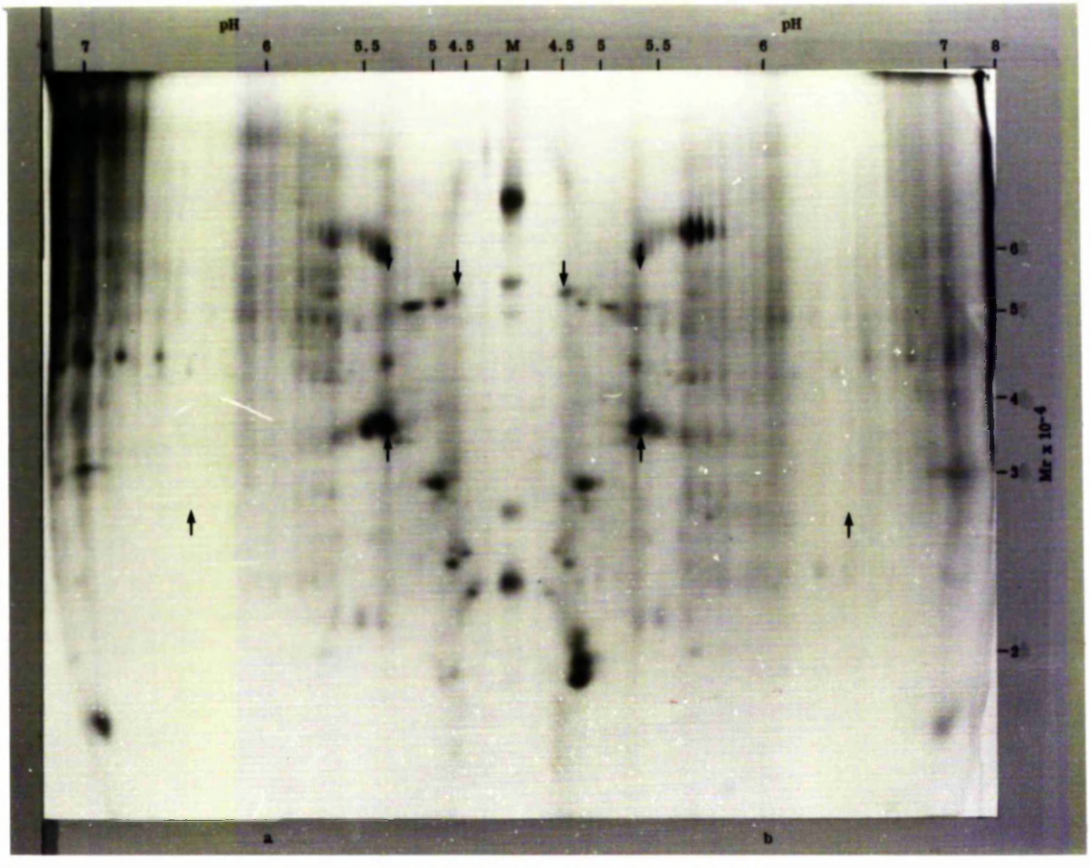
	Integrated Density (arbitrary units)			
pI/Mr x 10^3	MOPC	315.32	MOPC	315.40
	A	N	A	N
6.4/27	-	-	15	80
5.0/32	7	27	25	50
5.3/38	110	240	-	-
5.6-5.9/42	67	56	55	232
5.4/45	-	17	-	80
5.6/45	-	-	-	21
5.4/48	2	20	46	87
5.6/48	35	50	-	34
4.9/50	13	55	16	24
5.2/50	18	65	13	70
5.4/50	-	46	5	182
4.6/51	-	3	100	22
5.3/54	-	-	33	149

A = Nucleoplasmic proteins; N = NHCP

Selected spots from Figures 8 and 9 were scanned using a Joyce-Loebel densitometer and integrated spot densities measured in arbitrary units from the area under the peak.

similarity to those of MOPC 315.32, when allowances are made for differences in loading and pH gradients. All proteins present in MOPC 315.40 but absent in MOPC 315.32 are also absent in MOPC 315.35 and 36. In addition, protein 5.3/38 which is intensely labelled in MOPC 315/32 but barely discernible in MOPC 315.40 also is intensely labelled in MOPC 315.35 and MOPC 315.36 (arrowed).

Figure 11.



Non-histone chromatin proteins of plasmacytoma cell lines MOPC 315.35 and 36.

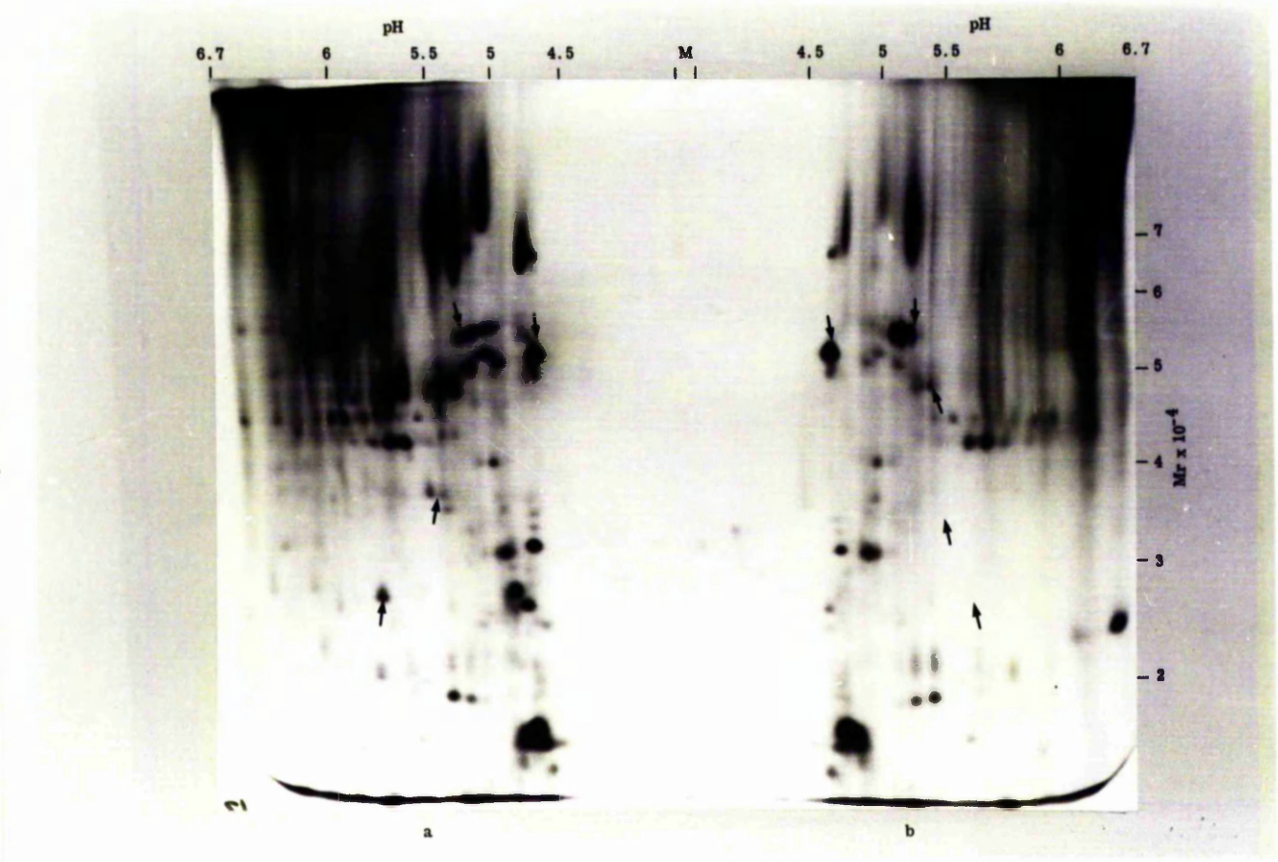
Plasmacytoma cell lines were incubated with L-[^{35}S]-methionine for 4 hrs at 37^{o}C and the nuclear proteins fractionated and analysed by 2-D polyacrylamide gel electrophoresis. a) MOPC 315.36; b) MOPC 315.35. The central track (M) contains marker proteins of known Mr. The positions of spots referred to in the text are indicated by arrows.

5.3 Comparison of cell lines producing different classes of immunoglobulin

The two dimensional electrophoretic profiles of MOPC 315.40 and Y5781.4 an IgM(K) producing plasmacytoma are shown in Figures 12 and 13. In some respects the profile of MOPC 315.40 appears to be more similar to that of Y5781.4 than to MOPC 315.32 (and MOPC 315.35 and 36). For example protein 5.5/44 is present in both fractions of MOPC 315.40 and Y5781.4 but absent in MOPC 315.32, MOPC 315.35 and MOPC 315.36. Furthermore, protein 5.3/38 which is intensely labelled in both fractions of MOPC 315.32, MOPC 315.35 and MOPC 315.36 is barely detectable in both fractions of MOPC 315.40 and Y5781.4. However, protein 6.4/27 found in both fractions of MOPC 315.40 is absent in both fractions of Y5781.4 as well as MOPC 315.32,35 and 36.

The situation with regard to protein 5.3/54 is more complex, this protein is present in both fractions of MOPC 315.40 (but predominantly in the NHCP fraction) but absent from the NHCP fraction of Y5781.4. However superimposition of the nucleoplasmic fraction of Y5781.4 on the NHCP fraction of MOPC 315.40 shows that protein 5.3/54 corresponds to the most basic of the chain of three spots (in Y5781.4) 5.1/54. This is different to

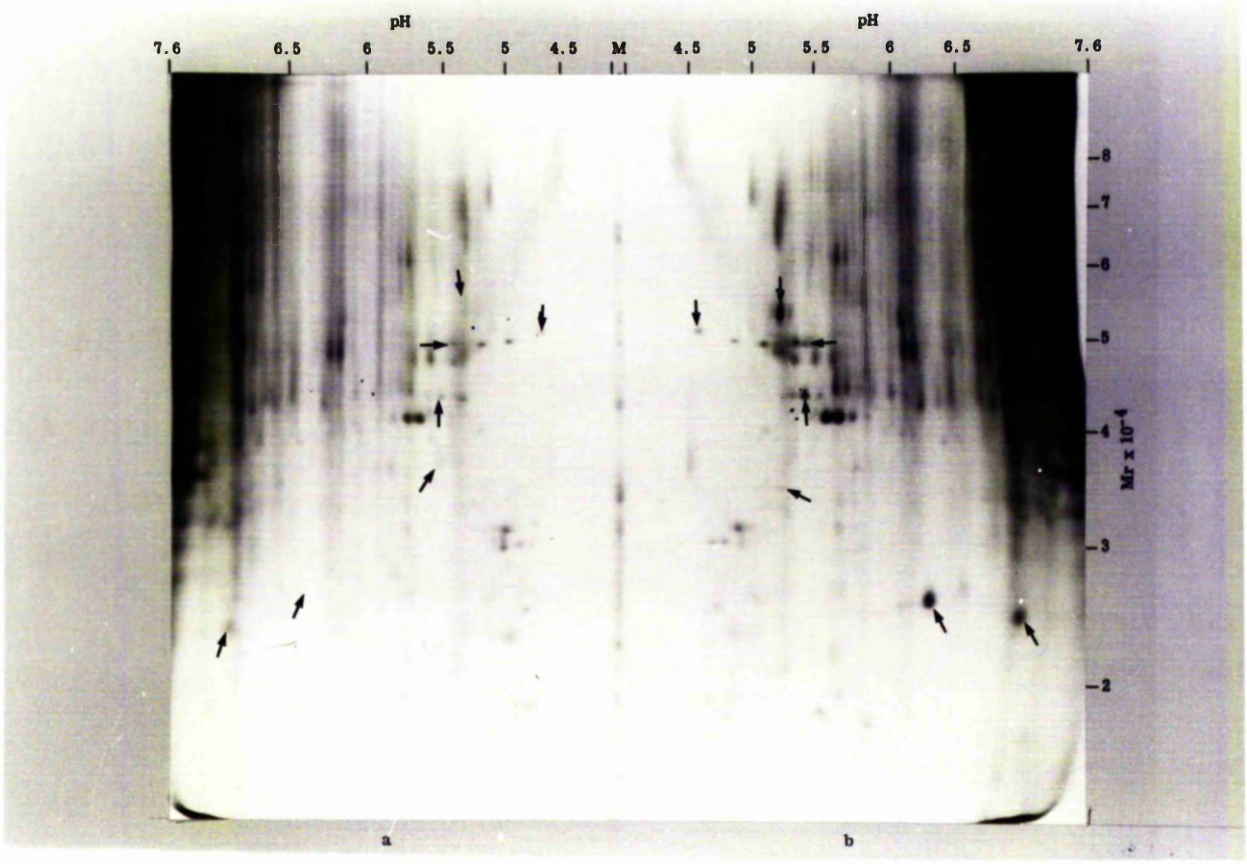
Figure 12.



Nucleoplasmic proteins of plasmacytoma cell lines MOPC 315.40 and Y5781.4.

Plasmacytoma cells were incubated with L-[^{35}S]-methionine for 4 hours at 37^{o}C and the nuclear proteins were fractionated and analysed by 2-D polyacrylamide gel electrophoresis : a) MOPC 315.40; b) Y5781.4. The central track (M) contains marker proteins of known Mr. The positions of spots referred to in the text are indicated by arrows.

Figure 13.



Non-histone chromatin proteins of plasmacytoma cell lines MOPC 315.40 and Y5781.4.

Plasmacytoma cells were incubated with L-[^{35}S]-methionine for 4 hrs at 37^{o}C and the nuclear proteins fractionated and analysed by 2-D polyacrylamide gel electrophoresis : a) Y5781.4; b) MOPC 315.40. The central track (M) contains marker proteins of known Mr. The positions of spots referred to in the text are indicated by arrows.

the situation in the nucleoplasmic fraction of MOPC 315.40 where it is the most basic of the chain 5.0/56 to 5.3/54. This indicates that the post synthetic modification giving rise to the charge heterogeneity may be different in the cell lines in that MOPC 315.40 adjacent spots have a molecular weight difference of the order of 500 Mr whereas in Y5781.4 adjacent spots are the same molecular weight. Alternatively,the difference in Mr could be due to a neutral post-synthetic modification.

The differences in protein composition between the cell lines discussed above are summarised in Table 9.

Table 9 Summary of protein differences between cell lines

Plasmacytoma cell line	Protein 6.4/27	5.3/38	5.5/44	4.6/51	5.3/54
Y5781.4	-	-	+	+	*
MOPC 315.40	+	-	+	+	+
MOPC 315.32	-	+	-	-	-
MOPC 315.35	-	+	-	-	-
MOPC 315.36	-	+	-	-	-

\+ indicates that a protein is present in the cell line

\- indicates that a protein is absent or found in negligible quantities in the cell line.

* This protein is absent from the NHCP fraction but present in the nucleoplasmic fraction.

6. PULSE-CHASE EXPERIMENTS

The turnover of nuclear proteins in some of the cell lines exhibiting differences in nuclear protein patterns was measured by labelling the appropriate cell line (MOPC 315.40, MOPC 315.32 and Y5781.4) for four hours and chasing with a large excess of unlabelled methionine for 20 hrs.

Briefly, the cells were labelled in the same way as in the comparison experiments. At the end of the labelling period the cells were washed twice in pre-warmed Hanks basal salt solution containing 5% foetal calf serum and 1 mM unlabelled methionine (to displace free [^{35}S]-methionine). The cells were then resuspended in fresh pre-warmed cell culture medium at a concentration of 5 x 10^5 cells/ml and incubated at 37^{o}C. The initial cell viability in these experiments was 90-98% which fell to 80-85% after the 20 hr. chase period. Because only 10^7 plasmacytoma cells were used for analysis at each time point (0, 1, 2, 4 and 20 hrs) it was necessary to add 4 x 10^7 mouse splenic lymphocytes to each sample, before preparation of nuclei, to provide enough material for the efficient preparation of nuclear protein fractions. Nuclear protein fractions were prepared and analysed by two dimensional polyacrylamide gel electrophoresis. Protein spots were detected by fluorography using a pre-exposed X-ray film (Laskey and

Mills,1975). Quantitation of the amount of radioactivity in selected spots was carried out by scanning the spots using a Joyce-Loebel densitometer. The scan of each spot was traced onto tracing paper of uniform thickness, the peaks were cut out and weighed, the weight being proportion to the area under the peak and thus the amount of radioactivity in the sample (Laskey and Mills, 1975). These experiments were carried out to determine the average rate of turnover of the nuclear proteins separated by our fractionation procedure and to examine the rate of turnover of individual protein species within the nuclear non-histone protein fractions with particular reference to those proteins which are specific to individual cell lines. It should be noted that in these experiments we are measuring the loss of label from protein fractions which is normally due to protein degradation.

6.1 Turnover of protein fractions

If there is no significant re-incorporation of label the amount of radioactivity in each individual protein species will decay exponentially. Thus, the half lives of individual proteins can be calculated from the following equation :

$$T^{1/2} = \frac{t \log 2}{\log \left(\frac{\text{CPM at time } t_0}{\text{CPM at time } t_1}\right)}$$

where $T^{1/2}$ = half life (hours)

$t = t_1 - t_0$ (hours)

and t_1 is any time after t_0

However this relationship does not hold for mixtures of proteins turning over at varying rates and was only applied to individual proteins; half lives of the protein fractions were measured directly from plots of the turnover (Figures 14-18) of the cytoplasmic and nucleoplasmic proteins. However the histones and NHCP turned over too slowly for their half lives to be measured directly so the above equation was used to provide an approximation.

SECRETED PROTEINS

Proteins secreted into the culture medium are not subject to turnover in the same sense as intracellular proteins. The amount of label present in the medium increases throughout the experiment (Figure 14). This is because there is a large intracellular pool of immunoglobulin and free light chains which continue to be released during the chase, hence the label continues to appear in the culture medium during the chase. In addition there may be a very small amount of cell lysis at the end of the experiment as the cell viability dropped (very slightly).

CYTOPLASMIC FRACTIONS

The cytoplasmic protein fraction turns over fairly quickly relative to the nuclear fractions and with the exception of MOPC 315.32 counts present in this fraction fell from the beginning of the experiment (figure 15, Table 10). There are a number of reasons which could account for the observed differences including differences in metabolic rate and rates of cell division.

NUCLEOPLASMIC FRACTION

The proteins in the nucleoplasmic fraction turn over fairly quickly at similar rates to those of the cytoplasmic fraction, with the exception of plasmacytoma Y5781.4(Table 10). It can be seen in Figure 16 that the counts in this fraction begin to fall immediately (MOPC 315.40) or after one hour (MOPC 315.32 and Y5781.4) the initial rise in this fraction is presumably due to an influx of proteins from the cytoplasm.

HISTONES

It can be seen (Figure 17 and Table 10) that the histones turn over very slowly and at comparable rates in all the cell lines. This is in keeping with their role as structural proteins.

NON-HISTONE CHROMATIN PROTEINS

The NHCP turn over more slowly on average than the cytoplasmic and nucleoplasmic proteins in all of the cell lines. In addition, this fraction shows an initial rise in the amount of label incorporated, presumably due to transport of labelled proteins from the cytoplasmic and nucleoplasmic fractions into the NHCP fractions (Figure 18 and Table 10).

Table 10 Average half lives of protein fractions(hours)

Cell line	Cytoplasmic proteins	Nucleoplasmic proteins	Histones	NHCP
MOPC 315.40	6	8.5	7.0	22
MOPC 315.32	7	3	50	19
Y5781.4	1	9	68	26

6.2 Turnover of individual proteins

The turnover of individual proteins within the nucleoplasmic and NHCP fractions of MOPC 315.40 and MOPC315.32 was investigated by analysing each fraction from each time point by 2-D gel electrophoresis. The integrated densities of selected spots from the fluorographs were measured using a Joyce-Loebel densitometer and plotted as shown in Figure 19. The half lives of some of the proteins were calculated and are given in Table 11.

Within each fraction individual proteins exhibited marked differences in their rate of turnover, for example, protein 4.7/19 turns over relatively quickly in both fractions of each cell line whereas protein 5.4/50 turns over so slowly that its radioactivity does not drop appreciably over the chase period. Protein 5.6-5.9/42 (a protein exhibiting large heterogeneity) turns over very slowly in all the fractions studied except for the nucleoplasmic fraction of MOPC 315.40. However, it can be seen that almost all of the proteins in this fraction turn over quickly. It is not obvious whether this is related to differences in gene expression or to general differences in metabolic rate between the cell lines.

Looking at proteins which are present only in MOPC 315.40 we see that proteins 6.4/27 and 5.5/54 turn over fairly quickly in the nucleoplasmic fraction but very slowly within the NHCP, in contrast protein 4.6/51 turns over very slowly within the nucleoplasmic fraction but at three times the rate in the NHCP.

Protein 5.3/38 which is found in very large amounts in MOPC 315.32, but in negligible amounts (too little to quantify using these techniques) in MOPC 315.40, turns over very slowly in the nucleoplasmic fractions but at a higher rate in the NHCP.

Most of the proteins studied show a lag period before the counts incorporated reach a maximum. In general this is shorter in the nucleoplasmic fraction than in the NHCP fraction and perhaps represents proteins being transported from the cytoplasm to the nucleoplasm followed by subsequent incorporation into the NHCP fraction.

Table 11 Half lives of individual proteins

Cell line

	MOPC 315.40		MOPC 315.32	
Protein	**Nucleoplasmic**	**NHCP**	**Nucleoplasmic**	**NHCP**
4.7/19	7.9	7.6	7.8	4.2
6.4/27	4.7	21.4	-	-
5.0/32	7.6	16.1	15.0	10.8
5.3/28	-	-	28.8	10.4
5.6-5.9/42	6.6	750	*	*
4.9/50	7.6	6.2	12.1	10.9
4.6/51	42.7	14.7	-	-
5.5/54	7.4	55.8	-	-

Half lives were measured (in hours) from the amount of radioactivity present in each spot at t = 20 and the time and number of counts when the radioactivity present was at a maximum.

In the proteins marked * the counts were at a maximum at t = 20, thus we were unable to measure the half life using this method.

Figures 14-18.

Turnover of nuclear protein fractions

Triplicate samples of each of the proteins were taken, precipitated with TCA (as in materials and methods) and counted using a scintillation fluid cocktail containing PPO/POPOP. The total counts in each fraction were then calculated and plotted against time.

□ Y5781.4

\+ MOPC 315.40

◇ MOPC 315.32

Figure 14.

Secretion of Proteins into Culture Medium

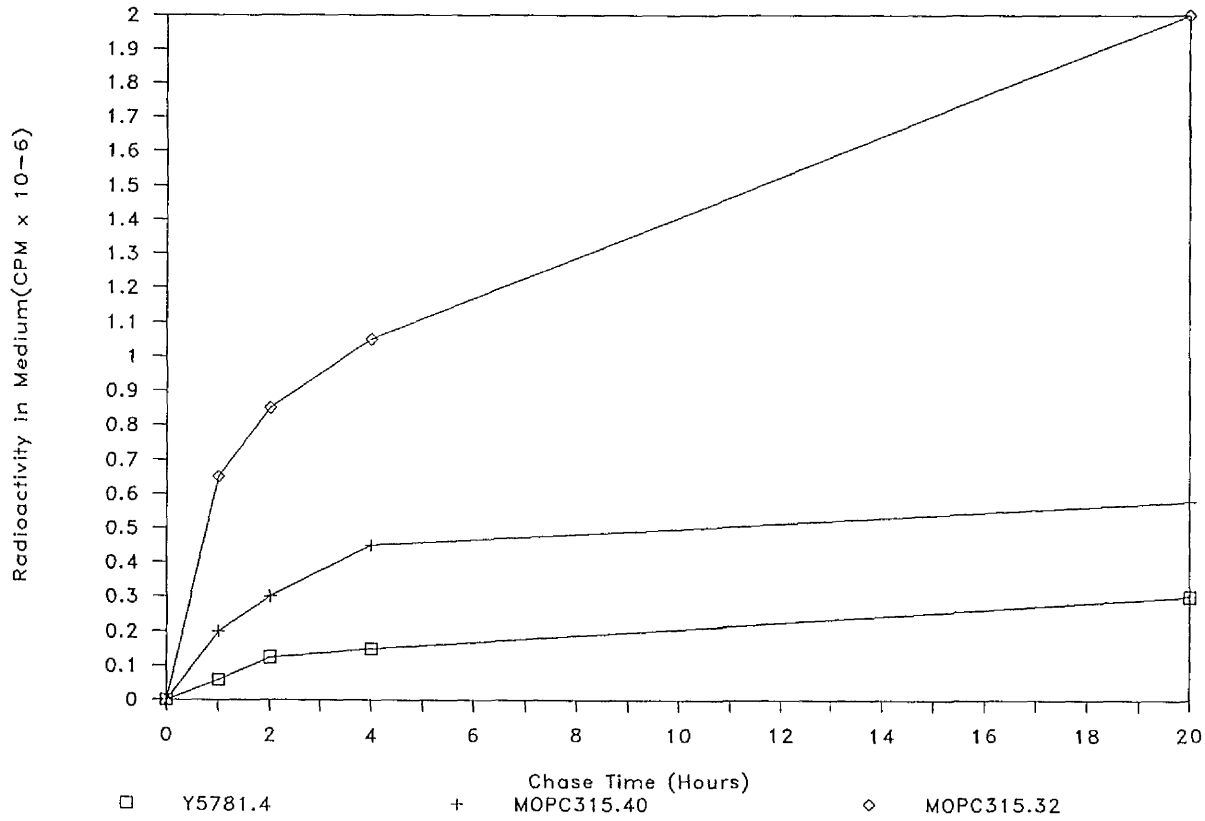


Figure 15.

Turnover of Cytoplasmic Proteins

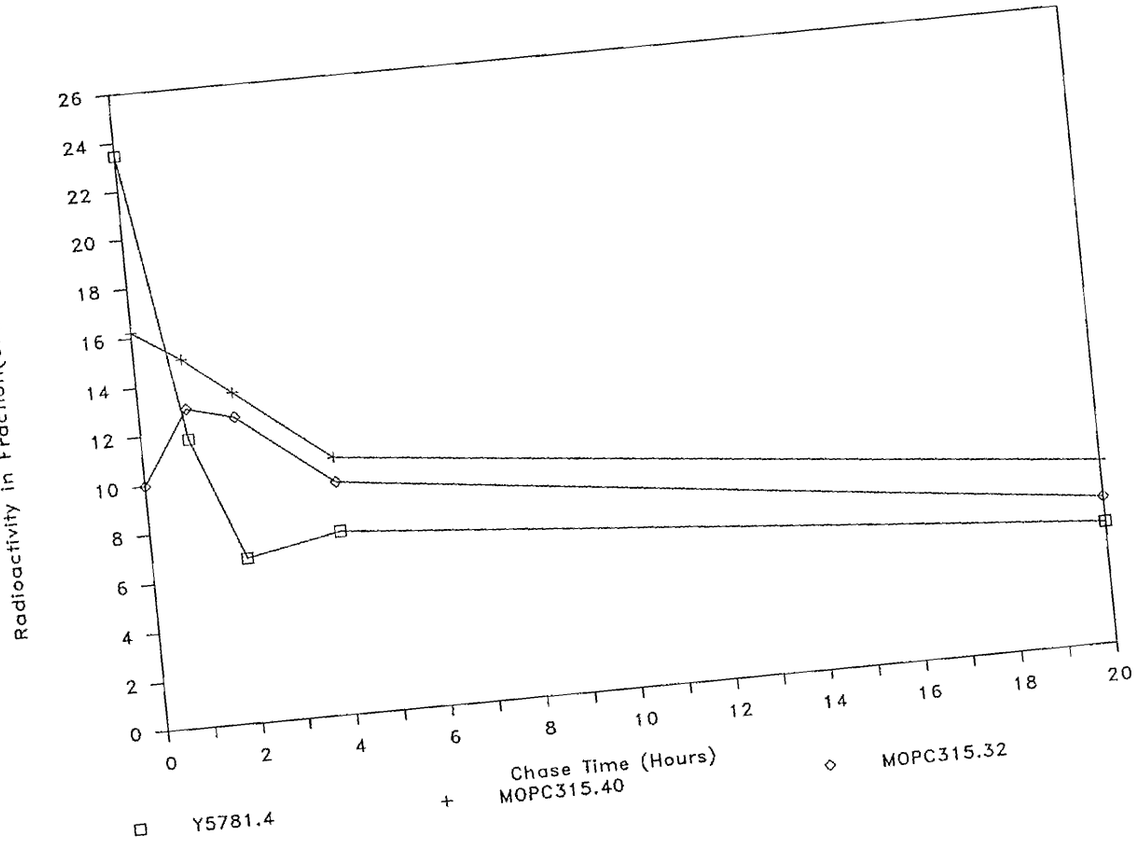


Figure 16.

Turnover of Nucleoplasmic Proteins

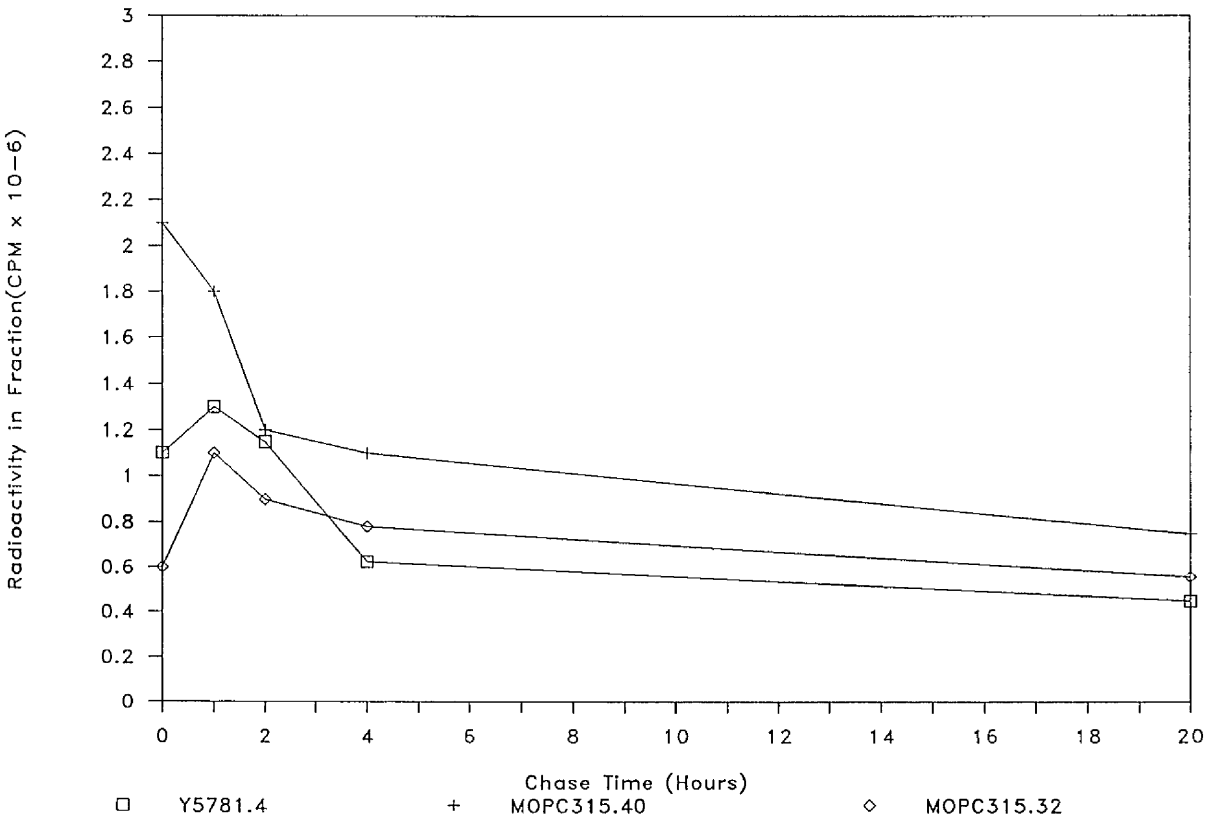


Figure 17.

Turnover of Histones

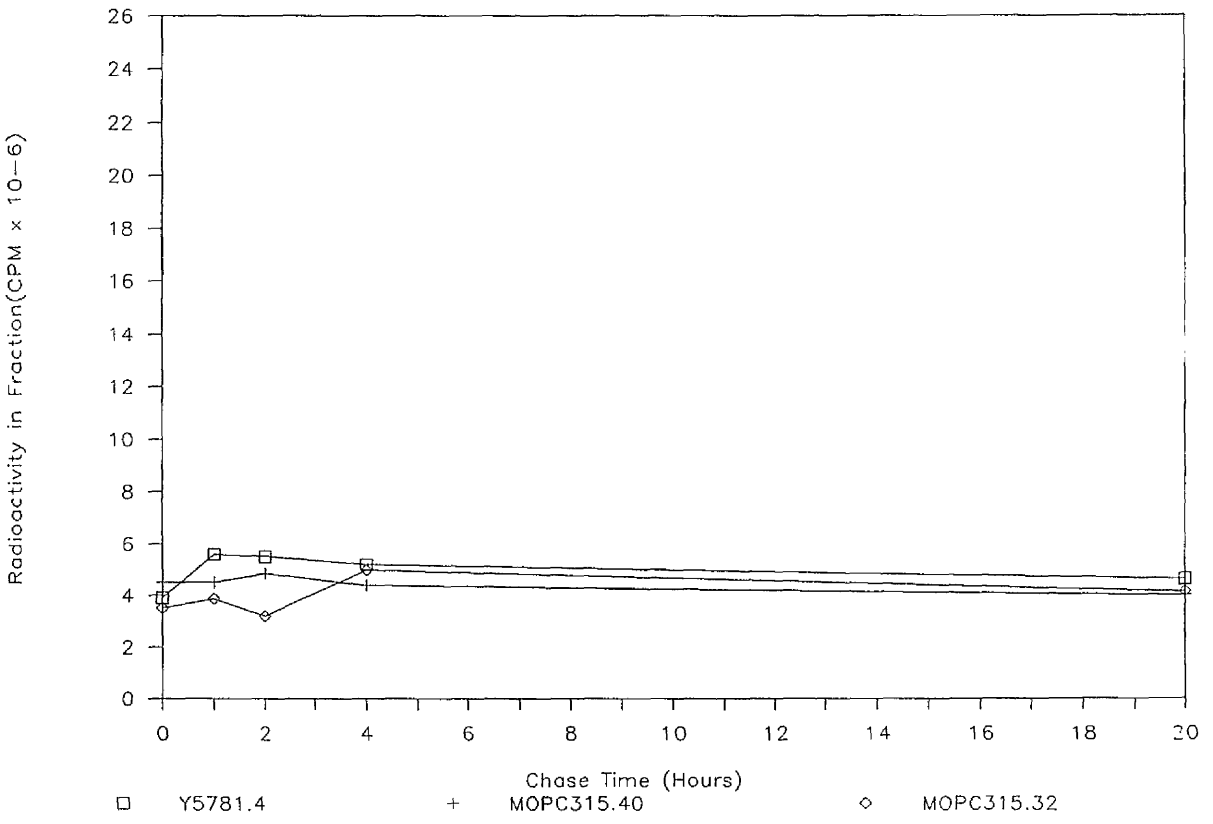


Figure 18.

Turnover of Non-histone Chromatin Proteins

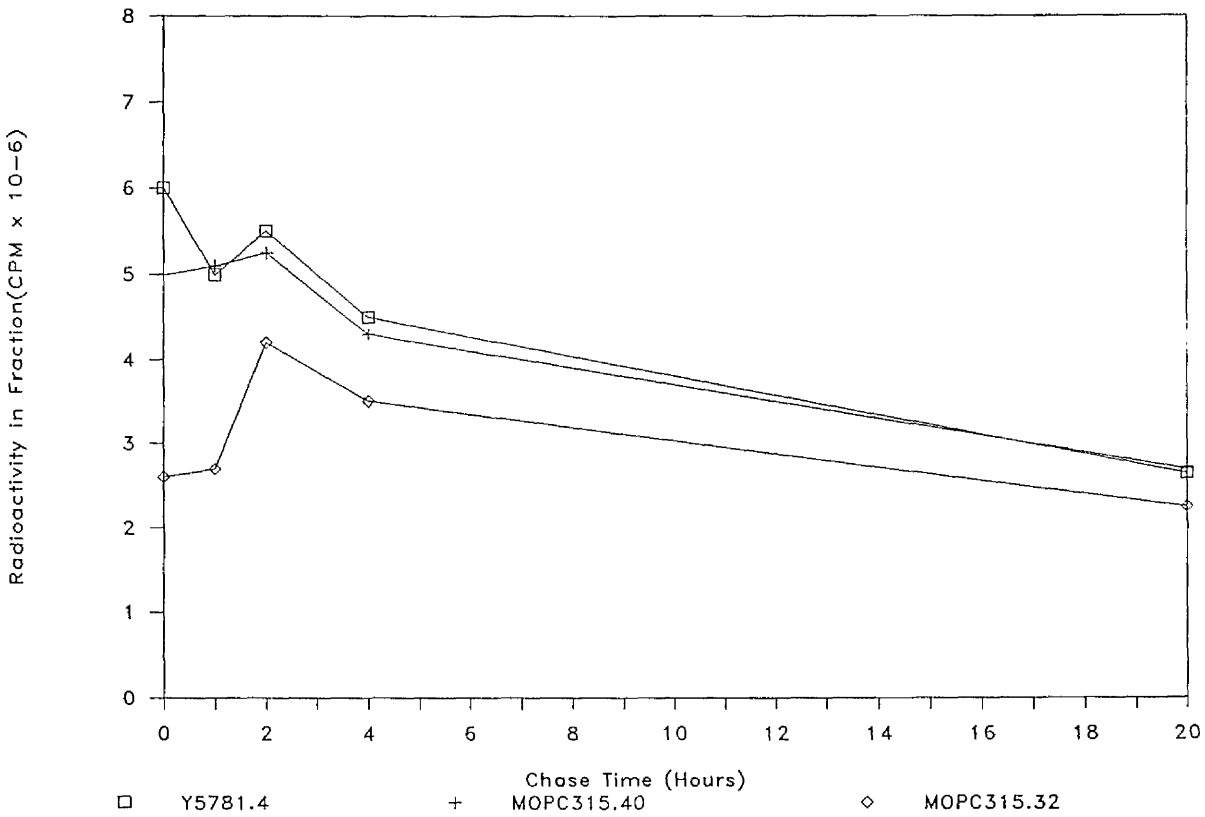


Figure 19.

Turnover of individual nuclear proteins

Plasmacytoma cells were incubated with L-[^{35}S]-methionine for 4 hr. at 37^{o}C and then chased with unlabelled methionine. Samples were taken at 0, 1, 2, 4 and 20 hr. after the beginning of the chase, nuclear proteins were fractionated and analysed by 2-D polyacrylamide gel electrophoresis. Selected spots from the fluorographs were scanned and the area under the peak,which is proportional to the amount of radioactivity in the spot,was calculated.

Spots are designated by their co-ordinates (pI/Mr x 10^{-3}):

A and B - MOPC 315.32; C and D - MOPC 315.40;

B and D - non- histone chromatin proteins;

A and C - Nucleoplasmic proteins.

○——○ 4.6/19; ●——● 4.6/25; □——□ 6.4/27;

■——■ 5.0/32; △——△ 5.3/38; ▲——▲ 5.6-5.9/42;

○-----○ 4.9/50; ●-----● 5.2/50; □-----□ 5.4/50;

■-----■ 4.6/51; △-----△ 5.5/54.

Figure 19.

Turnover of individual nuclear proteins

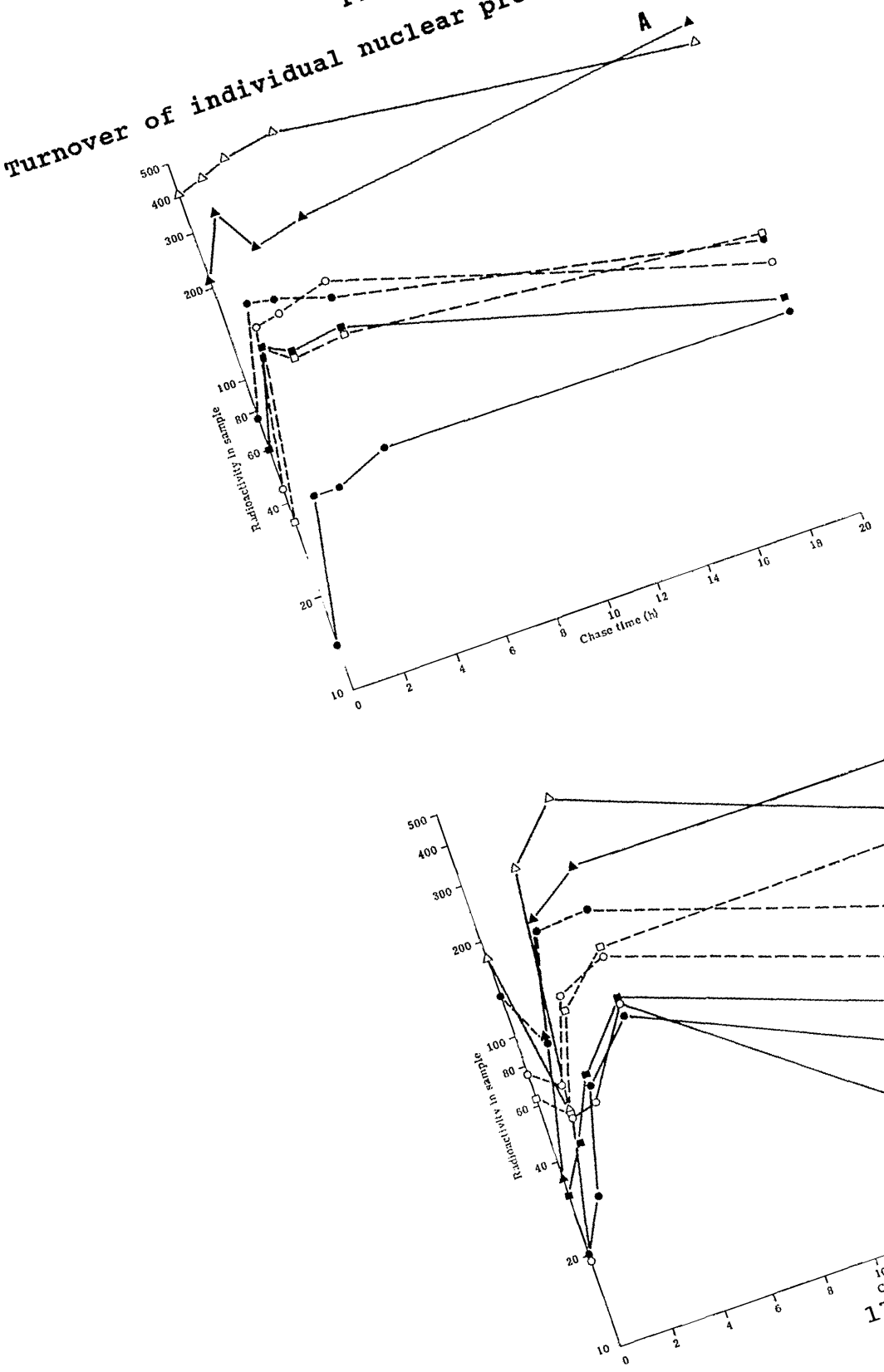
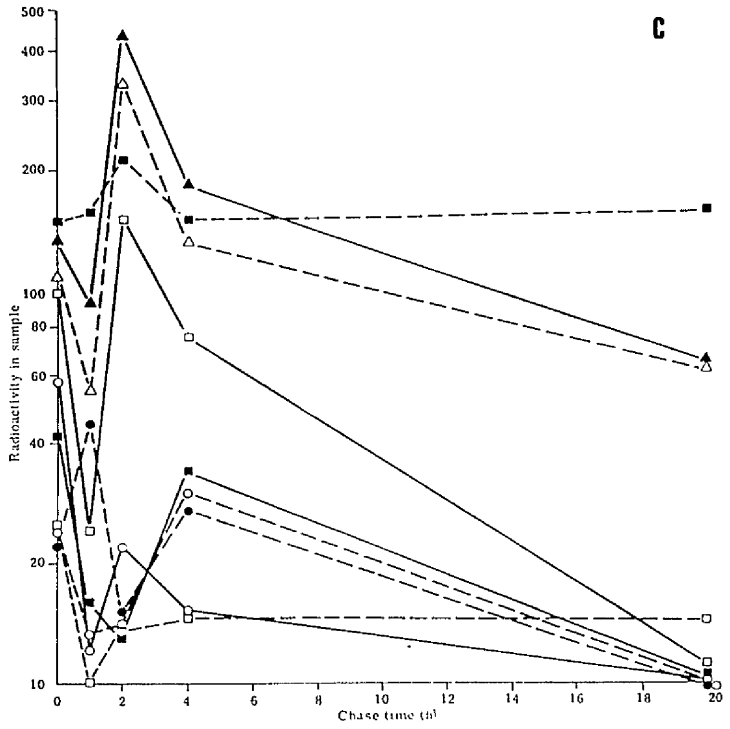
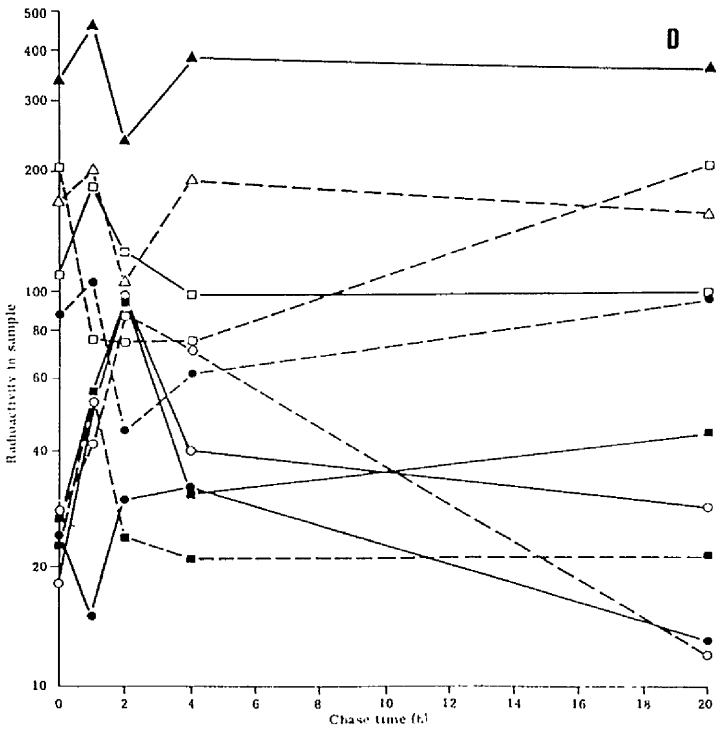


Figure 19.

Turnover of individual nuclear proteins





7. DISCUSSION

7.1 The binding of acetylaminofluorene to nuclear macromolecules

Early studies by Sporn and Dingman (1966) on the effects of carcinogens (including AAF) and hormones on chromatin showed that the total nuclear protein content of animals fed carcinogens showed significant variations from normal. However, no significant changes were observed in the histone protein content of any animals. They also found that intense stimulation with pituitary hormones increases the RNA and total protein content of liver chromatin but not the histone protein content.

In later experiments Barry et al. (1968) studied the binding of [^{14}C]-AAF to rat liver nuclear proteins after up to 7 injections of labelled carcinogen. Using gel filtration and ion exchange chromatography they isolated stable labelled protein adducts. Their results indicated a two-fold greater binding of carcinogen to the arginine rich histones than to the remaining basic nuclear proteins. Jungmann and Schweppe (1972) undertook similar studies using a number of labelled carcinogens and found a degree of specificity of binding to the nuclear proteins. They found that pretreatment of rats with nonradioactive carcinogen significantly reduced labelling by chemically identical

radioactive carcinogen while not markedly altering the uptake of a chemically different carcinogen. They found the greatest uptake of radioactivity into acidic nuclear protein fractions 60 minutes after administration of AAF. In their experiments approximately 12 pmol of carcinogen was bound per mg of "acidic protein" (NHCP) 60 minutes after injection. In the experiments reported in chapter 4 the values obtained were 55, 43 and 16.4 pmol/mg for the urea soluble NHP, residual proteins and histones respectively. However, in the experiments reported here the sample values were obtained 24 hr. after the second injection of carcinogen. In addition more carcinogen was administered (2 x 50 μCi/100 g body weight in comparison with 1 μCi/100 g body weight) in our experiments and at a higher specific activity (50 mCi/mM compared to 12.7 mCi/mM).

Binding to rat liver DNA was recorded by them to be 26 p mol/mg of DNA (60 minutes after injection) falling to 15 p mol after 3 days, in the experiments described here the values were 19.6 pmol/mg of DNA 24 hr. after the second injection falling to 2 p mol after 14 days. This corresponds to a half life of binding to DNA of 3.9 days in our experiments, compared to 3.8 days in their results.

Lotliklar and Paik (1971) reported that approximately four times as many fluorene residues were bound to the NHP fraction than were bound to histones; the results reported here confirm these findings.

By analysing the histones further using S.D.S. gel electrophoresis it has been shown that isolated histone species contained much less bound carcinogen than the initially isolated crude histone mixture. Furthermore the bound carcinogen was distributed evenly among the five components. This may be the result of carcinogen binding to modified histone molecules with a different electrophoretic mobility or that the binding of carcinogen alters the electrophoretic mobility of the histones.

When the experiments described in this thesis were performed it was believed that activation of acetylaminofluorene proceeded via N-oxidation in the smooth endoplasmic reticulum followed by formation of the sulphate ester, which was believed to be the ultimate carcinogen. In order to examine the effects of increased N-oxidation and sulphate ester formation experimental animals were pre-fed sodium sulphate and phenobarbitone to examine their effects on the metabolism of AAF. In both experiments the specific activity of all nuclear fractions fell. The effect of

the administration of phenobarbitone to rodents is a rapid and marked enlargement of the liver which may to some extent account for the reduction in the specific activities of the nuclear macromolecules. However the reduction is probably too large to be accounted for by a simple increase in liver weight and the quantity of nuclear macromolecules. Other effects of phenobarbitone administration include an increase in part of the cytochrome p450 mixed function mono-oxygenase system and an increased level of glutathione S-Transferase (Pickett et al.,(1981). The stimulation of the liver cytochrome p450 mono oxygenase system may therefore generate an increased amount of the active N-hydroxy-acetylaminofluorene metabolite. However this activated metabolite may be detoxified at an increased rate by first binding to glutathione S-transferase and then reacting with glutathione or simply by reacting with the protein itself. Therefore the reduction in the specific activity of nuclear macromolecules in phenobarbitone treated rats may be due to an alteration in the balance of detoxification and activation. This change being brought about by an increase in the 'scavenger' activity of glutathione S-transferase. In addition the relative levels of activation (via N-oxidation) and detoxification (via ring oxidation) of acetylaminofluorene may be altered in favour of the

latter leading to a further reduction of binding to nuclear macromolecules.

Current evidence suggests that the binding of AAF proceeds via de-acetylation rather than sulphate esterification (Schut et al., 1978, Sakai et al., 1978, Stout et al., 1980). Sulphate esterification is now believed to be responsible for the alkylation of cytoplasmic components. Thus phenobarbitone may alter the balance of detoxification and metabolic activation resulting in more efficient removal of carcinogen from the liver, with the possibility of increased binding to cytoplasmic macromolecules (unfortunately the binding of AAF to cytoplasmic components was not measured in these experiments).

The reduction of specific activities of sulphate treated animals was more puzzling at the time as the then available evidence (Kriek, 1974, Weisburger et al., 1972, Gutmann et al., 1972) pointed to the sulphate ester of AAF as the ultimate carcinogen. However, in the light of present evidence (Schut et al., 1978, Sakai et al., 1978, Stout et al., 1980), it can be seen that this is likely to lead to an increase in cytoplasmic binding of AAF (unfortunately this was not measured) possibly reducing the AAF available for binding to nuclear macromolecules.

Although it is now widely accepted that DNA is the critical target in the initiation of carcinogenesis by chemicals, the role of protein, particularly nuclear protein carcinogen adducts, may be important and cannot be ignored. These experiments show that the bulk of nuclear macromolecule-bound AAF is bound to non-histone proteins. Some of these proteins have been identified by separation using isoelectric focusing (Fig. 2) in particular in those bands of pI 5.2, 7.3 and 7.5. Furthermore, some of these proteins have been shown to be very long lived, particularly those bound to DNA. Elucidation of the role of these proteins as modifying factors in the early stages of chemical carcinogenesis is important if we are to understand how carcinogenesis takes place and hence how gene expression is controlled.

7.2 Comparison of nuclear proteins in plasmacytoma cell lines

The results reported in chapter 5 illustrate the differences in the nuclear protein patterns of closely related cell lines derived from the IgA secreting plasmacytoma cell line MOPC 315. These cell lines differ in their ability to express known genes (α, J and λ_2) on different chromosomes. In this work it was hoped that by looking at cells which were 'frozen' at one stage of differentiation, viz. the plasma cell, that it would be possible to both limit the number of differences between the cell lines and correlate particular proteins with phenotypic differences. To this end high resolution two dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975, O'Farrell and O'Farrell,1978) combined with fluorography (Bonner and Laskey, 1974, Laskey and Mills, 1975) were used to analyse these extremely heterogeneous protein fractions.

The MOPC 315 cell line secretes an IgA (λ_2) antibody and has been used extensively in experiments on B-Cell regulation and differentiation (reviewed by Lynch et al.,1978 and Abbas,1979). MOPC 315.40 is a sub clone of MOPC 315 which produces and secretes IgA. MOPC 315.32 produces and secretes λ_2 light chains. MOPC 315.35 and MOPC 315.36 are non-secreting clones of MOPC 315.32

derived by ultra-violet irradiation. Y5781.4 cells represent native ,IgM secreting plasma cells , 5563T is a tissue culture adapted IgG(k) secreting cell line.

Plasmacytomas MOPC 315.35 and 36 (non producers) are phenotypically almost identical and this is reflected in the electrophoretic profiles of their nuclear proteins in which all detectable nuclear proteins are common to both cell lines (there appear to be one or two quantitative differences which may be due to small differences in extraction conditions). The only apparent phenotypic difference between these cell lines and plasmacytoma MOPC 315.32 is that they are unable to produce λ_2 light chains; again the two dimensional electrophoretic profiles of these cell lines are very similar. In contrast, the parent cell line MOPC 315.40 is different from these cell lines in a number of ways. It produces and secretes IgA (α , λ_2 and J chains) whilst MOPC 315.32, 35 and 36 have all lost the ability to produce α and J chains and have been selected to be 6-thioguanine resistant and HAT sensitive. In addition MOPC 315.35 and 36, have lost the ability to produce λ_2 light chains. These phenotypic differences are reflected in a number of differences in the 2-D electrophoretic profiles between MOPC 315.32, 35 and 36 and MOPC 315.40. A number of proteins (6.4/27, 5.5/44, 4.6/51 and 5.3/54) show a positive correlation

with the ability to produce α and J chains whilst one (protein 5.3/38) exhibits a negative correlation (being barely detectable in MOPC 315.40 and showing up very strongly in MOPC 315.32, 35 and 36), although it could be that there is a correlation with resistance to 6-thioguanine and HAT sensitivity. Of particular interest is that these correlations continue to hold good for protein 6.4/27 and 5.3/54 when MOPC 315.40 is compared with the IgM (K,J) producing plasmacytoma Y55781.4, suggesting that these proteins correlate with heavy chain production. Furthermore the negative correlation of protein 5.3/38 with immunoglobulin production is also seen when MOPC 315.32 is compared with Y5781.4.

It must be emphasised that the correlation of a particular nuclear protein with the expression of a particular gene cannot establish a causal relationship between the observed phenomena. In addition it would be expected that potential gene regulatory proteins would be present in a limited number of copies per cell. Although it is theoretically possible to identify proteins in such small quantities using the system employed here the actual resolution may be be lower. The theoretical sensitivity of detection for the fluorographic techniques used in these experiments is of the order of 1-10 molecules per cell. However for a number of reasons the resolving power of the system will be lower,for example the more common proteins may 'mask' rarer proteins with similar co-ordinates and degradation products may give a general background exposure obscuring less common proteins. Another problem is that not all proteins present in the nucleus will be detected,some may lack methionine and others will have been lost during separation. In particular proteins were lost from the basic end of the isoelectric focusing gel because of over-running.

However, the methods described here may provide a starting point for further work in the search for, and identification of, potential gene regulatory proteins.

In similar experiments to the ones described here,conducted independently and simultaneously with this work, Connett and Fleishman (1981) used two-dimensional polyacrylamide gel electrophoresis to examine NHCP from six related plasmacytoma cell lines which differed in their ability to synthesise one or both immunoglobulin chains, and a non-plasmacytoma cell line,in similar attempts to identify proteins which correlated with the production or non-production of immunoglobulin chains. In their experiments they used variants of plasmacytoma cell lines MPC 11Y γ_{2b} ,K and MOPC 460 (α,K) and mouse lung fibroblast L929 cells; they identified three proteins which correlated with MPC 11 γ_{2b} chain synthesis. Unfortunately it is not possible to make a direct comparison with the results of the experiments reported here; they used both a different labelling technique ([^{3}H]-leucine in serum free medium compared to [^{35}S]-methionine in medium containing foetal calf serum) and a different method of preparing NHCP (they prepared chromatin by centrifugation through sucrose gradient).

The half lives of individual proteins that correlated with immunoglobulin production were measured in the experiments described in chapter six. These were carried out to provide additional information on these proteins,which may give some clues as to their possible

roles,for example proteins performing regulatory roles might be expected to have a higher turnover rate than structural proteins. Of the proteins that showed a positive correlation with the ability to produce α and J chains proteins 6.4/27 and 5.5/54 had a much longer half life in the NHCP fraction compared to the nucleoplasmic fraction (21.4 hours compared to 4.7 hours for protein 6.4/27, 55.8 hours compared to 7.4 hours for protein 5.5/54), whilst protein 5.5/54 had a shorter half life (42.7 hours in the nucleoplasmic fraction and 14.7 hours in the NHCP fraction). Protein 5.3/38 which exhibited a negative correlation with immunoglobulin production had a longer half life in the nucleoplasmic fraction (28.8 hours) than in the NHCP fraction. These results, together with the experiments on the long term binding of the carcinogen acetylaminofluorene to nuclear proteins (chapter 4) show that a number of proteins bound to DNA have a very slow turnover (even when modified by bound carcinogen). This may make possible quite long term changes in gene expression by epigenetic means. However,it is more likely that proteins bound to DNA having a slow turnover are perfoming a structural role rather than a regulatory one.

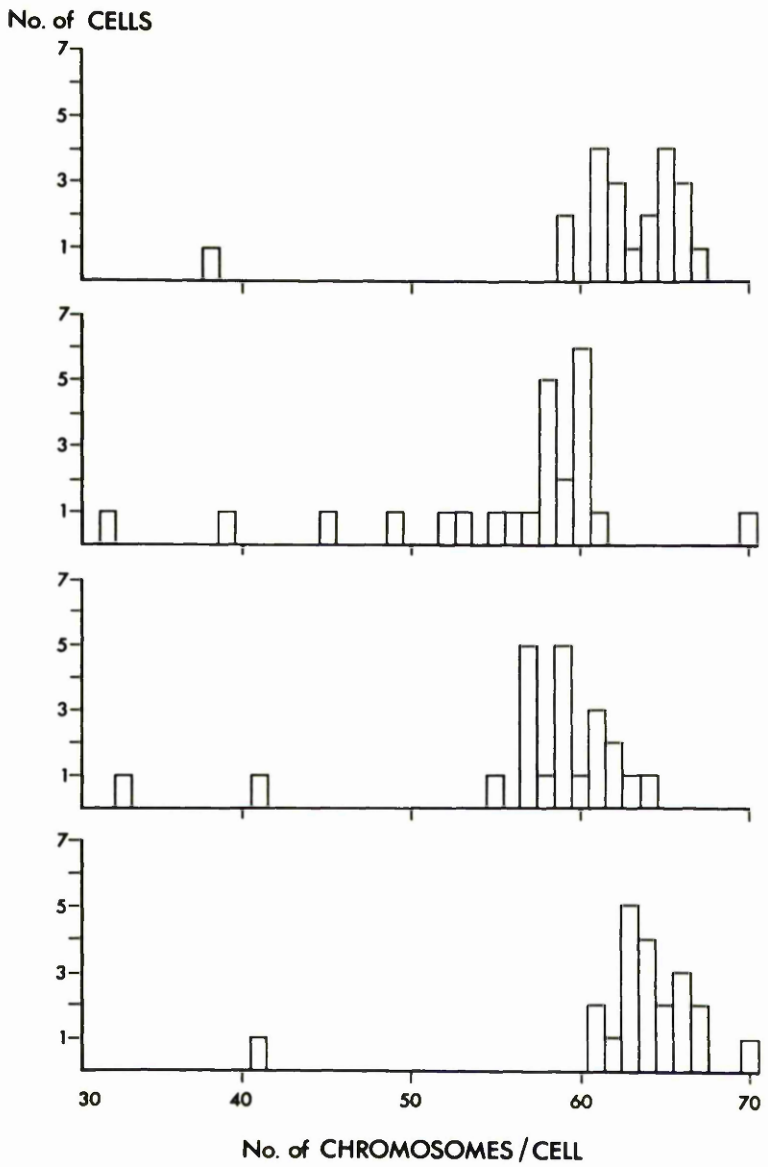
It is interesting to consider mechanisms by which the observed changes in gene expression could occur. The genetic material of cultured cell lines tends to be unstable (Lewin, 1980), chromosomal loss may occur even in cell lines that retain a constant number of chromosomes by unequal separation of chromosomes in mitosis. Chromosomal rearrangements are common and these factors make the genetic content of cultured cell lines uncertain. The karyotype of these cell lines is shown in Table 12 and Figure 20 (from Lincoln and Stott, 1983). It can be seen that there is a variation in chromosome numbers between individual cells of the same line and between the cell lines. The loss of α and J chain production in the case of MOPC 315.32 and the subsequent loss of λ_2 chain production in MOPC 315.35 and 36 could be the result of the loss of whole chromosomes or parts of chromosomes. In this case the differences in nuclear protein patterns could be due to the loss of chromosome specific proteins.

Another possible mechanism by which the observed changes in gene expression could occur is deletion of all or part of the DNA coding for α , λ_2 and J chain or of control sequences needed for their expression.

This is supported by the work of Hozumi et al. (1982),who when looking at the arrangements of light

chain genes in cell lines MOPC 315.34, 35, 36 and 37,found that MOPC 315.35 and MOPC 315.36 DNA does not contain sequences coding for the rearranged λ_2 light chain gene.

Figure 20.



Karyotype analysis of plasmacytoma cell lines

Karyotype analysis of a) MOPC 315.40; b) MOPC 315.36; c) MOPC 315.35; d) MOPC 315.32. Chromosomes from greater than 20 nuclei were counted for each cell line.

Table 12 Chromosome counts of cell lines

Cell line	Ig chains synthesised	Mode	Mean ± SD
MOPC 315.32	λ_2	63	64.4 ± 2.2
MOPC 315.35	none	57/59	59.4 ± 2.3
MOPC 315.36	none	58/60	58.6 ± 3.5
MOPC 315.40	λ_2,α, J	61/65	63.2 ± 2.4

The chromosomes of more than 20 nuclei from each cell line were counted.

ENHANCER SEQUENCES

Recently "enhancer sequences" which can stimulate the transcription of a gene have been found to be present in immunoglobulin genes; these sequences were first found in animal viruses (Khoury and Grass, 1983). Miller et al. (1983) have demonstrated that these sequences are associated with DNAse I hypersensitive sites and that a major hypersensitive site maps to a conserved region of the J_H-Cu-intron which Banerji et al. (1983) have shown to be a tissue specific enhancer of gene expression. Picard and Schaffner (1984) demonstrated the presence of a transcriptional enhancer within the large intron of the K light chain gene; however, no enhancer was found to be associated with the λ_1 light chain gene. Church et al. (1985) identified specific sequences of immunoglobulin genes that were protected from or had enhanced reactivity with dimethyl sulphate. By looking at DNA sequences and dimethyl sulphate reactivity patterns they were able to identify similarities in putative binding sites in MOPC 104E (μ, λ producer) and J558L (α, λ producer) myeloma cells which suggested binding by a single factor (protein?) in a tissue and sequence specific fashion.

The observation that enhancers lie in DNAse I hypersensitive regions suggests that they are involved in "opening out" local chromatin structure making it more accessible for transcription. Because immunoglobulin enhancers work in a tissue specific fashion, specific components, presumably proteins, are implicated in gene activation. The identification and characterisation of these factors that bind enhancer sequences (presumably NHCP) should aid our understanding of the mechanisms of immunoglobulin gene regulation. The techniques used in the experiments reported here are designed to identify such proteins. It is possible that the loss of α and J chain production could be due to the loss of enhancer region DNA or of proteins necessary for them to function.

7.3 Further Work

The work described in earlier chapters should be extended by a further characterisation and analysis of the nuclear proteins which were found to correlate with gene expression. One approach might be to identify the sub-nuclear location of the proteins identified,this could be accomplished using a variety of approaches.

It should be possible to establish whether any of the proteins described are present in nucleosomes or polynucleosomes. This could be accomplished by digestion of chromatin containing labelled proteins using micrococcal nuclease. The nucleosomes and polynucleosomes would then be prepared using sucrose density gradient centrifugation and the proteins present analysed by two dimensional polyacrylamide gel electrophoresis. If any of the proteins of interest were present in the preparations a further analysis could be performed using a more limited nuclease digestion with DNAse II or micrococcal nuclease to establish whether they were associated with active genes.

Another possible avenue of investigation would be to establish whether these proteins were associated with ribonucleoprotein particles within the nucleus containing heterogeneous nuclear RNA (hnRNP particles). These particles are involved in the processing,splicing

and transport of messenger RNA precursors (reviewed by Knowler,1983). These particles can be isolated from nuclei by a variety of methods,separated by centrifugation through a sucrose density gradient,and analysed by 2-dimensional polyacrylamide gel electrophoresis.

In addition it might be possible to make preparations of the nuclear envelope. This is made up of four distinct regions:

1) The outer nuclear membrane.

2) The inner nuclear membrane (separated from the inner nuclear membrane by a perinuclear cisterna.

3) The fibrous lamina attached to the nucleoplasmic side of the inner membrane.

4) The pore complexes

A number of procedures may be used to separate nuclear envelopes (MacGillivray and Birnie,1986);one method involves the isolation of nuclei,DNAse and RNAse treatment of nuclei,nuclear lysis and the extraction of chromatin,and finally the washing of nuclear envelopes. The protein content of the preparation could then be analysed by 2-dimensional polyacrylamide gel

electrophoresis. It is also possible to prepare further subfractions of the nuclear envelope.

An alternative approach to the ones described above might be to prepare monoclonal antibodies to these proteins in order to directly examine the intracellular location and function of these proteins.

It is possible to directly microinject flouresceinated proteins and antibodies into living cells. This permits the observation of the intracellular distribution of these molecules in living cells. Antibodies and antibody fragments (F(ab)2) retain their antigen binding activity and can be used to localise proteins and interfere with cellular functions (Einck et al.,1984). This technique has been used to localise HMG proteins in the nucleus of human fibroblasts (Einck et al.,1984) and might be used to study the distribution of the proteins identified in chapter 5 in similar fashion. Alternative techniques which could be employed include immunoelectron microscopy of cell sections stained using monoclonal antibodies to these proteins which have been conjugated to gold particles, and the study of proteins which bind to the enhancer sequences of cloned immunoglobulin genes or sequences on either side of the gene .

In conclusion: Although many recent advances in the understanding of how gene expression is controlled have resulted from the study of nucleic acids, particularly RNA and DNA sequence analysis, the methods described here might provide a basis for the identification of proteins which correlate with changes in gene expression (without necessarily being involved in the control of gene expression). Further work should research into the sub nuclear location of these proteins which may provide some clues to their function. This may then be extended to include the purification and functional analysis of such proteins.

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