Changes in HTC Cells' Nuclear Proteins Associated with Glucocorticoid Stimulation.

by

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To my wife, Margaret.

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Abbreviations.

The standard abbreviations, as recommended in revised "Instructions to Authors" (Biochem. J. (1976) 153, I-2I), are used throughout this thesis with the following additions:-

SDS	Sodium Dodecyl Sulphate
DTT	Dithiothreitol
HTC	Hepatoma Tissue Culture
Dx	Dexamethasone disodium phosphate
TAT	Tyrosine Amino Transferase (EC. 2.6.15.)
RSB	Reticulocyte Saline Buffer
NHCP	Non-Histone Chromosomal Proteins
PMSF	Phenyl Methyl Sulphonyl Fluoride
CBG	Corticosteroid Binding Globulin

TABLE OF CONTENTS

LIST OF FIGURES. Page.

LIST OF TABLES. Page.

Summary.

This thesis describes an investigation of the effects of the synthetic glucocorticoid, dexamethasone, on the nuclear non-histone chromosomal proteins of the Hepatoma Tissue Culture cell line. This cell line responds to dexamethasone by synthesising a small number of the proteins normally produced in the liver in response to glucocorticoids. Therefore it was hoped that a study of the non-histone chromosomal proteins in such a simplified system would provide some insight into the role of the chromosomal proteins in the hormonal control of transcription.

The bulk non-histone chromosomal proteins were isolated by two methods. One method used mild denaturants, so minimising damage to the proteins, while the other used more extreme conditions, including acid extraction, so allowing quick isolation with the minimum opportunity for proteolytic degradation. A preparation of 0.35 M saline soluble proteins and a preparation of tightly bound proteins which remained bound to DNA in urea-guanidine HCl, were separately investigated.

The isolated proteins were analysed by one and two dimensional polyacrylamide gel electrophoresis. The two dimensional gel system involved isoelectric focussing in the first dimension and SDS-urea polyacrylamide electrophoresis in the second dimension. The separated proteins were detected by staining and autoradiography or scintillation autoradiography, depending on the isotope used.

The three fractions gave a different one dimensional electrophoresis pattern, although some of the bands appeared common to more than one fraction. The number of major bands present in the bulk non-histone chromosomal proteins, the saline soluble proteins and the proteins tightly bound to DNA were 25, 20 and 7 respectively.

The glucocorticoids stimulate the induction of tyrosine amino transferase in the Hepatoma Tissue Culture cells. This enzyme was used as an indicator of the cells' responsiveness to the hormone and to obtain a time course of induction to compare with alterations in the nuclear

chromosomal proteins. It was found that the level of the enzyme was above the control level by 1 hour and it continued to increase until 8-12 hours, thereafter remaining fully induced.

The proteins were labelled with L-[^{3}H]-tryptophan to monitor their turnover and with [^{32}P]-orthophosphate to monitor phosphorylation. Dexamethasone (5×10^{-6}M) was found to affect the turnover and phosphorylation of various fractions. The turnover of the bulk non-histone chromosomal proteins decreased after 1 hour of hormone treatment and continued to do so up to 4 hours before returning to the control level by 24 hours. The turnover of the other protein fractions followed a similar time course. However, the phosphorylation of the bulk non-histone chromosomal proteins showed turnover rates with exactly the reverse pattern. Phosphorylation was increased for the first 4 hours before returning to the control level by 24 hours. The other protein fractions did not show any changes in phosphorylation rates over 24 hours.

The nuclear proteins, when analysed on the one dimensional electrophoresis system and detected either by staining or by L-[^{3}H]-tryptophan incorporation, were not found to be altered in response to dexamethasone. Dexamethasone was found to change the [^{32}P]-orthophosphate labelling pattern of the bulk non-histone chromosomal proteins by reducing the level of phosphorylation of one of the peaks. This change occurred 1 hour after the addition of dexamethasone and did not alter in magnitude by 24 hours. The alteration corresponded to a change in a protein species with a molecular weight of approximately 81,000. Any effects of dexamethasone on the other fractions were not observed at the level of resolution of one dimensional electrophoresis.

The separation of the bulk non-histone chromosomal proteins on two dimensional electrophoresis was carried out by using two different systems. In the first system, the proteins were detected by staining and autoradiography while in the second, the proteins were labelled for 4 days with L-[^{35}S]-methionine and detected by scintillation autoradiography. The

effect of dexamethasone on the bulk non-histone chromosomal proteins was observed in each system. The difference found in each system was shown not to be identical. In the first system, dexamethasone led to the appearance of a spot which was observed in the samples isolated 1 and 24 hours after hormone addition. The new spot migrated to a position corresponding to a protein of pI 5.4 and an approximate molecular weight of 86,000. Bulk non-histone chromosomal proteins separated by the second system showed that continuous dexamethasone treatment resulted in the appearance of a new spot at pI 5.7 with an apparent molecular weight of 80,000.

The levels of possible hormone-stimulated chromosomal regulator molecules are discussed with reference to their probability of being detected using techniques presently available and comparing the detectable levels with the results obtained.

INTRODUCTION.

1.1 The Eukaryotic Genetic Material.

Chromatin was originally defined as a nuclear material that stained with a basic stain (Flemming et al., 1882). In recent years the eukaryotic nucleoprotein complex containing the genetic material has become known to biochemists as chromatin. This differs from the original definition in that the chromatin has been isolated from its nuclear environment.

Problems arise in interpreting data derived from this material because current methodology cannot give precise information as to the extent to which isolation has resulted in degradation and loss of constituents or the extent to which contaminants have been incorporated. The following summary of current knowledge of chromatin structure and function must therefore be subject to these reservations.

1.1.1 Chromatin Structure.

The nucleoprotein complex of eukaryotic cells is a complex of DNA, histones and non-histone chromosomal proteins (NHCP) with some associated RNA, the importance of which is uncertain. The DNA of a single cell measures several metres and therefore has to be compacted so that it can be confined inside the nucleus. It is believed that some of the proteins condense the DNA, imposing on it the chromatin's structure.

It appears that there is only one DNA molecule per chromosome. Isolation of large DNA molecules from Drosophilia melanogaster (Kavenoff & Zimm, 1973) and yeast (Petes & Fangman, 1972; Blamire et al., 1972) gave results that were half as long, from Drosophila melanogaster, and equal, from yeast, to the length of DNA expected if each chromosome contained only one DNA molecule.

One of the first models of chromatin proposed was that of Pardon et al. (1967) and Pardon & Wilkins (1972). Their model was of a supercoiled DNA, held together by histones, with a diameter of 10 nm and a pitch of 12 nm.

Ideas on the structure altered after experiments using nuclease digestion (Hewish & Burgoyne, 1973; Ramsay Shaw et al., 1974) and electron microscope studies (Olins & Olins, 1974; Woodcock, 1973) on chromatin. Hewish & Burgoyne (1973) found that approximately 80% of the chromatin could be digested to give fragments of multiples of 200 base pairs of DNA. Van Holde (Ramsay Shaw et al., 1974) isolated st aphylococcal and micrococcal nuclease treated chromatin particles that were globular and held together by histones. The electron microscope work of Olins & Olins (1974) and Woodcock (1973) showed a chromatin structure consisting of a linear arrangement of spherical particles. The particle diameter was estimated to be between 7 and 10 nm.

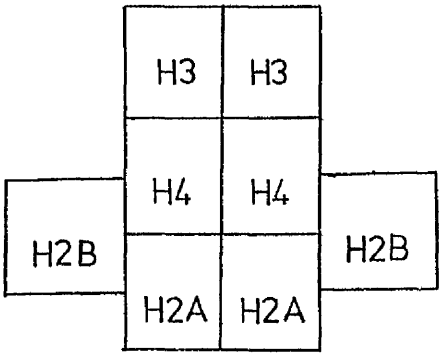
Neutron diffraction data (Baldwin et al., 1975) were consistent with the model of chromatin composed in part of "particles on a string".

The chromatin subunit has been called a nucleosome, "v" body, P-S particle, or bead and it has been shown to be composed of an octamer of histones in equal molar ratios of H2A, H2B, H3 and H4 and 200 base pairs of DNA (Histone nomenclature according to Bradbury, 1975). Current evidence suggests that the chromatin subunit contains 200 base pairs of DNA within which is a nuclease resistant core of 140 base pairs (Sollner-Webb & Felsenfeld, 1975). DNase 1 digestion of rat liver chromatin (Noll, 1974) gave products which were multiples of 10 base pairs from 10 to 220 base pairs. This led to the supposition that the DNA was coiled or kinked on the outside of the chromatin subunit.

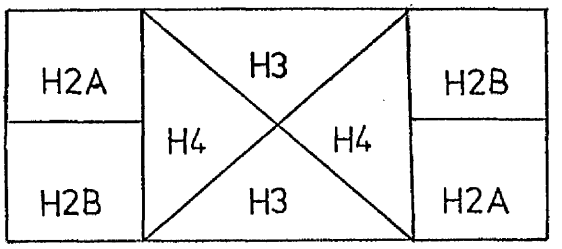
The relationship of the histones within the chromatin subunit was inferred by cross-linking experiments using dimethylsuberimidate (Kornberg & Thomas, 1974; Kornberg, 1974; Thomas & Kornberg, 1975). It was found that the octamer of histones consisted of a core tetramer of $(H4)_2(H3)_2$ which had contact with the other histones (Kornberg & Thomas, 1974). Cross-linked products H2A,H4; H4,H2B; and H2A,H2B were found. These findings led Thomas & Kornberg (1975) to propose the

following possible arrangement of histones within the octamer.

i



ii



A recent report stated that no NHCP could be found associated with the isolated chromatin subunit monomer (Augenlicht & Lipkin, 1976).

H1 is not required for the chromatin subunit structure and it has been suggested that H1 may help in the super-structure of the chromatin. The super-structure of the chromosome is thought to be a supercoil or "solenoid" of chromatin subunits (Finch & Klug, 1976) in which each turn contains six chromatin subunits. The solenoid was inferred, by electron microscopy, to have a diameter of 30 nm and a pitch of 11 nm, the direction of the "thread" of the filament could not be deduced from their pictures. It was postulated that the solenoid existed in short lengths of 4-10 turns with a length of naked DNA passing down the centre of the segment and on to the next section of solenoid. H1 is not required for the chromatin subunit structure but removal causes the relaxation of small pieces of chromatin.

1.2 Chromatin Components.

1.2.1 Histones.

Histones were discovered by Kossel (1884) as basic proteins that were combined with DNA. The histones seem to act, in chromatin, as gross repressors of transcription and as structural components.

There is only a small number of histone species and these have highly conserved amino acid sequencesbut the possibility exists that the histones may have a variability of structure due to modifications.

The modifications could lead to changes in the electrostatic interaction with the DNA.

Phosphorylation would cause a reduced attraction between the histones and DNA. The phosphate is covalently bound as phospho-serine and phospho-threonine (Ord & Stocken, 1966; Kleinsmith et al., 1966), N^6-phosphoryllysine and 3-phosphohistidine (Chen et al., 1974). Most of the work to date has monitored only the acid-stable phosphorylated histones.

Langan (1968) showed that cyclic AMP would stimulate the phosphorylation of rat liver histones by a cyclic AMP dependent histone kinase. A Cell cycle-dependent pattern of HI phosphorylation was reported in Physarum polycephalum (Bradbury et al., 1973) in which, at the point when the chromosome began to condense in late G2, there was a peak in HI phosphorylation. A hypothesis was forwarded that the initiation of mitosis is partly controlled by the phosphorylation of HI (Bradbury et al., 1974). It also was shown by Balhorn et al. (1971), using regenerating rat liver, that there is a link between histone phosphorylation and DNA replication.

The replacement of histones during spermatogenesis by protamines is also thought to be dependent on the phosphorylation of HI and H3 (Louie & Dixon, 1973). At the time of removal of HI the protamines are phosphorylated to allow them to migrate into the nucleus (Marushige et al., 1969).

The function of methylation of histones has not been established. N-methyllysine was first discovered in calf thymus histones by Murray (1963) and it was shown by Kim & Paik (1965) that the donor of the methyl group was S-adenosylmethionine. Other modified amino acid products which were found included N^6-dimethyllysine (Paik & Kim, 1967), N^6-trimethyllysine (Hempel et al., 1968) and N-methylarginine (Paik & Kim, 1970).

Changes have been found to occur in the thiol content of histones. Chromatin containing a lower thiol content was found less

efficient at synthesising RNA in vitro (Hilton & Stocken, 1966). It was shown by Sadgopal & Bonner (1970a,b) that HeLa metaphase chromatin had a higher content of the disulphide derivatives of H3 than interphase chromatin. A relationship between increased thiol content and DNA synthesis was observed in sea urchin embryos (Ord & Stocken, 1970).

Acetylation would reduce the electrostatic interaction between histones and DNA. The acetate is covalently bound to lysine to give N^6-acetyllysine. Increased acetylation of histones has been shown to coincide with increased RNA synthesis (Mukherjee & Cohen, 1969; Pogo et al., 1968).

The lack of tissue and species specificity of histones suggests that they could only act as gross regulators and/or structural proteins. The modifications of histones would not give them the degree of varibility needed for them to function as fine regulators. The expected role of modification would either be to direct the histones to the correct position on the DNA or to influence gross changes in the chromatin structure such as may occur in mitosis or the packing of DNA in the sperm head.

1.2.2 Non-Histone Chromosomal Proteins.

Nuclear chromosomal proteins, other than histones, were first demonstrated by Mirsky & Ris (1951). The term non-histone chromosomal proteins (NHCP) defines all chromosomal proteins except the histones. In experimental situations the proteins isolated as "NHCP" are only a subset with a degree of histone contamination. Each method of isolation will tend to give a slightly different spectrum of proteins.

NHCP are synthesised throughout the cell cycle (Stein & Baserga, 1970; Cave, 1968). It has been shown that they are synthesised in the cytoplasm (Stein & Baserga, 1971) and are not affected by inhibition of DNA replication (Stein & Thrall, 1973). The turnover and phosphorylation of NHCP were shown to alter throughout the cell cycle (Karn et al., 1974; Tsansev et al.,1974; Le Stourgeon et al., 1973).

Various groups have been trying to isolate individual NHCP and are investigating their molecular role (Shooter et al., 1974; Goldknopf et al., 1975; Kostraba & Wang, 1975; Chiu et al., 1975a; Le Stourgeon et al., 1975).

1.2.2.1 Non-Histone Chromosomal Proteins as Regulators.

Over the past decade evidence has accumulated suggesting that, while the histones function as the non specific repressors of gene activity at least some of the NHCP are specific inducers. The evidence for the NHCP as regulators of gene activity is as follows:-

1. Greater heterogeneity:- There are more NHCP than histones. Peterson and McConkey (1976) have recently detected 450 components in the NHCP of HeLa cells.

2. Turnover rates:- The NHCP have a faster turnover rate in general than histones (McClure & Hnilica, 1972) although some NHCP do turnover at the same rate as histones (Tsansev et al., 1974).

3. Species differences:- Even relatively crude fractionation techniques, such as one dimensional polyacrylamide gels of total NHCP, have been used successfully to detect differences in the NHCP between species. (Wu et al., 1973; Bekhor et al., 1974; Wilhelm et al., 1972).

4. Tissue differences:- One dimensional fractionations show few differences but such separations would only be expected to detect proteins present in sufficient quantity to be structural or major enzyme species. However two dimensional separation on polyacrylamide gels begins to reveal tissue dependent differences (MacGillivray & Rickwood, 1974; Suria & Liew, 1974a) and the sensitivity of immunological methodology reveals differences both in different tissues (Zardi, 1975) and in a tissue undergoing differentiation (Chytil & Spelsberg, 1971).

The proteins found tightly bound to DNA, after various extraction procedures with high salt, urea, and guanidine HCl, have been implicated in tissue specificity. Wakabayaski et al. (1974) and Chiu et al. (1975b) demonstrated immunologically that rat liver tightly bound chromatin proteins

shared tissue specificity with the non-tightly bound NHCP. Wakabayaski et al. (1974) also showed that the tightly bound proteins only bound to homogeneous DNA and that the immune response between the tightly bound proteins of normal and neoplastic tissues were different. A difference in the stainable pattern of proteins separated on one dimensional polyacrylamide gels after isolation from normal (WI-38 Fibroblasts) and transformed (SV40) cells has been reported (Stein,1976). It was demonstrated by Pederson & Bhorjee (1975) using DNAase 11, which was assumed to selectively digest chromatin containing nascent RNA transcripts, that the tightly bound proteins were associated with the template inactive region.

5. Changes during differentiation:- Numerous examples have been published of a change in the NHCP spectrum observable after a change in the state of the tissue. These include hormone stimulation and induced differentiation (Spelsberg et al., 1971a; Enea & Allfrey, 1973; Kadoma & Turkington, 1974; Dierks-Ventling & Jost, 1974), Lymphocyte maturation (Levy et al., 1973), slime mold differentiation (LeStourgeon & Rusch, 1971), tissue regeneration (Yeoman et al., 1975), echinoderm metamorphosis (Cognetti et al., 1972), neoplastic change (Kadoma & Turkington, 1973).

A novel method demonstrating the role of the NHCP in gene expression involves reconstitution of the chromatin components. Chromatin can be dissociated using high concentrations of salt and urea. If, then, the salt and urea are slowly and sequentially dialysed from the chromatin, it will reconstitute and Stein et al. (1975a) have shown that the reconstituted product can show almost identical properties to that of native chromatin. By conducting hybrid reconstitutions, in which the NHCP of one tissue is reconstituted to the histone DNA complex of another tissue, it has been shown that NHCP control the binding of progesterone to its target cell chromatin (Spelsberg et al., 1972), the synthesis of globin mRNA in erythropoetic chromatin (Gilmour & Paul, 1975) and the cell cycle dependent synthesis of histone (Stein et al., 1975b; Stein et al., 1976).

The possible effect of modification of NHCP on their activity will be discussed in section 1.3.

1.2.2.2 Structural and Enzymic Activities of Non-Histone Chromosomal Proteins.

Evidence suggests that some of the NHCP are enzymes and chromatin structural components. Proteins of similar identity to contractile proteins have been found in NHCP (LeStourgeon et al.,1975; Douvas et al., 1975).

Actin, myosin and tropomysin-like proteins were isolated from the NHCP of Physarum polycephalum and HeLa cells and it was found that the amount of actin increased during chromatin condensation and inactivation (LeStourgeon et al.,1975). Douvas et al.(1975) isolated contractile proteins from rat liver chromatin that accounted for 38% of the NHCP. The proteins identified were actin, myosin, tropomyosin and possibly tubulin.

The enzymes isolated with the NHCP are either involved in nucleic acid metabolism or protein metabolism. The enzymes of nucleic acid metabolism include, DNA polymerase, RNA polymerase, poly (A) polymerase, DNA endonuclease, and DNAase. The protein metabolising enzymes include, poly (adenosine diphosphate ribose) polymerase, histone acetyltransferase, protein kinases, histone methylase, proteases and phosphatases.

The proteases have been investigated in a number of systems as their activity can modify the isolated NHCP. Protease activity was demonstrated by Phillips & Johns (1959) in their isolated histones. The role for the proteases has not been established as yet; Bartley & Chalkley (1970) proposed an autolytic function rather than a gene regulatory role. Chong et al. (1974) have purified a neutral protease from rat liver chromatin and found it to be sensitive to phenyl methyl sulphonyl fluoride (PMSF). It was found that while the histones were bound to DNA only HI could be rapidly degraded but if the histones were free from DNA the protease could attack all the histones with HI degrading

the slowest. It has been suggested that the protease would help remove the histones and so cause derepression. Marushige & Dixon, (1971) suggested that the chromatin bound protease digested histones from DNA during spermatogenesis. Conversely, experiments using Xenopus laevis and rat liver by Destree et al. (1975) indicated that protease isolated with chromatin could be a cytoplasmic contaminant.

1.2.3 DNA.

Differentiation could be controlled by alteration in the DNA by either addition or rejection of DNA sequences. Certain experiments have disproved this hypothesis. Frog intestinal cells' nuclei were placed inside enucleated unfertilised oocytes and in some cases they grew to form normal frogs (Gurdon & Laskey, 1970). A similar experiment was performed using a single differentiated carrot cell from which a fully developed carrot could be grown (Joshi & Ball, 1968).

These experiments lead to the conclusion that there is no elimination of DNA and that addition of DNA is unlikely during differentiation.

Amplification of ribosomal genes occurs in developing oocytes of Xenopus laevis and the new transcripts are transferred to an extra chromosomal site. However the amplified genes are degraded in the mature oocyte (Brown & Dawid, 1968).

Methylation of DNA in prokaryotes seems to have a role in host recognition but in eukaryotes a role in differentiation has not been proposed. The methylation occurs immediately after replication and is independent of cellular differentiation (Turkington & Spielvogel, 1971).

1.3 Modification of Non-Histone Chromosomal Proteins.

NHCP have been found to be post-synthetically modified by the covalent binding of a moiety to an amino acid residue. The precise effect each modification endows upon the NHCP has not yet been definitely established.

The modifications of the NHCP found to occur are phosphorylation, acetylation, glucosylation, poly adenosine diphosphate ribosylation and

alteration of thiol content. These alterations would cause changes in the charge distribution of the proteins and would therefore alter their characteristics of binding to the DNA, histones and other NHCP.

1.3.1 Phosphorylation of Non Histone Chromosomal Proteins.

The NHCP are phosphorylated to much higher degree than the histones (Teng et al., 1971; Rickwood et al., 1973) although MacGillivray & Rickwood (1974) showed that not all the NHCP can be labelled with [^{32}P]. Pederson & Bhorjee (1975) have investigated the NHCP tightly bound to DNA and have found them to be phosphorylated at a much lower level than either the non-tightly bound NHCP or histones.

Kish & Kleinsmith (1974) demonstrated the existence of various protein kinases some of which had specificity to NHCP and which were stimulated by cyclic AMP.

The level of phosphorylation seems to determine the NHCP ability to interact with DNA (Teng et al., 1971; Wang, 1967; Van den Broek et al., 1973; Kleinsmith, 1973). Phosphoproteins were isolated by Teng et al. (1971) and Kleinsmith (1973) and they found them to be species specific with respect to DNA binding. Ezrailson et al. (1976) have shown that growing tissues, regenerating liver and Novikoff hepatoma cells have a higher level of phosphorylation than normal liver. The level of phosphorylation can be shown to alter at different stages of the cell cycle increasing during GI and early S while decreasing during late S, G2 and M phases of the cell cycle (Marty De Morales et al., 1974; Karn et al., 1974). The phosphorylation of NHCP seems to correlate with the synthesis of RNA (Platz et al., 1973; Pogo & Katz, 1974) and to be linked to cellular changes evoked by external stimuli.

Phosphorylation of NHCP has been shown to alter in differentiating chick muscle cells (Man et al., 1974); androgen-stimulated testis and epididymis (Kadoma & Turkington, 1974); and in concanavalin A stimulation of lymphocytes (Johnson et al., 1974).

Kleinsmith et al. (1976) demonstrated that the level of

phosphorylation of NHCP had an effect on the expression of a specific gene. A reconstituted chromatin, prepared with dephosphorylated NHCP, was found to possess 50% reduced template activity when compared with normal chromatin and when transcripts were monitored using histone mRNA cDNA as a probe, dephosphorylation reduced the expression of the gene by 70%.

1.3.2 Poly Adenosine Diphosphate Ribosylation of Non-Histone Chromosomal Proteins.

A polymer of adenosine diphosphate ribose units, formed from NAD by the enzyme poly adenosine diphosphate ribose polymerase, can be covalently linked to the NHCP (Dietrich & Siebert, 1974). DNA is essential for the synthesising enzyme's activity (Chambon et al., 1966; Nishizuka et al., 1969). Poly adenosine diphosphate ribose formation is stimulated in vitro by exogenous DNA and the degradation is also inhibited by DNA in vitro (Chambon et al., 1966; Yoshihara, 1972). The amount of mono adenosine diphosphate ribose bound to protein varies with different growth rates (Adamietz et al., 1974). These results indicate that the polymer might have a role in the regulation of DNA synthesis and cell proliferation.

1.3.3 Acetylation of Non-Histone Chromosomal Proteins.

A post synthetic acetylation has been observed in NHCP. Suria and Liew (1974a) analysed [^{3}H]-acetate labelled NHCP and reported the occurrence in NHCP of the amino acid residues N^2-acetylserine, N^2-acetyllysine with a small amount of N^6-acetyllysine. They also reported that the acetylation of rat liver NHCP was decreased after partial hepatectomy. The acetylation of ovarian NHCP increases with gonadotrophin stimulation (Jungmann & Schweppe, 1972). A role for acetylation of NHCP has not been demonstrated.

1.3.4 Glucosylation of Non-Histone Chromosomal Proteins.

Stein et al. (1975c) found glycoproteins in isolated chromatin and it was suggested that the isolated chromatin from HeLa cells was not contaminated by non-chromosomal glycoproteins by isolating chromatin in the presence of labelled plasma membrane and in other experiments in the

presence of trypsinised labelled cells. The role of the glycoproteins in chromatin has not been shown. The glycoprotein, heparin, has been demonstrated in rat nuclei to activate endogenous DNA polymerase (Cook & Aikawa, 1973).

1.3.5 Thiol Content of Non-Histone Chromosomal Proteins.

Cortisol has been shown to alter the thiol content of isolated NHCP from rat liver (Doeneche et al., 1972). This type of modification has not been investigated sufficiently to determine a role for it.

1.4 Transcriptional Control Systems.

1.4.1 Prokaryotic Systems.

The most studied system of prokaryotic transcriptional control involves an interaction between a metabolite and a regulatory protein. The metabolite-protein complex can either enhance or depress the synthesis of an operon product acting either by association with or by dissociation from the DNA. There are therefore four possible control systems, examples of three of which are known.

1. Enhanced synthesis of operon structural gene product by the dissociation of the regulatory protein.

The best example of this is the lac operon of E. coli (Jacob & Monod, 1961). Lactose regulates its own utilisation by inducing the lac operon. Only in the presence of lactose, or a similar β-galactoside, are the operon's three structural genes expressed. The lac operon order of genes is control gene, promoter gene, operator gene, β-galactosidase, β-galactoside permease and thiogalactoside transacetylase. The inducer (β-galactoside) interacts with the repressor, thereafter an alteration in the repressor's conformation occurs which lessens the association between the repressor and the operator. This allows the RNA polymerase bound to the promoter sequence to transcribe the cistrons coding for the enzymes.

11. Enhanced synthesis of an operon by the augmented association of the metabolite activator complex.

An example of this system is the arabinose operon. The

enzymes transcribed are the controlling enzymes of arabinose catabolism. The organisation of the operon is similar to the lac operon. The only difference is that the combination of the inducer (arabinose) and the activator causes enhanced transcription of the cistron.

111. Depressed transcription of an operon by the augmented association of the metabolite-regulator complex.

An example of this type is the histidine operon (Kasai, 1974). Histidine (co-repressor), complexed to the regulatory protein, increases the association between the co-repressor-regulator complex and the operator. This association decreases the transcription of the histidine operon.

Other prokaryotic transcriptional control mechanisms do not involve metabolite interaction with a regulatory protein. An overriding control system occurs in bacteria whereby glucose causes decreased synthesis of other sugar metabolising enzymes. The glucose decreases the bacterial cyclic AMP concentration so that in the sugar's absence the cyclic AMP can complex with the catabolising activator protein (CAP) and bind to the promoter of operons such as lac and arabinose. The combination of cyclic AMP, CAP and promoter allows the transcription of the lac and the arabinose operons. This causes the preferential breakdown of glucose even though other sugars may be present in concentrations which could cause induction of their specific metabolising enzymes.

A further bacterial control system is autoregulation. It has been shown that L-threonine deaminase from Salmonella typhimurium regulates its own synthesis (Hatfield & Burns, 1970; Calhoun & Hatfield, 1973). The holoenzyme leucyl-$tRNA^{leu}$ complex was reported to regulate the ilv-ADE operon (coding for enzymes required in the biosynthesis of the branched chain amino acids).

1.4.2 Eukaryotic Systems.

Eukaryote Transcriptional Control.

Though the details are less completely understood, eukaryotes probably exhibit similar control mechanisms to those of prokaryotes.

However, eukaryotes must show additional control systems by which different cells and tissues of the organism can influence each other. Two such systems are currently subject to much study:- nervous control and hormonal control. Hormonal control is the subject of this thesis.

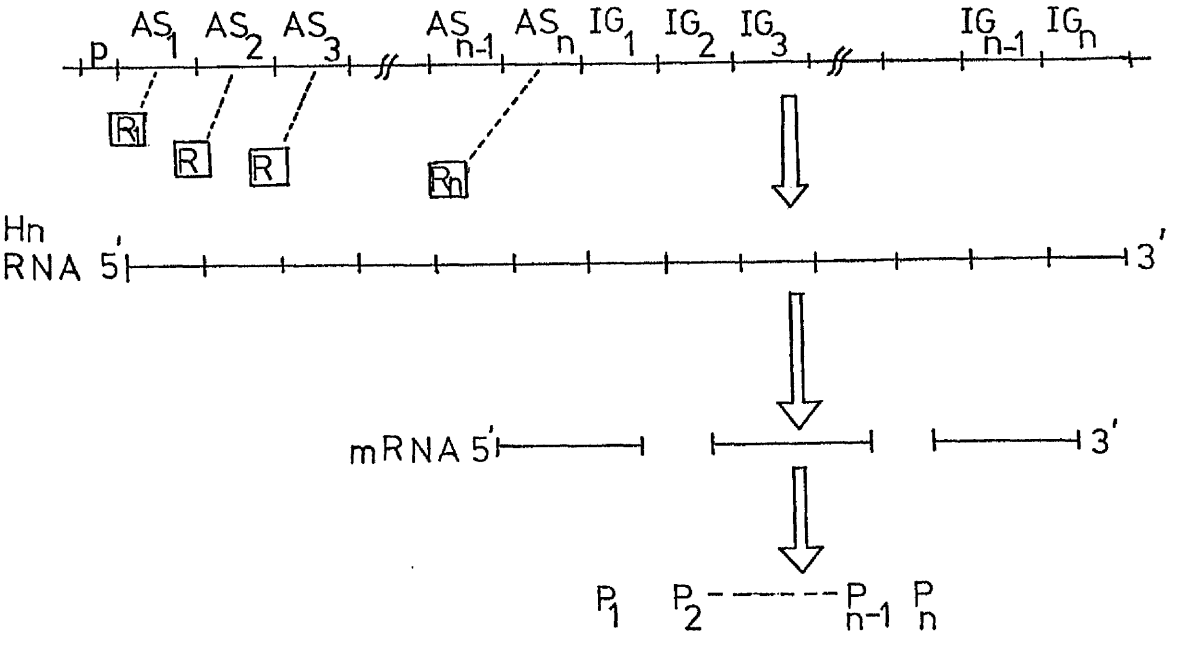
It is also clear that the eukaryote genome is more complicated than that of the prokaryotes, both in the amount of genetic material and in the number of chromosomal components. Thus models for the control of eukaryote transcription should take into account the apparent DNA redundancy, the chromatin proteins and RNA, the synthesis of nucleoplasmic RNA in sizes far greater than occur in the cytoplasm and of the need to transport mRNA across the nuclear membrane. Some of the models put forward are the following:-

Models of Transcriptional Control.

1. "Eukaryotic Operon Model" (Georgiev, 1969).

This model is similar to the prokaryotic operon system (Jacob & Monod, 1961) in that each operon contains a non-informative and an informative region between the promotor and the terminator. The non-informative part would be proximal and the informative part would be distal to the direction of transcription. The non-informative section would consist of a zone or zones that could be recognised specifically by regulatory proteins. The informative section would code for the structural genes and regulatory genes. The non-informative region could have a number of different acceptor sites which could interact with specific regulatory proteins. Different operons could contain identical or similar acceptor sites. Transcription would be affected by the interaction of the acceptor site with its regulatory protein. Georgiev's hypothesis is that the whole operon is transcribed and that post-transcriptional activity would cleave the high molecular weight RNA into the mRNA species which are observed in the cytoplasm.

A Schematic Diagram of the "Eukaryotic Operon Model" (Georgiev, 1969).



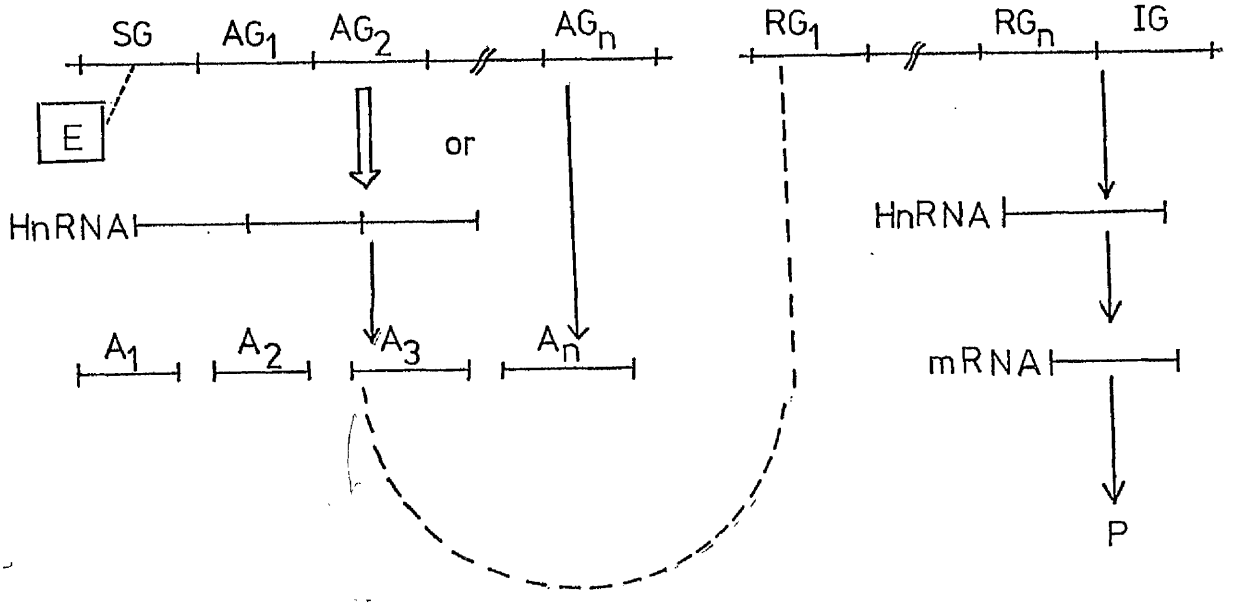
Components of the diagram:- R-Regulatory protein; AS-Acceptor site; IG-Information gene; P-Products of the information gene.

11. "Gene Battery Model" (Britten & Davidson, 1969).

The model envisages redundancy of genes so that multiple activation could occur. The major constituents of the model are unlinked, thus giving a flexibility of regulation. It would allow coincident transcription of related information genes without their having to be polycistronic. The model structure postulates that sequences act as acceptor sites (sensor genes) that would bind an excitatory macromolecule. Linked to the sensor gene would be either a single activator gene or a series of different activator genes, each of which would produce an activator. The activator would interact with another base sequence, separate from the sensor and activator genes, the receptor genes. The interaction of the product from the activator genes and the receptor gene would cause the transcription of the information gene linked to the receptor gene. Each information gene could have a series of receptor

genes attached to it.

A Schematic Diagram of the "Gene Battery Model" (Britten & Davidson, 1969).



Components of the diagram:- E-Excitatory molecule; SG-Sensor gene; AG-Activator gene; A-Activator; RG-Receptor gene; IG-Information gene; P-Product of the information gene.

111. "Cascade Model" (Scherrer & Marcaud, 1968).

4-7% of the avian erythroblast genome was found, by hybridisation, to be transcribed. This was much greater than the predicted transcription of haemoglobin messenger, ribosomal and transfer RNA should have given. This led to a model of regulation acting at several levels. At each level from transcription to translation a selected "labile" fraction would be degraded.

It is not known at which sites (or loci) regulation would occur. The result would be a multi-step reduction in the number of RNA molecules until those RNA species required for the individual cell's phenotype remained.

IV. "Analogue Computer" (Paul, 1976).

A model of regulation of transcription favoured by recent data

is that proposed by Paul (1976) in comparison with an analogue computer. The model is similar to the "cascade model" (Scherrer & Marcaud, 1968) in that a much greater proportion of the genome would be transcribed than necessary. The whole genome would be transcribed at a low level. The cell's phenotype would be expressed as a result of the stimulation of the transcription of particular genes and the degradation of unwanted gene products. The work of Humphries et al., (1976) supports this model in that globin mRNA has been found in the nuclei of a variety of non-erythroid cell types.

The production of high molecular weight nuclear RNA with a high rate of turnover, of which only a small percentage enters the cytoplasm is now well established. The three other models (1-111) could be incorporated in this model to add to its complexity.

The role of the chromatin subunit in regulation is unknown as yet. It remains to be established whether the chromatin subunits are stationary or mobile on the DNA chain in vivo and whether the particles repress or stimulate transcription. The degree to which the chromatin subunits associate with NHCP is not known.

Kuo et al. (1976) have found RNA sequences in chromatin subunits which hybridise to cytoplasmic polyadenylated RNA. Kinetic considerations revealed that the chromatin subunit included sequences of single copy mRNA.

This cannot be taken as conclusive as Bloch & Cedar (1976) demonstrated that the chromatin subunits moved during isolation.

1.4.2.1 Hormonal Control of Transcription.

The hormones are defined as molecules that interact with a target cell which is distinct from the cell of synthesis. Since the glucocorticoids are the subject of this thesis, priority will be given to them in this discussion. However a great deal of evidence is accumulating in support of the conclusion that all steroid hormones act by essentially the same mechanism.

The glucocorticoids are synthesised in the fascicular and reticular zones of the adrenal cortex. The major cortico-steroids in mammalian systems are cortisol and corticosterone. The glucocorticoids influence a broad spectrum of biochemical and physiological functions. They affect metabolism of carbohydrate, lipid and protein, neuromuscular irritability, allergic response and resistance to noxious stimuli. They are essential for the maintenance of life, and they play an important part in resistance to shock and infection. Most of the glucocorticoids have some mineralocorticoid activity.

The levels of secretion of cortisol and corticosterone are controlled by adrenocorticotropic hormone (ACTH). Secreted glucocorticoids are transported in the blood bound to an α-globulin, known as transcortin or corticosteroid binding globulin (CBG). Small quantities are also transported bound to albumin and a small amount can be found loosely bound to the cell membrane of erythrocytes (Kornel et al., 1970). CBG is thought to be synthesised in the liver (Westphal, 1971) and its level is regulated by thyroid hormone and oestrogen (Sandberg & Slaunwhite, 1959). The synthetic corticosteroid dexamethasone does not bind to CBG and is transported free in the blood (Rousseau et al., 1972; Lippman and Thomson, 1974).

The biological activity of CBG-bound steroid seems to depend on the different affinities of the target tissue binding proteins. In some systems only the free hormone has been shown to be active (Kawai & Yates, 1966; Slaunwhite et al., 1962; Matsui & Plager, 1966; Blecher, 1966; Lippman & Thompson, 1974) while in other systems the bound hormone is also used (Keller et al., 1969). Werthamer & Amaral (1971), working with leukaemic lymphocytes, demonstrated a differential effect between free and CBG bound cortisol.

The corticosteroids are catabolised to form water soluble conjugates and some evidence indicates that CBG may be involved (Sandberg & Slaunwhite, 1971). The HTC cell line has a reduced ability to

metabolise cortisol and cannot degrade the synthetic hormone dexamethasone (Baxter & Tomkins, 1970).

In the target cell, steroid hormones bind to specific cytoplasmic receptor proteins and this is a prerequiste of hormone action. The combination of steroids with receptors was first predicted by Jensen & Jacobson, (1962) from studies involving immature rat uteri. They showed that the uteri could accumulate tritiated oestradiol from the blood against a concentration gradient. The first cytoplasmic receptor to be isolated was the oestrogen receptor from rat uteri (Toft & Gorski, 1966).

In rat liver, five different corticosteroid-binding proteins have been found. They can be separated by ion-exchange chromatography on DEAE Sephadex and each has a different specificity. The work of Litwack and coworkers (Litwack et al., 1971; Litwack et al., 1973; Morey & Litwack, 1969; Litwack & Rosenfield, 1975) suggested different roles for each one. At lease one of them (Litwack et al., 1973; Litwack & Rosenfield, 1975) has the characteristics of the corticosteroid receptor. The others appear to be intracellular transporting proteins mediating the metabolism of the corticosteroids (Litwack et al., 1971; Morey & Litwack, 1969) and one appears to be identical to CBG. The "receptor" that is identical to CBG could be a contaminating serum protein and/or newly synthesised CBG ready for export out of the cell.

The experimental work of this thesis has been carried out on the tissue culture cell line HTC. The HTC cell line was derived from a hepatoma (7288c) formed in male buffalo rats by feeding them a diet containing N,N'-2,7-fluoroenylene-bis-2,2,2-trifluoroacetamide for 12.4 months (Morris, 1963). An ascitestumour was produced, and from this the HTC cell line was cultured to form a stable epithelial cell line (Thompson et al., 1966).

The dexamethasone cytoplasmic receptor complex of HTC cells has been isolated and a number of different sedimentation coefficients

have been reported. The variation was found to be dependent on the ionic strength of the buffers used and on the receptor concentration. Under the conditions found in the cytoplasm the sedimentation coefficient of the receptor was estimated to be 4S (Baxter & Tomkins, 1971).

Before entry into the nucleus the extranuclear receptor-hormone complex has to be transformed by a temperature-dependent process (Baxter et al., 1972) after which the hormone is translocated to the nucleus. The transfer is dependent on the presence of the cytoplasmic receptor.

The receptor has been shown to be involved in the binding of the hormone to the target tissue chromatin (Baxter et al., 1972). In many steroid hormone systems the non-target cell chromatin binds less hormone receptor complex than target cell chromatin (King & Gordon, 1972; Mainwaring & Peterken, 1971; Liao et al., 1973; Spelsberg et al., 1971 a,b; Steggles et al., 1971a,b). The high affinity binding of hormone to chromatin requires the hormone to be bound to the receptor and involves the chromatin proteins.

Reconstitution experiments have shown that the NHCP isolated from chick oviducts are the components of chromatin responsible for the specificity of binding of the progesterone-receptor complex to chromatin (Spelsberg et al., 1971c; Spelsberg et al., 1972; O'Malley et al., 1972).

A fraction with a high affinity for oestradiol and corticosterone has been found in rat liver NHCP by Defer et al. (1974 a,b). Spelsberg et al. (1972) have isolated a fraction of NHCP that has a specific binding capacity for the hormone-receptor complex and in recent work Spelsberg et al. (1976) have demonstrated that nuclear proteins tightly bound to DNA contain the progesterone-receptor "acceptor".

Conversely, a number of workers have demonstrated that the hormone receptor complexes would bind to naked DNA without the need for chromatin proteins. Thus, Baxter et al. (1972) demonstrated that the

corticosteroid-receptor complex of HTC cells would bind to DNA. Furthermore the binding showed some steroid specificity in that the binding of various glucocorticoids and anti-inducers mirrored their activity in enzyme (TAT) induction.

The answer to this paradox may lie in the finding of Schrader et al. (1972). They used DEAE cellulose to separate two progesterone receptors, termed A and B, from chick oviduct. Progesterone receptor A non-specifically bound DNA from several sources but did not bind to chromatin. Conversely receptor B specifically bound to target cell chromatin and did not bind to DNA.

Additional evidence supporting the requirement of the chromosomal proteins is that of Simons et al. (1976) who compared the binding of dexamethasone-receptor complex to naked DNA from HTC cells and DNA associated with chromosomal proteins. It was shown that the chromosomal proteins appeared to reduce the number of acceptor sites while increasing the affinity of the complexes for the DNA.

The binding of the hormone-receptor complex to the target cell chromatin initiates, in a little understood manner, a series of transcriptional events specific to the target cell. In one hormonal system this process can actually be visualised. This is the stimulation, by the insect hormone ecdysone, of RNA synthesis in the salivary gland polytene chromosomes. The chromosomes can be seen to puff or condense as a result of ecdysone administration. The synthesis of RNA has been shown to occur at the site of a puff (Ashburner, 1970). Ecdysone causes three types of puffs during larval development: intermoult puffs; early puffs; and late puffs.

The very rapid initiation of the early puffs is insensitive to protein synthesis inhibitors and it is suggested that it is a primary response to ecdysone. The repression of the early puffs and initiation of the late puffs is sensitive to protein synthesis inhibitors and appears to be a secondary response requiring protein synthesis.

No such visualisation is possible for glucocorticoids and the situation is rendered much more complicated by various aspects of their action:-

1. Glucocorticoids exhibit a number of direct and indirect, so called "permissive effects", on nucleic acid synthesis and enzyme activities.

2. The direct interactions appear to depend on the particular tissue. In some tissues,such as the liver, they are mainly anabolic and stimulatory while in others, such as lymphocytes, they are catabolic and inhibitory. Under both circumstances DNA synthesis seems always to be repressed by glucocorticoids.

3. There is considerable controversy surrounding some of the direct effects, particularly the anabolic induction of amino acid metabolising and gluconeogenic enzymes, as to whether the induction is a result of transcriptional or translational control.

Permissive Effects.

The permissive effects are brought about in conjunction with other factors. Such cooperativity is shown by the joint effects of corticosteroids with catecholamines or glucagon in stimulating gluconeogenesis. Thus the degree to which gluconeogenesis can be induced by catecholamines and cyclic AMP in rat liver is decreased by adrenalectomy. It has been shown in HTC cells that the level of TAT induced by pretreatment of the cells with dexamethasone could be augmented by cyclic AMP (Manganiello & Vaughan,1972). TAT and phosphoenol pyruvate carboxykinase have been shown to be induced by cyclic AMP in Reuber H35 cells, while in HTC cells, cyclic AMP induced neither of the enzymes (Van Rijn *et al.*, 1974). HTC cells were found to have a much lower level of adenyl cyclase and cyclic AMP than rat liver (Granner, 1974).

Cyclic AMP has been shown to act at the translational level (Wicks, 1974) but the particular step at which this occurs has not yet been demonstrated. However, cyclic AMP has been shown to stimulate the

release of TAT from polysomes (Chuah & Oliver, 1971).

Direct Effect.

The glucocorticoids are now assumed to have a direct effect on gene expression but it has yet to be shown by what means the alteration of gene expression is achieved.

As stated previously the glucocorticoids have an anabolic and stimulatory effect in some tissues and in others, a catabolic and inhibitory effect, DNA synthesis being inhibited in both cases.

Glucocorticoids' Effect on DNA Synthesis.

Glucocorticoids have been shown to have an effect on growth at the level of DNA synthesis. The synthesis of DNA in regenerating rat liver was inhibited by glucocorticoids (Einhorn et al., 1954; Jones & Irvin., 1972) but the DNA replication in mitochondria was unaffected by corticosterone acetate (Kimberg & Loeb, 1971). Concentrations of dexamethasone greater than 10^{-4}M have been reported to inhibit growth of HTC cells (Thompson & Lippman, 1974). DNA polymerase activity from livers of young rats was observed to decline after injection of corticosterone (Henderson & Loeb, 1970).

Catabolic and Inhibitory Effects.

The action of cortisol in rabbit lymph nodes has been investigated by Kidson (1967). One minute after cortisol administration there was a partial inhibition of RNA synthesis. After a lag period there was a transient increase in protein synthesis followed by an inhibition of protein synthesis. With the aid of protein synthesis inhibitors the action of glucocorticoids can be shown to require protein synthesis. Thus the decreased glucose and uridine uptake in thymocytes (Makman et al., 1970), the decreased amino acid transport in muscle (Kostyo & Redmond, 1966), the reduced uptake of deoxyglucose in lymphosarcoma (Rosen et al., 1972), the diminished amino isobutyrate transport in human leukaemic lymphocytes (Baran et al., 1972) and the reduced accumulation of RNA precursors by bone (Peck et al., 1969) can

all be shown to depend on protein synthesis. An exception to this is the decrease in RNA polymerase activity in rat thymus which does not require protein synthesis (Nakagawa & White, 1966). In general, all of the glucocorticoid-induced inhibitory effects in target tissues occur with a time lag sufficient to allow binding and synthetic steps to occur.

Anabolic and Stimulatory Effects.

The catabolic and anabolic effects have many similarities, both requiring the synthesis of an intermediate. One of the best characterised systems is the induction of glutamine synthetase in the embryonic chick retina in response to glucocorticoids. This has been shown to be due to the synthesis of glutamine synthetase mRNA (Schwartz, 1972). Other systems include cortisol induced accumulation of exportable enzymes in the pancreas (Yalovsky et al., 1969) and the accumulation of rough endoplasmic reticulum in mammary gland in response to glucocorticoids (Tokami & Topper, 1972). It is the liver, however, that corticosteroids appear to exert their most profound anabolic effect. The activity of many enzymes involved in gluconeogenesis and the metabolism of amino acids are activated and those which have been subject to extensive study appear to be subject to transcriptional control involving the synthesis of new mRNA. Liver enzymes activated by glucocorticoids include:-

Phosphoenolpyruvate carboxykinase	Shrago et al. (1963)
Phosphoglucomutase	Weber et al. (1961)
Phosphohexoisomerase	Weber et al. (1961)
Lactate Dehydrogenase	Weber et al. (1963)
Glucose-6-phosphate dehydrogenase	Weber et al. (1961)
Glucose-6-phosphate gluconate dehydrogenase	Weber et al. (1961)
Tyrosine amino transferase	Lin & Knox (1957)
Tryptophan oxygenase	Labrie & Korner (1968)
Alanine amino transferase	Segal et al. (1964)
Arginase	Ashmore et al. (1963)

Methionine adenosyl transferase	Pan et al. (1968)
Ornithine decarboxylase	Park & Kenney (1971)
Glutamine synthetase	Kulka & Cohen (1973)
Homogentisic oxidase	Lin & Knox (1958)
Phenylalanine hydroxylase	Haggerty et al. (1973)
Serine dehydrase	Greengard & Dewey (1967)

A number of these systems have found favour as experimental research systems, particularly the one which is the subject of a part of this thesis; the induction of hepatic tyrosine amino transferase (TAT) to up to 15 times the basal level.

1.4.2.2 Corticosteroid Induction of Tyrosine Amino Transferase.

Tyrosine amino transferase (TAT) has a molecular weight of 91,000. Three chromatographically distinguishable forms have been reported (Hayashi et al., 1967) and it has been shown to be phosphorylated (Lee and Nickol, 1974). Cortisol induces one of the isoenzymes of TAT in rat liver (Mertvetsov et al., 1973), although the validity of the different isoenzymes has been questioned (Johnson & Grossman, 1974).

The first demonstration of TAT induction in rat liver by glucocorticoids was by Lin & Knox (1957). In this liver system, induction of gluconeogenic enzymes proceeds, together with a gross stimulation of RNA synthesis (Wicks et al., 1965; Greenman et al., 1965). Inhibitor studies reveal that TAT induction does require RNA synthesis but in experiments with HTC cells, TAT induction is not accompanied by gross changes in either RNA (Gelehrter & Tomkins, 1967) or protein synthesis (Tomkins et al., 1966; Tomkins et al., 1969). The only RNA species found to be increased in HTC cells by dexamethasone was phenylalanine tRNA (Lippman et al., 1974) but it has not yet proved possible to monitor the synthesis of TAT mRNA directly.

Work with HTC cells and another hepatoma cell line, the

Reuber H35 line, has led to the postulation of two opposing theories to account for the mechanism of TAT induction.

The post-transcriptional model of Tomkins et al. (1966) proposes that a labile RNA species interacts either directly or indirectly via its product with the TATmRNA resulting in suppression of the TATmRNA translation. The hormone removes the repression by the labile RNA or its product and allows the accumulation of TATmRNA. Tomkins' theory is based on work that suggested that TATmRNA could be inactivated by another RNA or protein. The various results on which the theory is based are:- enucleation does not cause a reduction in the levels of TAT (Ivarie et al., 1975). and treatment with actinomycin D does not lead to a reduction in the level of induced TAT and can cause superinduction (an elevation of an induced level) even when the steroid has been removed at the time of actinomycin D treatment (Tomkins et al., 1966).

The transcriptional model was inferred from experiments that allowed the calculation of the rates of TATmRNA synthesis and TAT degradation and the values were substituted into a mathematical model. The data led to the conclusion that cortisol acts at the transcriptional level.

There is much evidence to support the transcriptional model. The work of Baxter et al. (1972) and Defer et al. (1974a,b) has shown specific binding of glucocorticoids to target cell chromatin and NHCP respectively. Potter and coworkers (Butcher et al., 1972; Bushnell et al., 1974a; Bushnell et al., 1974b) demonstrated a transcriptional level of stimulation by glucocorticoids. They studied the action of cordycepin, an inhibitor of polyadenylation which is thought to be essential for mRNA production, on Reuber H35 cells and HTC cells (Butcher et al., 1972) and with camptothecin, an inhibitor of mRNA synthesis, on Rueber H35 cells (Bushnell et al., 1974a). These inhibitors blocked TAT induction and caused deinduction in the presence of cortisol. Also both inhibitors had no effect on the superinduction of TAT by actinomycin D.

Since both cordycepin and camptothecin inhibit RNA synthesis, without causing superinduction, the validity of the model proposed by Tomkins et al. (1966) has to be questioned. Further experiments using ultraviolet light to inhibit transcription caused deinduction of TAT in the presence of cortisol (Bushnell et al., 1974b).

Actinomycin D has been shown to have effects other than the inhibition of RNA synthesis. Its role in superinduction has been investigated by two groups of workers who found that superinduction was due to an artifical disturbance of either the balance of protein metabolism or the relative proportion of mRNA species. Kenney et al. (1973) and Reel & Kenney (1968) have investigated the action of actinomycin D on TAT induction by cortisol in Reuber H35 cells. They labelled the enzyme and then used a sensitive radioimmunoassay to follow its degradation. They concluded that actinomycin D acted by inhibiting the degradation of proteins. Superinduction has also been studied in the oviduct where oestradiol and progesterone induce ovalbumin synthesis (McKnight et al., 1975; Palmiter & Schimke, 1973). Using DNA complementary to ovalbumin mRNA as a probe these workers found that the induction was dependent on the appearance of ovalbumin mRNA. It is not understood, however, why there is a delay of 90 min with progesterone and 3 hours with oestrogen, between the binding of the hormone receptor complex to chromatin and the appearance of ovalbumin mRNA. Actinomycin D was found to enhance polypeptide elongation and initiation but had no effect on the degradation of proteins. A model was proposed in which the mRNAs had different half lives and that superinduction was an expression of the slow decay of ovalbumin mRNA compared with the total mRNA.

Studies on receptors and their steroid specificity demonstrated a link with TAT induction. The binding of different steroids to their receptors correlated with the steroids' ability to induce TAT (Rousseau et al., 1972). A variant of HTC cells, in which TAT was not inducible by glucocorticoids, was found to have a decreased receptor capacity (Levisohn

& Thompson, 1972).

These results have not ruled out the possibility of a combination of transcriptional and translational control mechanisms. The steroid–receptor complex bound to the chromatin may block the transcription of the Tomkins "repressor", so allowing induction of TAT. Steinberg et al. (1975) extended the studies of cordycepin effects on TAT induction in HTC cells and their results do not exclude the possibility of a labile RNA regulating the TATmRNA. It seems possible that a translational control system is superimposed on the transcriptional mechanism.

The HTC hepatoma cell line provides a system in which it appears that corticosteroids exert some transcriptional control and it has been demonstrated in many experimental systems that transcriptional control involves the NHCP. The picture in most of these systems, however, is clouded by the fact that the hormone initiates many changes in association with a general differentiation of the target tissue. Conversely, HTC cells exhibit very few known changes in response to corticosteroids and it is possible that any changes detected in the components involved in transcription, such as the spectrum of NHCP, could be related directly to the synthesis of known proteins. Such a concept forms the basis of the research in the following sections.

Materials and Methods.

2.1 Materials.

Fine chemicals were obtained as follows:-

β-mercaptoethanol	Koch-Light Laboratories Ltd., Colnbrook, England.
Agarose	"
Toluene (AR grade)	"
Triton X-100	"
2,5-diphenyl oxazole (PPO)	"
N,N,N',N'-Tetramethylene diamine (TEMED)	"
Calf Serum	Bio-Cult Laboratories Ltd., Paisley, Scotland.
Foetal calf serum	"
Amino acids	"
Vitamins	"
Trypsin	Difco Laboratories, Michigan, U.S.A.
Streptomycin	Glaxo Pharmaceuticals, London.
Penicillin	"
p-Bis(o-methyl-styryl) benzene (Bis-MSB)(Scintillation grade)	Kodak Ltd., Manchester, England.
Kodirex KD 54T (35x43 cm) X-ray film	"
DX-80 developer	"
FX-40 X-ray liquid fixer	"
Urea	Carlo Erba, Divisione Chimica Industriale, Milano, Italy.
Xylene Brilliant Cyanin G	Gurr, Searle, High Wycombe, England.
Coomassie Brilliant Blue R	"
Guanidine HCl	Cambrian Chemicals Ltd., Croydon, England.

AG 501-X8(D)	Bio-Rad Laboratories, Richmond, U.S.A.
Bio-Rex 70	"
QAE-Sephadex A25	Pharmacia, Upsala, Sweden.
Triton X-100	Rohm & Haas, Croydon, England.
Ampholines pH range 3.5-10	LKB Instruments Ltd., Croydon, England.
Ampholines pH range 5-7	"
Phenyl Methyl Sulphonyl Fluoride (PMSF)	Sigma, Kingston-upon-Thames, England,
Pyridoxal-5'-Phosphate	"
Sodium Diethyldithiocarbamate	"
Dexamethasone Disodium Phosphate (Dx)	Merck, Sharp & Dohme Ltd., Hoddesdon, England.
∝-keto glutarate	Boehringer, Mannheim, Germany.

Polyacylamide Gel Electrophoresis Molecular Weight Calibration Proteins: Aldolase, Chymotrypsinogen, Cytochrome C, Hen Serum Albumin, Bovine Serum Albumin and Catalese were supplied by Boehringer, Mannheim, Germany.

All other chemicals, whenever possible, were AnalaR reagents supplied by B.D.H. Chemicals Ltd., Poole, Dorset.

Radiochemicals:

[^{32}P]-orthophosphate([^{32}P]; 10 mCi/ml) carrier - free (supplied in a solution of dilute HCl)	Radiochemical Centre, Amersham, England.
[1,2,6,7(n)-^{3}H]-Corticosterone (91 Ci/mmol)	
D.L. Tryptophan (methylene-[^{14}C]) (57mCi/mmol)	"
L-[G-^{3}H]Tryptophan (9.0Ci/mmol)	"
[^{35}S]Sulphur (10 mCi/ml)	"
[methyl-^{3}H]Thymidine (20 Ci/mmol)	"

5-[^{3}H]Uridine (5 Ci/mmol)	Radiochemical Centre, Amersham, England.
Enzymes:	
DNAase (Pancreatic)	Sigma, Kingston-upon-Thames, Surrey, England.
RNAase (Pancreatic)	"

2.2 Standard Solutions

Scintillation Spectrometry Solutions

Toluene based scintillation fluid consisted of

A) 0.5g 2,5 diphenyloxazole (PPO) per litre of toluene.

B) Triton/Toluene scintillation fluid consisted of 5g PPO, 0.5g p-Bis(o-methyl-styryl) benzene (Bis-MSB), 350 ml of Triton X-100 and 650 ml of toluene.

Low Sulphate Medium.

1 mM $MgCl_2$, 0.05 mM Na_2SO_4, 22.1 mM KH_2PO_4, 18.7 mM NH_4Cl, I% (^{w}/v) glucose, adjusted to pH 7.2-7.0 with Na_2HPO_4.

Reticulocyte Saline Buffer (RSB)

0.0I M Tris-HCl, pH 7.4, 0.0I M NaCl, 0.00I5 M $MgCl_2$.

Sodium Dodecyl Sulphate Urea Polyacrylamide Electrophoresis Buffer.

24.8 mM Tris, I92 mM Glycine, 3.5 mM SDS.

8 M Urea Stock Solution.

All urea solutions were made using a stock 8 M urea solution that was deionised immediately before use by passage through a column of AG 50I-X8(D) mixed bed resin.

Polyacrylamide Electrophoresis Gel Solutions.

SDS Gel Electrophoresis Solutions.

A:3.0 M Tris-HCl, 0.23% (^{v}/v) TEMED, pH 8.9.

B:20 % (^{w}/v) Acrylamide, 1.2 % (^{w}/v) N'N'-Methylene Bisacrylamide.

C:0.15 % (^{w}/v) ammonium persulphate, 0.2 % (^{w}/v) SDS, 8 M urea (Freshly made).

D:0.49 M Tris-HCl, 0.46 % (^{v}/v) TEMED, pH 6.7.

E: 12 % (^{w}/v) Acrylamide, 1.2 % (^{w}/v) N'N'-Methylene Bisacrylamide.

F: 0.004 % (^{w}/v) Riboflavin.

G: 8 M urea, 0.2 % (^{w}/v) SDS.

SDS/urea/Polyacrylamide Gels

The gels were made as follows:-

The lower gel constituents for the 7.5 % (^{w}/v) acrylamide gels were stock solutions A: B: C in the ratio 1: 3: 4 and for the 5 % (^{w}/v) acrylamide gels were stock solutions A: B: C: H_2O in the ratio 1: 1: 2: 4. The 3 % (^{w}/v) acrylamide stacking gel constituents were stock solutions D: E: F: G in the ratio 1: 2: 1: 4. The running gel measured 95 mm long and the stacking gel, on top of the running gel, measured 5mm.

Each gel stage was overlayed with water to form a level surface. The stacker gel was polymerised by a light held at a distance of 10 cm for 15 min. After polymerisation the water layer and parafilm from the bottom of the tubes were removed and the electro-phoresis buffer added to the tanks. The proteins were placed on the top of the gels and electrophoresed from the cathode to the anode.

2.3 Conversion of [^{35}S] Sulphur to L-[^{35}S]- Methionine.

Labelled [^{35}S]- methionine was synthesised using a modification of the technique of Bretscher & Smith (1972).

0.1ml of E.coli ML 308, grown overnight in 20 ml of low sulphate medium (page 31) was inoculated into a similar flask containing 10mCi [^{35}S] sulphur and shaken at 37^{o}C overnight. The cells were pelleted (2500g for 10min, MSE 18, 8x50) and the supernatant discarded (61% incorporated). The pellet was transferred to a hydrolysis tube in 0.5 ml water, together with a further 0.5 ml 12 M HCl used to wash out the tube. 100 µl of thioglycollic acid was added and the contents were hydrolysed under vacuum at 105^{o}C for 18 hours. The hydrolysate was dried down by rotary evaporation and the residue dissolved in

0.I M β-mercaptoethanol.

The L-[^{35}S]- methionine was separated from the other components by descending paper chromatography. The developing solvent used was the upper phase of a thoroughly equilibriated mixture of N-butanol: acetic acid: water in the ratio of 4: 1: 5. The L-[^{35}S]-methionine (R.F.=0.55) was cut out, eluted with 5 mM β-mercaptoethanol and stored at -20°C, (2.5 mCi L-[^{35}S]-methionine produced). Approximately 2.5 mCi of L-[^{35}S]-methionine was purified after an input of 10 mCi of [^{35}S] sulphur.

Table A.

Constituents of Minimal Essential Medium (Dulbecco's Modification)-(DMEM).

DMEM amino acids	mg/litre	DMEM vitamins	mg/litre
L-alanine	35.6	D-calcium pantothenate	4.0
L-arginine	84.0	choline chloride	4.0
L-asparagine	60.0	folic acid	4.0
L-aspartic acid	53.2	i-inostol	8.0
L-cystine	48.0	nicotinamide	4.0
L-glutamic acid	58.8	pyridoxal HCl	4.0
L-glutamine	580.0	riboflavin	0.4
glycine	30.0	thiamine HCl	4.0
L-histidine HCl	38.4		
L-isoleucine	105.0		
L-leucine	105.0		
L-lysine	146.0		
L-methionine	30.0		
L-phenylalanine	66.0		
L-proline	46.0		
L-serine	42.0		
L-threonine	95.0		
L-trytophan	16.0		
L-tyrosine	72.0		
L-valine	94.0		

Inorganic salts and other components	mg/litre
$CaCl_2.6H_2O$	393.0
KCl	400.0
$MgSO_4.7H_2O$	200.0
NaCl	6800.0
$NaH_2PO_4.2H_2O$	140.0
D-glucose	4500.0
$NaHCO_3$	2240.0

Phenol red	15.3
streptomycin	100.0
penicillin	100,000 units/litre

2.4.1 Cell Culture Solutions.

A) Balanced Saline Solution (BSS)

II6 mM NaCl, 5.4 mM KCl, I mM $MgSO_4$, ImM NaH_2PO_4, I.8 mM $CaCl_2$, 0.002% (^{w}/v) phenol red, adjusted to pH 7.0 by the addition of 8.4% (^{w}/v) $NaHCO_3$.

B) Minimum Essential Medium (Dulbecco's modification)-(DMEM)

Constituents shown in Table A.

C) Versene

0.54mMNa_2EDTA, 0.17 M NaCl, 3.4 mM KCl, I0 mM Na_2HPO_4, 2.4 M KH_2PO_4, pH 7.4-,0.002% (^{w}/v) phenol red.

D) Trypsin/Citrate

0.25% (^{w}/v) trypsin, I0.5 mM NaCl, I.0 mM sodium citrate and 0.002% phenol; adjusted to pH 7.8 with NaOH.

E) Trypsin/Versene

Trypsin/Citrate I part, Versene 4 parts (^{v}/v).

F) Dulbecco's Medium

88% DMEM, 10% calf serum, 2% foetal calf serum.

2.4.2 Propagation of Cells.

HTC cells were grown in monolayers in 80oz Winchester bottles (burlers) according to the method of House & Wildy (1965).

Cells in the logarithmic phase of growth were used in all experiments. Figure A shows the growth characteristics of the HTC cell line grown in conditions described on page 38. The cells were considered to be in the logarithmic stage of growth between 24-96 hours.

Passaging.

Cells were removed from the glass by treatment with trypsin/versene, and suspended in Dulbecco's Medium, counted and aliquots of 20-25 x 10^6 cells were dispensed into sterile burlers containing 180 ml of Dulbecco's Medium. The burlers were then gassed to give them an atmosphere of 5% CO_2, 95% air. The cells were allowed to adhere to the glass by revolving the burlers slowly (0.2 r.p.m.) between 3 and 18 hours after which they were rotated at the normal speed (0.5 r.p.m.).

2.4.3 Contamination Checks.

All sterile media and passaged cells were checked regularly for bacterial, fungal and Pleuro-Pneumonia-like-Organisms (PPLO) infections as follows:

Bacterial Contamination: aliquots were placed on blood agar plates and in brain-heart infusion broths at 37°C. Results were considered to be negative if growth was absent within 7 days.

Fungal Contamination: a small sample was added to Sabouraud's medium and incubated at 32°C. The sample was considered free from contamination if no growth was observed for 7 days.

PPLO infection: passaged cells were seeded on the surface of an agar plate. The plates were incubated in an atmosphere of 5% CO_2 in N_2 at 37°C. After 8 days the plates were examined under a microscope. If PPLO were present, the cultures were discarded.

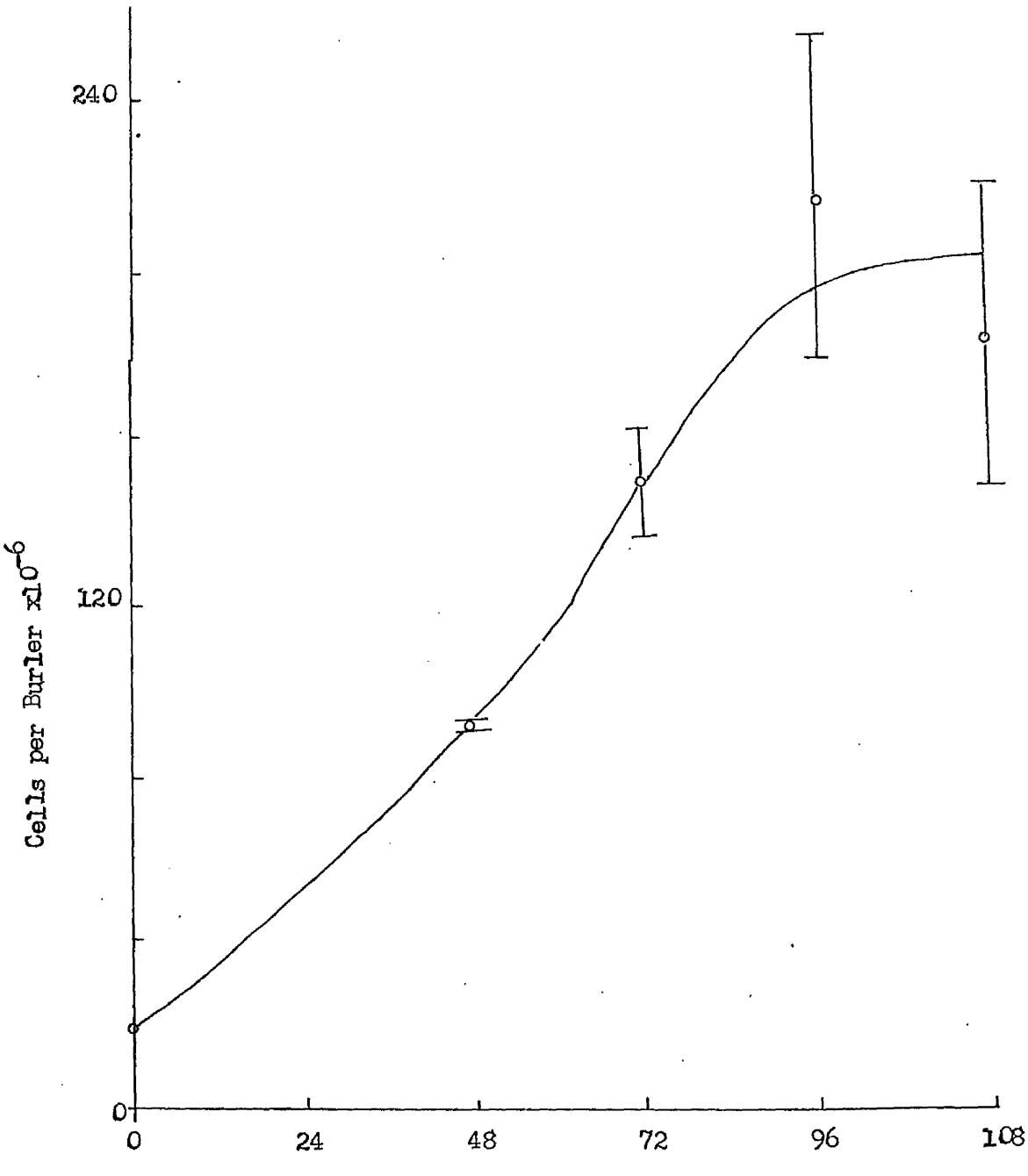
2.4.4 Continuity of TAT induction.

To ensure that the cells continued to respond to hormone,

Figure A.

Growth of HTC Cells in Burlers.

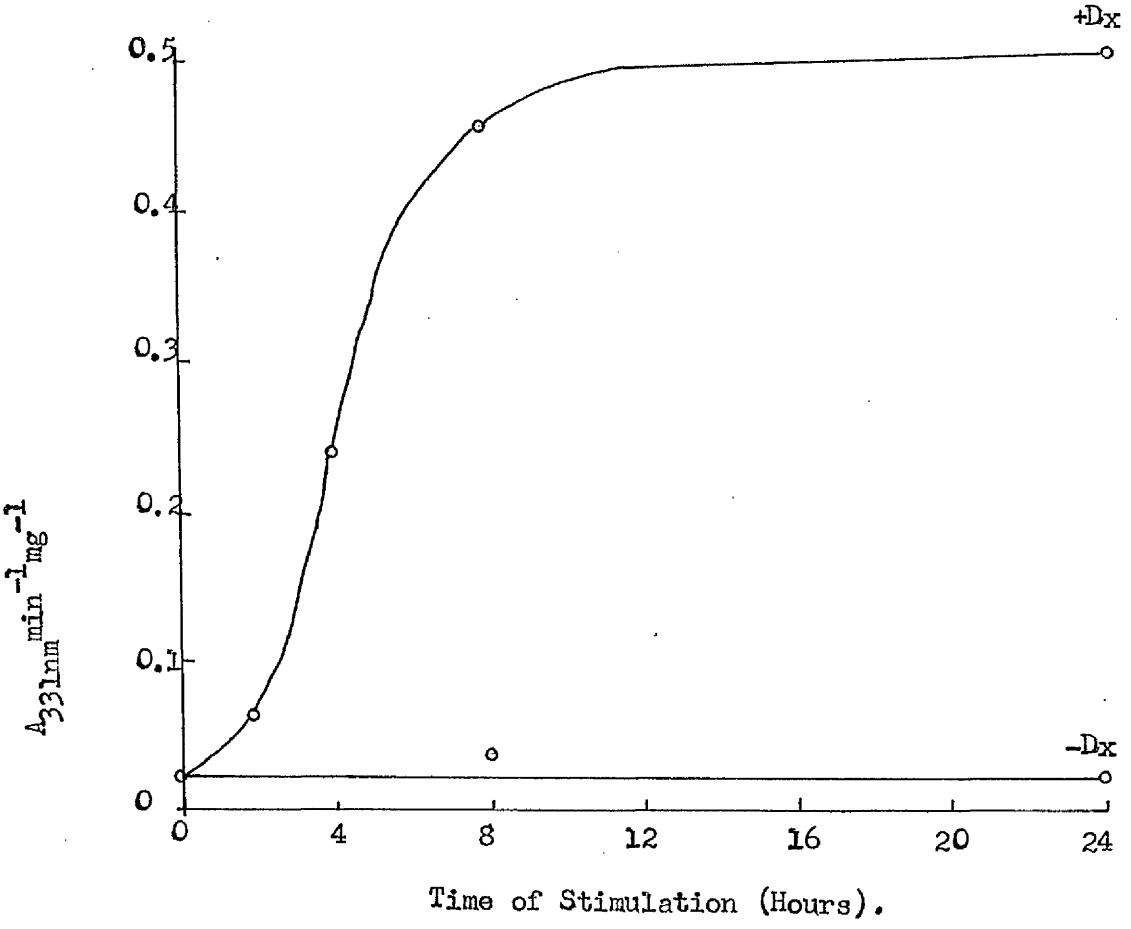
20×10^6 HTC cells were added to burlers containing 180 ml of Dulbecco's Medium. At one day intervals cells were harvested by the trypsin/versene technique and the number of cells measured using a heamatocytometer. The results are shown as the mean and the limits of the standard error of the gross number of cells/burler.


240
120
0
Cells per Burler x10⁻⁶
0
24
48
72
96
108
Time after Passage (Hours).

checks were carried out at regular intervals (2-3 months) for inducibility of TAT (assay on page 41). Figure B illustrates a typical time course of the induction of TAT by 5×10^{-6}M dexamethasone. The degree and kinetics of the hormonal response remained essentially constant throughout the period of research.

Figure B.

HTC cells growing in the exponential phase of growth were stimulated with 5×10^{-6} M Dx. Each point represents 1 burler of cells and all burlers were terminated coincidentally. The control burlers were mock stimulated using medium alone without Dx.


+Dx
-Dx
0.5
0.4
0.3
0.2
0.1
0
0
4
8
12
16
20
24
$A_{331nm}\ min^{-1}\ mg^{-1}$
Time of Stimulation (Hours).

2.5.1 Chemical Measurements.

Protein Estimation.

Protein concentration were determined by the method of Bramhall et al. (1969).

DNA Determination.

DNA was measured by the method of Burton (1956).

2.5.2 Enzymic Estimation.

Tyrosine Amino Transferase (E.C.2.6.1.5.) Activity.

Tyrosine amino transferase (TAT) activity was assayed by the method of Diamondstone (1966).

The fraction to be assayed for TAT activity was isolated by the method of Thompson et al. (1966). After pouring off the induction medium, 10 ml of ice cold Dulbecco's medium was added to the roux and the cells removed using a rubber scraper. The cells were pelleted by centrifugation at 800 g for 5 min at 0-4°C, washed with 2x5 ml of 0.15 M sodium phosphate, pH 7.9, and recentrifuged. The washed pellet was resuspended in 0.5 ml of the phosphate buffer and the cells were ruptured by sonication using two 10 s bursts at 2 A (Dawes Sonic Probe), keeping the suspension at 0-4°C. The broken cell suspension was centrifuged at 20,000 g for 10 min (MSE 18, 8x50) and the supernatant was assayed for TAT activity.

Reaction Mixture: 1300 µl 0.2 M potassium phosphate, pH 7.3 7.38 mM L-tyrosine; 50 µl 1.2 mM pyridoxal-'5 phosphate, 0.2 M potassium phosphate, pH 7.3; 100 µl 0.06 M sodium diethyldithiocarbamate in water; 50 µl 0.3 M∝keto glutarate, 0.2 M potassium phosphate pH 7.3; 100 µl enzyme solution. The mixture was incubated at 37°C for 1-10 min and the reaction was stopped by the addition of 100 µl 10 M NaOH and the tube shaken immediately. After 30 min the solutions were read at 331nm on a Gilford 240 spectrophotometer. The zero time value was obtained by adding the NaOH immediately before the ∝keto glutarate.

2.6 Charcoal Treatment of Calf Serum.

Calf serum was mixed with 2% (w/v) charcoal and allowed to

stand overnight. The serum was decanted and centrifuged three times at 17,000g for 10 min at 0-4°C. The final supernatant was used for the experiments.

2.7 Labelling and Hormone Treatment of HTC Cells.

The cells used for all experiments were taken between 72 and 96 hours after subculturing so that they were in the logarithmic phase of growth (see Figure A). To maintain the maximal uniformity between samples they were terminated coincidentally, i.e., stated times of hormone treatment are times prior to harvesting, except when cells were labelled with L-[^{35}S]- methionine. The medium was changed 24 hours prior to harvesting for new medium in which the calf serum had been charcoal treated.

Tryptophan labelling of the NHCP was accomplished by changing the medium 1 hour prior to the end of the experiment to a reduced volume (50 ml) of new medium containing one-tenth the normal amount of unlabelled tryptophan and 5 μCi/ml of L-[^{3}H]-tryptophan.

Labelling with [^{32}P] - orthophosphate was carried out by changing the medium 1½ hours prior to the end of the experiment to a reduced volume (50 ml) of new medium containing one-tenth (0.1 mM) the normal amount of unlabelled phosphate, although the calf serum was undialysed. 1 hour prior to the harvesting 1.5 mCi of [^{32}P]-orthophosphate (i.e., 30 μCi/ml) was added per burler.

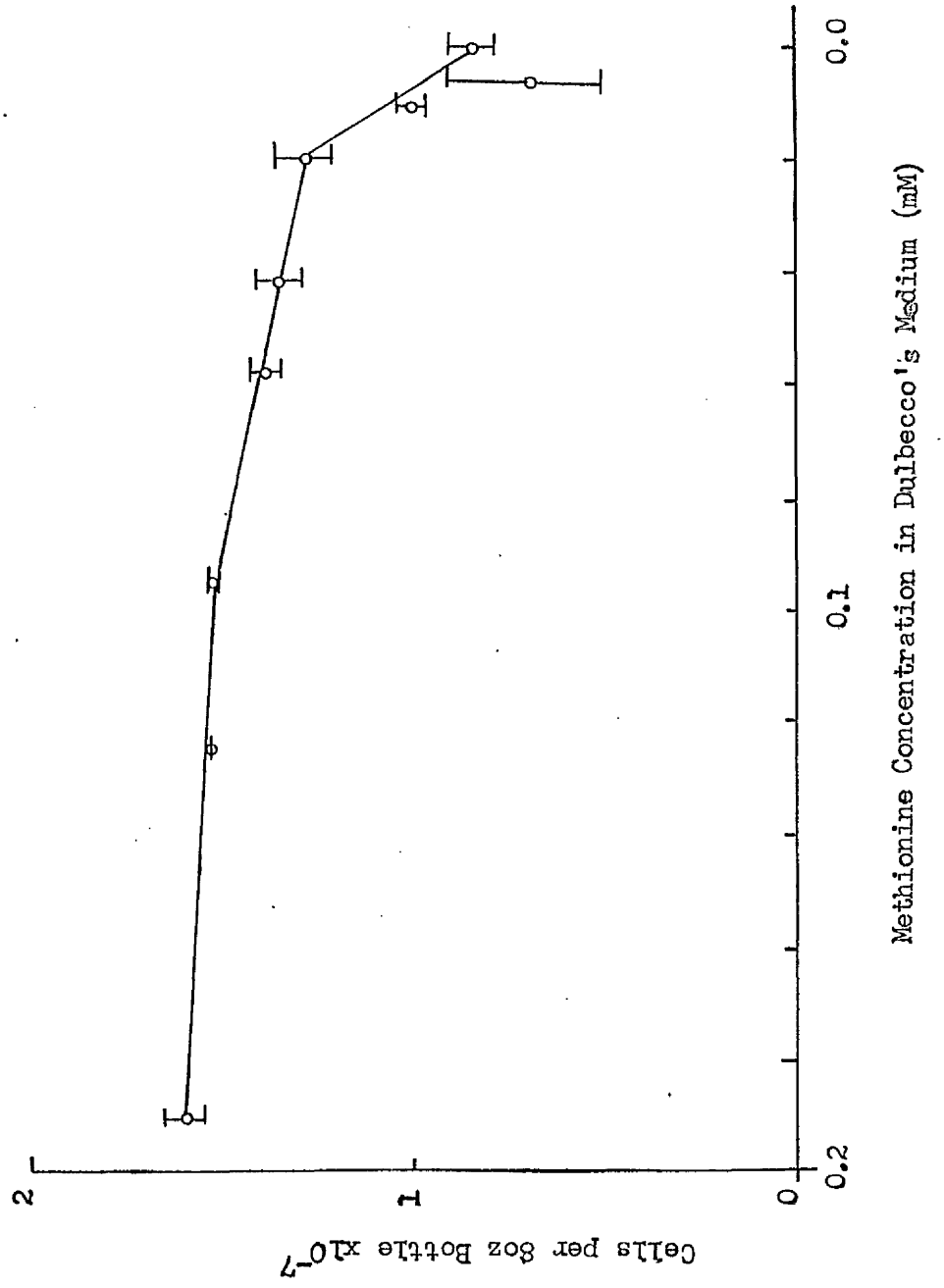
The labelling with L-[^{35}S]-methionine was carried out by growing the cells for 4 days in medium which contained one-tenth (0.02 mM) the normal concentration of unlabelled L-methionine and in which the calf serum had been charcoal treated. Figure C shows that reducing the methionine level to 0.02 mM reduces cell growth by only 20%. 0.5 mCi L-[^{35}S]-methionine (i.e., 2.7 μCi/ml) was added to each burler 3 days before harvesting. The hormone was added at the same time as the label.

2.8.1 Harvesting of Cells.

The growth medium was decanted and 20 ml of ice-cold Dulbecco's Medium was then added to the burler. The monolayer of cells was removed from the glass using a rubber scraper. The

Figure C.

5×10^6 cells were placed into 20 ml of medium in 8 oz bottles. The media contained differing concentrations of methionine. After 4 days of growth the cells were trypsin/versene treated and counted.


Cells per 8oz Bottle x10⁻⁷
2
1
0
0.2
0.1
0.0
Methionine Concentration in Dulbecco's Medium (mM)

dislodged cells were then pelleted (800 g for 5 min) and either used immediately or stored at -20°C.

Isolation of Nuclei, Chromatin and NHCP.

All homogenisation steps were carried out using a tight-fitting Teflon/glass homogeniser. Volumes added were equivalent to 5 ml/burler of original material. The protease inhibitor phenyl methyl sulphonyl fluoride (PMSF) was present at a concentration of 1 mM (from a 50 mM PMSF stock solution in isopropyl alcohol) during all stages of the isolation of chromatin and NHCP.

2.8.2 Isolation of Nuclei.

Pelleted cells were thoroughly suspended in 1% (w/v) Triton X-100, allowed to stand for 10 min and then centrifuged at 800 g for 5 min. The lysis procedure was repeated, the pellet was washed three times with RSB and centrifuged at 800 g for 5 min after each wash.

2.8.3 Isolation of Chromatin.

Chromatin was isolated by a modification of the method of Spelsberg et al. (1971c).

The nuclei were homogenised, using a Teflon/glass homogeniser, in 0.08 M NaCl, 0.02 M EDTA, pH 6.3. The resulting suspension was pelleted at 17,000 g for 5 min. The process of homogenisation and centrifugation was repeated twice. Breakage of nuclei was checked by phase contrast microscopy and if incomplete the homogenisation and centrifugation were repeated. The resulting pellet was homogenised in 0.35 M NaCl to extract the saline soluble chromosomal proteins. After 15 min the homogenate was centrifuged at 17,000 g for 5 min and the supernatant kept for analysis and referred to as the 0.35 M NaCl soluble proteins. The chromatin was homogenised in hypotonic buffer (2 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) and centrifuged at 25,000 g for 10 min. When the NHCP were isolated by the methods of Chaudhuri (1973); Levy et al. (1972) or MacGillivray et al. (1972) the chromatin was taken on to the next stage immediately after this step.

When the NHCP were prepared by the procedure of Wilson & Spelsberg (1973) the chromatin pellet was firstly homogenised in hypotonic buffer (2 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) and allowed to swell for 30 min. If the NHCP were to be isolated immediately the homogenate was then used without further treatment. Otherwise, the suspension was sedimented at 25,000 g for 10 min and the chromatin pellet was stored at $-20^{o}C$.

2.8.4.1 Non-Histone Chromosomal Protein Isolation.

1) Wilson & Spelsberg (1973):

This method of NHCP isolation is based on acid extraction of the histones followed by nuclease treatment to remove nucleic acids.

The chromatin was either homogenised in 0.2 M H_2SO_4 or mixed with 2.2 M H_2SO_4 to make a final concentration of 0.2 M H_2SO_4, depending on whether the chromatin had been previously stored as a pellet or freshly made. The histones were extracted for 15 min and then the solution centrifuged at 17,000g for 5 min. The supernatant (histones) was discarded and the process of extraction repeated with 0.2 M H_2SO_4. The dehistonised nucleoprotein complex was homogenised in 0.I M Tris-HCl,pH 7.5, 2mM $MgCl_2$, 2 mM $CaCl_2$, containing 25µg DNAase/mg DNA (approximately the amount of DNA isolated from one burler) (25 µg RNAase/mg DNA was also included in experiments with $[^{32}P]$-labelled NHCP) and incubated at $37^{o}C$ for 30 min. The NHCP were then precipitated with perchloric acid (addition of one-tenth volume of 4.4 M $HClO_4$). The NHCP were either dissolved in SDS (2% SDS, 0.0I M sodium phosphate, pH 7.5) for analysis by one dimensional poly-acrylamide gel electrophoresis or dissolved in the required solution for isoelectric focussing.

Fractionation of Non-Histone Chromosomal Proteins by Column Procedures.

2.8.4.2 Separation of Non-Histone Chromosomal Proteins from Histones using Bio-Rex 70, (Levy et al., 1972).

The isolated chromatin was suspended in 0.1 M sodium phosphate, pH 7.0, 0.1% (^{v}/v) β-mercaptoethanol and solid urea and guanidine HCl were added to make the solution 6 M and 0.4 M respectively. The final volume was adjusted to 0.25 mg DNA/ ml. The DNA was removed by centrifugation at 105,000g for 40 hours (65Ti rotor, MSE 65 Superspeed). The supernatant was diluted with 6 M urea, 0.1 M sodium phosphate, 0.1% (^{v}/v) β-mercaptoethanol, pH 7.0 to give a concentration of 0.35 M guanidine HCl. It was then loaded on to a 1.5 cm x 9 cm column of Bio-Rex 70 which had been previously equilibrated with the initial elution buffer, 0.35 M guanidine HCl, 6 M urea, 0.1 M sodium phosphate, 0.1% (^{v}/v) β-mercaptoethanol, pH 7.0.

The column was eluted with0.35 M guanidine HCl, 6 M urea, 0.1 M sodium phosphate, pH 7.0, 0.1% (^{v}/v) β-mercaptoethanol at an elution rate of 10-15 ml/hour and 2.7 ml fractions were collected. The NHCP were eluted in the void volume leaving the histones bound to the column. The latter were eluted from the column using 4 M guanidine HCl, 6 M urea, 0.1 M sodium phosphate, pH 7.0, 0.1% (^{v}/v) β-mercaptoethanol. A typical elution pattern is illustrated in Figure D (A). The trace of [^{3}H]-tryptophan indicates some spill-over of NHCP into the histone peak (histones contain no tryptophan residues(Wilhelm et al., 1971)).

In this method it was found that about 10% of the proteins remained bound to the DNA. These could be recovered by resuspending the DNA pellet in 0.1 M Tris-HCl, pH 7.5, 2.: mM $MgCl_2$, 2 mM$CaCl_2$ containing 25 μg DNAase/mg DNA and incubating at 37°C for 30 min. RNAase was also added to a concentration of 25 μg/ml when phosphorylation of the proteins was being investigated. After digestion, the NHCP were precipitated with 0.4 M perchloric acid and dissolved in 2% SDS, 0.01 M sodium phosphate, pH 7.5 for subsequent polyacrylamide gel analysis.

2.8.4.3 Fractionation of Dissociated Chromosomal Proteins using QAE Sephadex A 25, (Chaudhuri, 1973).

The QAE-Sephadex was equilibrated with three washes in 5 M urea, 0.01 M Tris-HCl, pH 8.3, 1 mM β-mercaptoethanol. The column was packed and before use, 100-150 ml of the wash solution was allowed to flow through the column.

The chromatin was dissociated in 7 M urea, 3M NaCl, 1 mM β-mercaptoethanol, 0.01 M Tris-HCl, pH 8.3. The DNA was removed by sedimentation at 105,000g for 40 hours (65Ti rotor, MSE 65 Superspeed). The supernatant was dialysed against three changes of 5 M urea, 0.01 M Tris-HCl, pH 8.3, 1 mM β-mercaptoethanol and the dialysed sample was loaded onto a 1.5 cmx 9 cm column of QAE-Sephadex A 25. The column was eluted with 5 M urea, 0.01 M Tris-HCl, pH 8.3, 1 mM β-mercaptoethanol, at a flow rate of 10-15 ml/hour and 2.7 ml fractions were collected. The histones were eluted after the void volume while the NHCP remained bound to the column. They were eluted with 3 M NaCl, 5 M urea, 0.01 M Tris-HCl, pH 8.3, 1 mM β-mercaptoethanol.

Figure D (B) shows a typical elution profile of L-[G-^{3}H]-tryptophan-labelled dissociated chromosomal proteins.

2.8.4.4 Fractionation using Hydroxyapatite (MacGillivray *et al.*, 1972).

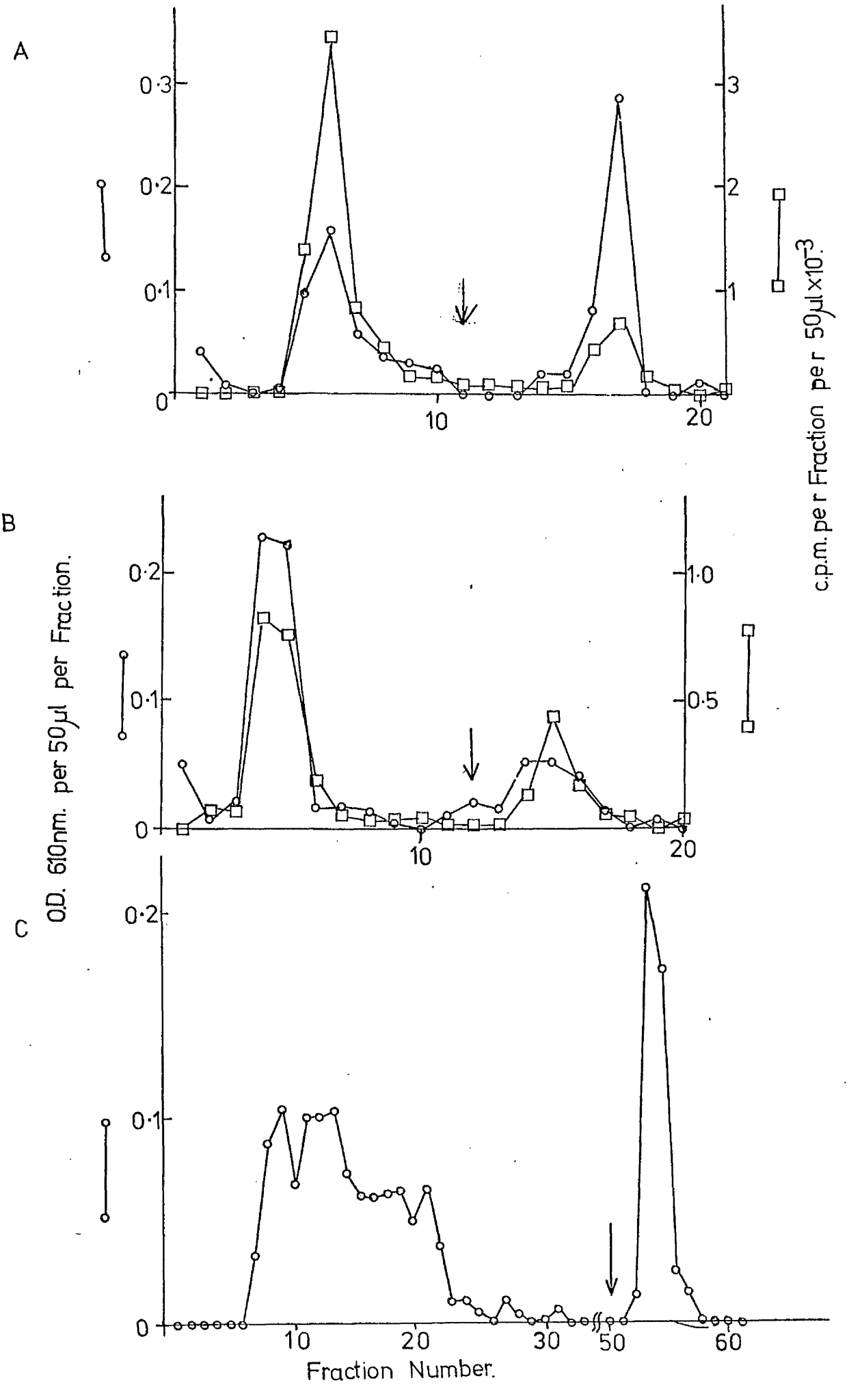
The hydroxyapatite was washed three times with 2 M NaCl, 5 M urea, 0.001 M sodium phosphate, pH 6.8 and was used to pack a column 20 cmx 2 cm. The column was pre-run, before use, by allowing 100 ml of the wash solution to flow through. The column was run at a rate of 10-15 ml/hour and 2.7 ml fractions were collected.

The chromatin was dissociated by homogenisation with 10 volumes of 2 M NaCl, 5 M urea, 0.001 M sodium phosphate, pH 6.8. The solution was sonicated using a Dawes Sonic Probe with two 15 sec pulses at 2 A and then centrifuged at 17,000g for 15 min to remove residual material. The resulting dissociated chromatin was applied to the column.

Figure D.

Typical elution profiles of the separation of histones from NHCP using: A) Bio-Rex 70, B) QAE-Sephadex A 25, C) Hydroxyapatite.

The NHCP were labelled with L-[G-^{3}H]-tryptophan (as described on page 43) except 25 μCi was used for B. The arrows indicate where the change in elution buffers occurred (elutions described pages 47-48).


A
B
C
0·3
0·2
0·1
0
3
2
1
10
20
0·2
0·1
0
1·0
0·5
10
20
0·2
0·1
0
10
20
30
50
60
Fraction Number.
O.D. 610nm. per 50µl per Fraction.
c.p.m. per Fraction per 50µl x10⁻³.

The proteins were fractionated using a step gradient, the histones being eluted after the void volume with 2 M NaCl, 5 M urea, 0.001 M sodium phosphate, pH 6.8. The phosphate concentration was then increased to 200 mM, while keeping the other constituents constant, to elute the NHCP.

Figure D(C) shows a typical elution pattern of dissociated chromatin on hydroxyapatite.

Analysis of Column Fractions.

The elution profiles were assayed by taking 50 µl samples for protein assay (Bramhall et al., 1969) and 50 µl for radioactivity determinations (made up to 200 µl with water and counted in 2 ml of Triton/toluene scintillant).

The fractions containing the NHCP and histones were dialysed against water, lyophilysed and the resulting protein was solubilised for assay or fractionation.

2.9 Separation of Proteins by Polyacrylamide Gel Electrophoresis.

2.9.1 SDS-Urea Polyacrylamide Electrophoresis.

The gel system is similar to that of Laemmli (1970) in which SDS was added to the discontinuous gel system of Davis (1964).

Gels were prepared from stock solutions as described in section 2.2. The % acrylamide used varied with the experimental situation.

The proteins to be analysed were dissolved in 2% ($^{W}/v$) SDS, 0.01 M sodium phosphate, pH 7.5, 10% ($^{W}/v$) glycerol overnight. Bromo-phenol blue was added as a tracker dye (at a level of 25 ul per ml of protein solution). The SDS-protein complex was electrophoresised at 2mA, for approximately 5 hours, on (5 mmx 910 mm) column gels until the bromo-phenol blue was approximately 0.5 cm from the bottom.

The proteins were fixed in 50% ($^{V}/v$) methanol, 10% ($^{V}/v$) acetic acid overnight and then stained with 0.25% ($^{W}/v$) coomassie

brilliant blue R in the same solution for 2-3 hours. The gels were then destained using fixative solution until the background was clear and stored in 7% (^{V}/v) acetic acid.

2.9.2 Two Dimensional Electrophoresis.

The proteins were separated by two similar techniques (Suria & Liew, 1974b; O'Farrell, 1975) that consisted of separation according to charge followed by separation according to molecular weight.

2.9.2.1 1st Isoelectric Focussing Technique (Suria & Liew, 1974b).

The proteins were dissolved in 8 M urea, 0.1 M Tris-HCl, pH 8.4, 0.0I M EDTA, 0.I4 M β-mercaptoethanol and dialysed against this same solution overnight. They were subsequently dialysed against 8 M urea, 0.I25 M Tris-HCl, pH 9.8, 0.02 M methylamine, 0.006 M β-mercaptoethanol for 6-8 hours at room temperature. The resulting protein solution was included in the polymerisation stage in this method.

The gels measured 4 mm x 95 mm and were composed of a solution made up from 40% (^{W}/v) acrylamide, I.6% (^{W}/v) N'N'-methylenebisacrylamide, 40% ampholines, pH 3.5-I0, the sample solution and 0.I2% (^{W}/v) ammonium persulphate freshly made up in I0 M urea mixed in the ratio of I: 0.4: I.6: 5. The gels were overlayered with water and left to polymerise for 1 hour.

The upper compartment (cathode) contained 5% (^{V}/v) I,2-diaminoethane and the lower compartment (anode) contained 5% (^{V}/v) orthophosphoric acid. The focussing was carried out at a constant voltage of 100 V for 16 hours.

2.9.2.2 2nd Isoelectric Focussing Technique (O'Farrell, 1975).

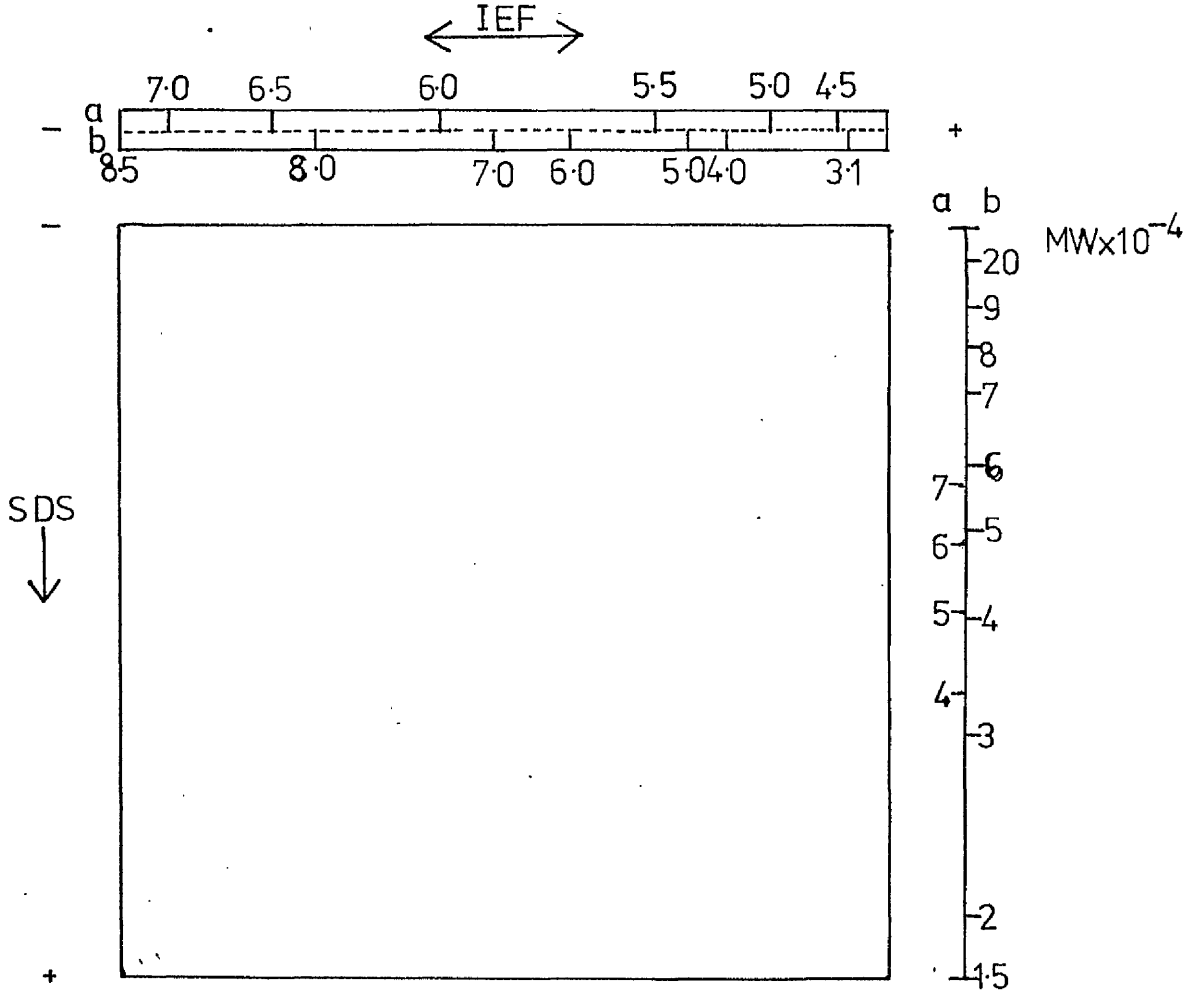
The proteins were dissolved in the solution used by Peterson & McConkey, (1976) for chromatin. They were homogenised in I0 M urea, 0.I% (^{W}/v) SDS, 0.00I M Tris-HCL, pH 7.4 to which was added one tenth the volume of 0.3 M lysine, pH 3.8, I.0% (^{W}/v) SDS, I0 M urea, 0.025 M $ZnCl_2$ and one-tenth the volume of I M Tris-HCl, pH 7.4,

Figure E shows diagrammatically the calibration of the pH gradient of the isoelectric focussing gels and the molecular weight spectrum of the second dimension.

The first dimension is split longitudinally to show the pH gradients of the systems of O'Farrell (1975) (top half a) and Suria & Liew (1974 b) (Bottom half b). The former pH gradient was measured using a flat nosed pH probe at 1 cm intervals, the latter pH gradient was determined by placing gel slices (2 x 1 mm) in 10 ml degassed 0.01 M KCl in scintillation vials; the pH was read after eluting the ampholines overnight.

The second dimension molecular weight calibration was estimated by running standards of known molecular weight.

igure E.


IEF
7·0
6·5
6·0
5·5
5·0
4·5
a
b
–
+
8·5
8·0
7·0
6·0
5·0
4·0
3·1
a b
MWx10⁻⁴
20
9
8
7
6
5
4
3
2
1·5
SDS
–
+

20% Nonidet P40, I.0% SDS. Pure β-mercaptoethanol and 40% ampholines pH 3.5-I0, were added to concentrations of 5% (^{v}/v) and 2% (^{v}/v) respectively.

The isoelectric focusing gels were made by mixing 5.5 g of urea, I.33 ml of 28.38% (^{w}/v) acrylamide + I.62% (^{w}/v) N'N'-methylene-.bisacrylamide, 2ml of I0% (^{w}/v) Nonidet P40, I.97 ml of water, 0.4 ml of 40% ampholines, pH 507 and 0.I ml of ampholines, pH 3.5-I0. This was then degassed and I0 µl of I0% (^{w}/v) ammonium persulphate and 7 ul of TEMED were added before the mix was pipetted into cylindrical gel tubes (130mm x 2.5mm). The gels were overlayered with 8 M urea and allowed to polymerise for 1-2 hours, after which the urea was replaced with 20 ul of 9.5 M urea, 2% (^{w}/v) Nonidet P40, 2% Ampholines (I.6% pH 5-7, 0.4% pH 3.5-I0) for a further 1-2 hours. This solution was then changed and the reservoir solutions added to the compartments (upper cathode reservoir, 0.02 M NaOH; lower anode reservoir, 0.0I M orthophosphoric acid). The gels were pre-electrophoresed at 200 V for $\frac{1}{4}$ hour, then 300 V for $\frac{1}{2}$ hour followed by 330 V for $\frac{1}{2}$ hour. The protein sample was placed on top of the gels and isoelectric focussing was carried out at a constant voltage of 330 V for 16 hours.

2.9.2.3 Second Dimension SDS-urea Polyacrylamide Electrophoresis.

The second dimension for both methods was a modification of the SDS-urea polyacrylamide gel electrophoresis already described (page 32). The proteins in the isoelectric focussing gels were equilibrated with SDS by successive washes for 2 x 15 min at 60°C with each of the following solutions:-

1). 8 M urea, I.0% (^{w}/v) SDS, I.0 mM DTT, 0.I M sodium phosphate, pH 7.2.

2). 8 M urea, I.0% (^{w}/v) SDS, I.0 mM DTT, 0.0I M sodium phosphate, pH 7.2.

3). 8 M urea, 0.I% (^{w}/v) SDS, I mM DTT, 0.0I M sodium phosphate, pH 7.2.

(Transferance to the Second Dimension).

The gels were annealled into position on the second dimension slab gel using I.0% ($^{w}/v$) agarose in electrophoresis buffer. The dimensions of the slab gels were 4 mm x 98 mm x 100 mm (Suria & Liew, 1974b) and 1.5 mm x 140 mm x 150 mm (O'Farrell, 1975). The gels were run at a constant current of 30 mA per slab for 4-5 hours until the bromophenol blue marker was approximately 0.5 cm from the bottom. The slabs produced by the method of Suria & Liew (1974b) were stained by the same method as one dimensional gels while those produced by the method of O'Farrell (1975) were used for [^{35}S] labelled proteins and the spots were visual ised by scintillation autoradiography (page 55).

2.10 Slicing Column Polyacrylamide Gels.

Gels were sliced transversely, using a multiblade cutter, into 1 mm discs and placed in 5 ml insert vials. The discs were dried at 60^{o}C for 1-1$\frac{1}{2}$ hours and then 0.2 ml hydrogen peroxide was added and the gel hydrolysed overnight at 60^{o}C. After cooling, 2 ml Triton/toluene scintillant was added and the radioactivity measured on a Phillips or Intertechnique Scintillation counter set for [^{3}H] and [^{32}P] labelled material respectively.

2.11 Autoradiography.

To detect the [^{32}P]- labelled proteins. in the two dimensional electrophoresis system of Suria & Liew (1974b), the slab gels were sealed in plastic bags and then exposed to Kodirex X-ray film for 3-7 days.

2.12 Scintillation Autoradiography.

The visualisation of L-[^{35}S]-methionine-labelled proteins after two dimensional electrophoresis (O'Farrell, 1975) was carried out by the method of Bonner & Laskey (1974). The gels were impregnated with 2,5-diphenyl oxazole (PPO) for scintillation autoradiography by successive washes in the following solutions:-

1. 20 min in 50% (^{v}/v) methanol, 10% (^{v}/v) acetic acid.
2. 2 x 20 min in dimethyl sulphoxide.
3. 30 min in 4 volumes per gel of 20% (^{w}/w) PPO in dimethyl sulphoxide.
4. 30 min in water (several changes).

The results were detected using Kodirex KD 54T X-ray film at -70°C.

Results.

3 In a preliminary study of the isolation of HTC cells' NHCP a number of procedures were examined in order to ascertain the best method for routine use.

The NHCP were isolated by the various procedures described in the Materials and Methods. Table B and Figure D show the resolution of the NHCP from the histones. All methods of chromosomal protein preparation used chromatin prepared by the method of Wilson & Spelsberg (1973). The final chromatin preparation represented a recovery of 30 % as judged by the recovery of DNA. The chromatin had a protein to DNA ratio of 1.4 and a NHCP to histone ratio of 0.64

3.1 The Separation of Non-Histone Chromosomal Proteins from Histones by Column Techniques.

Table B shows the recoveries, contamination and specific activities of the histones and NHCP recovered by the various methods of isolation. Separation using the method of MacGillivray et al. (1972) with hydroxyapatite was unsuitable as the column was run at room temperature and there was only a 55.8 % recovery of protein from the column with an apparent ratio of NHCP to histones of 2.52. This is probably a result of proteolytic activity which would reduce the recovery and increase the ratio of NHCP to histones. The histones are more readily degraded by proteases.

The method of Chaudhuri (1973) involved the separation of salt-urea dissociated chromosomal proteins on QAE-Sephadex. The DNA was removed from the solution of dissociated chromatin by centrifugation but only 82 % of the DNA was pelleted. The dissociation solution (7 M urea, 3 M NaCl, 1 mM β-mercaptoethanol, 0.01 M Tris-HCl, pH 8.3) gave a high and reproducible percentage of dissociation of the chromosomal proteins from the DNA (94 %). The recovery from the column was 79 % with a ratio of NHCP to histones of 3.65. The histones were contaminated by tryptophan-containing proteins; 26 % of the eluted L-[^{3}H]-tryptophan label was found in the histone peak.

Table B.

Techniques for the Separation Non-Histone Chromosomal Proteins from Histones.

(A) Separation by the method of MacGillivray *et al.* (1972).

(B) Separation by the method of Chaudhuri (1973).

(C) Separation by the method of Levy *et al.* (1972).

The values are shown as the mean ± standard error (number of samples in brackets). The results of the percentage of DNA pelleted were calculated by two different techniques; (a) assayed by Burton (1956) diphenylamine reaction; (b) the cells were labelled with 100 μCi [^{3}H]thymidine 1 hour prior to harvesting and the result is shown as the percentage of counts in the pellet.

The labelling by L-[^{3}H]-tryptophan was carried out as described on page 43. RNA distribution was estimated by measuring the incorporation of 100 μCi [^{3}H]-uridine into chromatin during a 1 hour pulse prior to harvesting. The percentage contamination of the histones by the NHCP was estimated from the overflow of L-[^{3}H]-tryptophan containing proteins into the histone peak and is represented as a percentage contamination derived from the ratio of counts in peak 1 and peak 2. The separation of Chaudhuri (1973) yields histones in peak 1 while in that of Levy *et al.* (1972) the histones are in peak 2.

Table B

	A	B	C
Protein Dissociation from DNA (%)	-	94.5±0.7(7)	88.7±1.8(10)
Pelleted DNA (%)	-	81.8(a)	100(a)99.9±0.1(2)(b)
Pelleted RNA (%)	-	-	98.5
Recovery of Protein from Columns (%)	55.8	79±8.5(5)	81±4(4)
Ratio of NHCP to Histones ($^{w}/_{w}$)	2.52	3.65	1.13±0.11(3)
Peak 1 c.p.m./mg $x10^{-4}$	-	2.84±1.07(5)	24.2±3.5(4)
Peak 2 c.p.m./mg $x10^{-4}$	-	7.90±2.03(5)	2.7±0.6(4)
Contamination of Histones (%)	-	26.4	10

The results show that, although the percentage dissociation and the percentage recovery are reasonable, the percentage contamination by DNA of the dissociated chromosomal proteins, the percentage of L-[^{3}H]-tryptophan in the histones and the ratio of NHCP to histones make the method unsuitable for experimentation.

An improved technique by Levy et al.(1972) in which the chromatin was dissociated in a urea/guanidine solution and the DNA was removed by centrifugation resulted in 88 % of the chromosomal proteins being dissociated from the DNA whilst 100 % of the DNA was pelleted. When chromatin RNA was labelled with [^{3}H]-uridine, 98 % of the labelled material was found to pellet with the DNA. The amount of protein recovered from the Bio-Rex 70 was 81 % with a ratio of NHCP to histones of 1.1. The L-[^{3}H]-tryptophan labelled protein contamination of the histone peak was only 10 %.

The profile of protein fractionation by all three methods is illustrated in Figure D of the Materials and Methods. Figure F illustrates the consistency of the NHCP (peak 1) isolated by the method of Levy. On rechromatography of peak 1 no peak 2 (histones) was seen. The reproducibility, level of recovery and low contamination of the NHCP achieved by this method made it the chromatographic method of choice. It also had an added advantage in yielding a fraction of proteins tightly bound to DNA. All three methods used much milder denaturing conditions than the acid extraction of Wilson & Spelsberg (1973) but took much longer and were more vulnerable to protease degradation.

3.2 Isolation of Non-Histone Chromosomal Proteins by the method of Wilson & Spelsberg (1973).

The histones were isolated from purified chromatin by acid extraction and were found to contain between 8-15 % of the total L-[^{3}H]-tryptophan labelled chromosomal proteins. In the subsequent DNAase treatment, 91 % of the DNA was digested when assayed by the

Figure F.

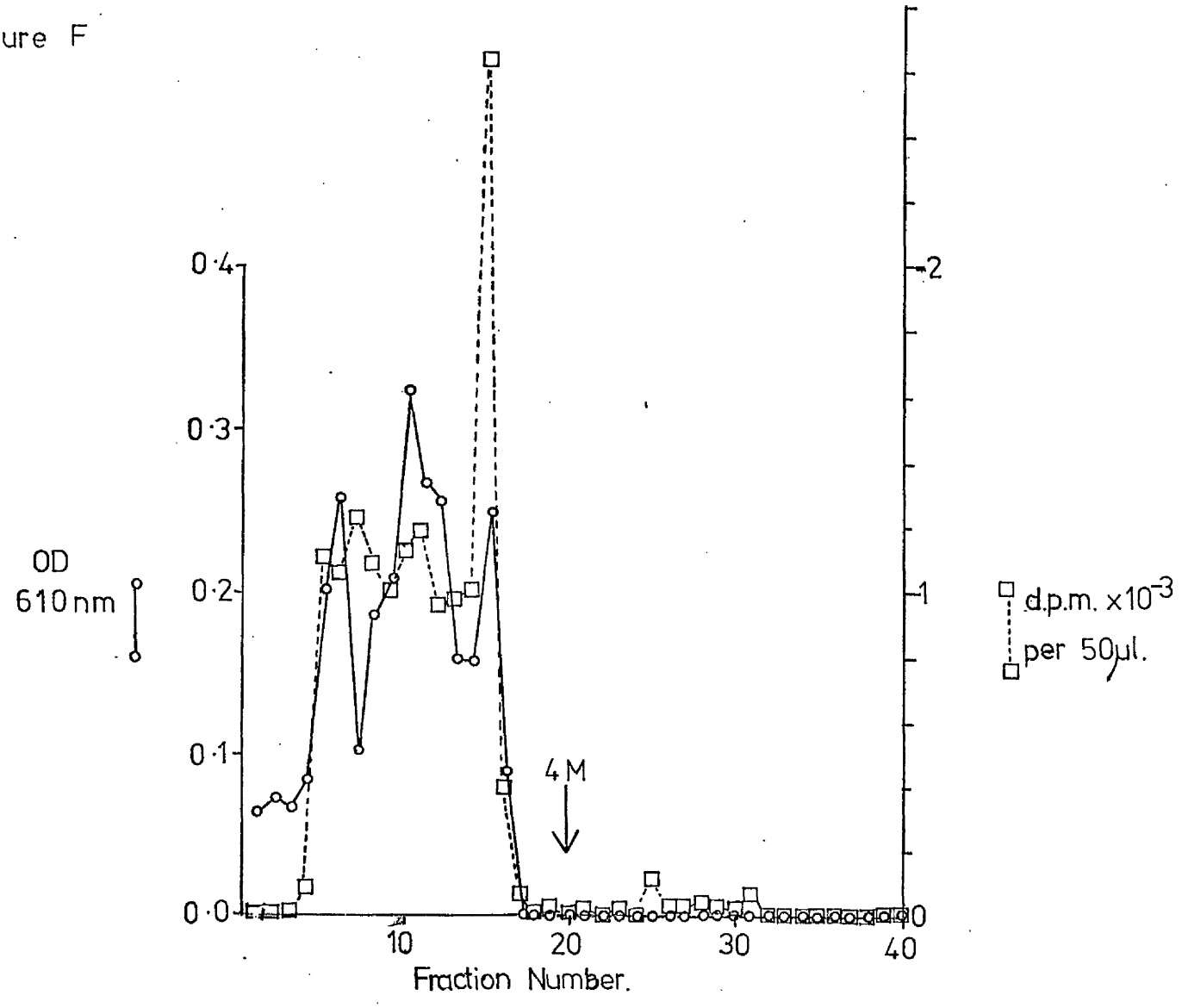
Peak 1 from Bio-Rex 70 Re-chromatographed on Bio-Rex 70.

The NHCP peak from Bio-Rex 70 labelled with L-[^{3}H]-tryptophan (labelling as described page 43) was re-chromatographed (as described page 47) on a second column containing Bio-Rex 70. The arrow shows where the elution buffer was changed to 4 M guanidine HCl, 6 M urea, 1 % (^{v}/v) β-mercaptoethanol, 0.1 M sodium phosphate (pH 7.0).

o——o the amount of protein per fraction (O.D.610 nm per 50 ul).

□------□ the amount of radioactivity per fraction (d.p.m. per 50 ul).

gure F


0·4
0·3
0·2
0·1
0·0
OD
610nm
2
1
0
d.p.m. x10⁻³
per 50µl.
4M
10
20
30
40
Fraction Number.

method of Burton (1956) and 95 % when monitored by solubilisation of [^{3}H]-thymidine labelled material. When RNA digestion was also performed at this stage, the RNAase digested 97 % of the [^{3}H]-uridine labelled material (labelling as described in Table B legend). DNAase and RNAase were found not to be inhibited by the PMSF included in the buffers at a concentration of 1 mM to inhibit protease activity.

This fractionation procedure has the advantage that the proteins are rapidly isolated, so reducing the time in which degradation could occur. The main disadvantage to the method is that the extreme denaturing conditions result in solubilisation problems in the later analysis of the NHCP product. Furthermore, it has been shown by Sonnenbichler & Nobis (1970) that acid treatment causes a variable proportion of the histones to become bound to the DNA, so fractionating as "NHCP". Spelsberg et al. (1973) showed by immunological techniques that the NHCP could also be altered by acid treatment.

As a result of the above analysis of the available methods of preparing NHCP, it was decided to carry out a study of the NHCP of the HTC cells by employing the methods of both Levy et al. (1972) and Wilson & Spelsberg (1973) on a comparative basis.

3.3 Optimal Growth Conditions for Investigating the Effect of Corticosteroids on HTC Cell Non-Histone Chromosomal Protein Synthesis.

The growth medium was altered to allow labelling of NHCP to an optimal specific activity and to maximise the effect of corticosteroid-induced enzyme synthesis.

The growth of HTC cells was found to be seriously affected if they were grown in the absence of serum but the serum was found to contain sufficient endogenous corticosteroid to give an induced level of TAT (10-20 % of the maximum induced levels). Charcoal treatment of the

Table C

The calf serum was treated with 2 % ($^{w}/_{v}$) charcoal (as described on page 41). The effect on the glucocorticoid level was measured by two different techniques.

1.5 µCi of [^{3}H] Corticosterone was added to 25 ml of calf serum and left to stand overnight. The serum was then charcoal treated and the results expressed as d.p.m./50 µl of calf serum before and after charcoal treatment, (column I).

2. The amount of total glucocorticoid in the calf serum was analysed, by the M.R.C. Blood Pressure Unit of the Western Infirmary, Glasgow, for cortisol plus corticosterone. The results in column II are the levels before and after charcoal treatment, expressed as µg/100 ml.

Table C

Extraction of Corticosteroids with Charcoal.

	I	II
	d.p.m./50 µl	µg/ 100 ml
Calf Serum	21856 (100%)	3.07 (100%)
Treated Calf Serum	3245 (15%)	0.75 (25%)

serum was found to lower the endogenous corticosteroid concentration by 75-85 % down to $2x10^{-9}$M (Table C). The work of Samuels & Tomkins (1970), which was conducted in serum-free conditions, showed that $2x10^{-9}$M is below the threshold of TAT induction so it was decided to use charcoal-treated serum. The decay of TAT to endogenous levels after the removal of corticosteroids from the medium takes approximately 5 hours (Steinberg et al., 1975). It was therefore decided to grow the HTC cells in medium containing normal serum but to change to one containing charcoal-treated serum 24 hours before harvesting.

Labelling of the HTC cells' NHCP with L-[^{3}H]-tryptophan and [^{32}P]-orthophosphate was most efficient if the growth medium was depleted of tryptophan and phosphate respectively. Tryptophan labelling was carried out with 250 μCi of L-[^{3}H]-tryptophan per burler in medium containing one tenth the normal concentration of tryptophan. Cells labelled with [^{32}P]-orthophosphate were grown for $1\frac{1}{2}$ hours in medium containing one tenth the normal concentration of phosphate and were labelled with 1.5 mCi of [^{32}P]-orthophosphate per burler for the final hour of incubation. Neither of these depleted media had any detectable effect on the growth of the cells over the period of time in which they were used.

To efficiently label the NHCP of HTC cells with L-[^{35}S]-methionine, the cells had to be grown in the presence of L-[^{35}S]-methionine over 4 generations (4 days). A series of experiments was conducted to ascertain the extent to which the medium could be depleted of unlabelled methionine over this period of time. Figure C shows that when cells were grown in a medium containing one tenth the normal concentration of methionine and charcoal-treated serum for 4 days, growth was only inhibited by 20 %. Furthermore, TAT induction was unaffected by these conditions. Therefore these growth conditions were used in studying the hormone effects of methionine labelling of NHCP throughout the cell cycle.

3.4 The Effect of RNAase and DNAase.

To remove interference by nucleic acids in the isolation and fractionation of the nuclear proteins, digestion with nucleases was carried out. RNAase was only employed when the labelling was with [^{32}P]-orthophosphate.

Figure G1a shows that without DNAase treatment, the NHCP is contaminated with DNA largely of a molecular weight too high for it to enter the gel. After DNAase treatment (Figure G1b), most of the residual DNA now runs with the marker dye.

Figure G2a and G2b illustrate the effect of RNAase on the contamination of NHCP by RNA. Without RNAase treatment (Figure G2a),RNA was distributed right across the gel and in [^{32}P] labelling experiments this had a profound affect on the labelling profile. After RNAase treatment (Figure G2b), any residual RNA migrated with the marker dye.

3.5 Comparison of the Different Nuclear Fractions Analysed by One Dimensional Polyacrylamide Gel Electrophoresis.

The different fractions vary in their staining profile Figures H, I and J. The number of major bands present in the NHCP, 0.35 M NaCl soluble proteins and the proteins tightly bound to DNA are 25, 20 and 7 respectively. Some bands are common to both the NHCP and the 0.35 M NaCl soluble proteins, although there are fewer components in the high molecular weight range in the latter. The proteins tightly bound to DNA give fewer bands on electrophoresis and the bands tend to be more diffuse. The large peak (R.M.,0.61) in the staining pattern of the proteins tightly bound to DNA was only slightly labelled with either L-[^{3}H]-tryptophan or [^{32}P]-orthophosphate and co-electrophoresed with histone H1 (R.M.,0.63, 0.65), DNAase (R.M., 0.62) and one of the RNAase bands (R.M., 0.62), indicating the possibility of protein contamination. The work of Pederson & Bhorjee (1975) on the proteins tightly bound to DNA demonstrated that this fraction did not contain any 0.2 M H_2SO_4 soluble proteins i.e. histones. This still leaves the possibility that the large band with a

Figure G

The Effect of RNAase and DNAase on the Removal of the Contaminating Nucleic Acids.

The HTC cells' DNA and RNA were labelled with 100 μCi of [^{3}H]-thymidine and 100 μCi of [^{3}H]-uridine respectively for 1 hour prior to harvesting. 1a and 1b show the different labelling pattern of the contaminating DNA without and with DNAase respectively. 2a and 2b show the different labelling pattern of the contaminating RNA without and with RNAase respectively. The arrow denotes the direction of electrophoresis.

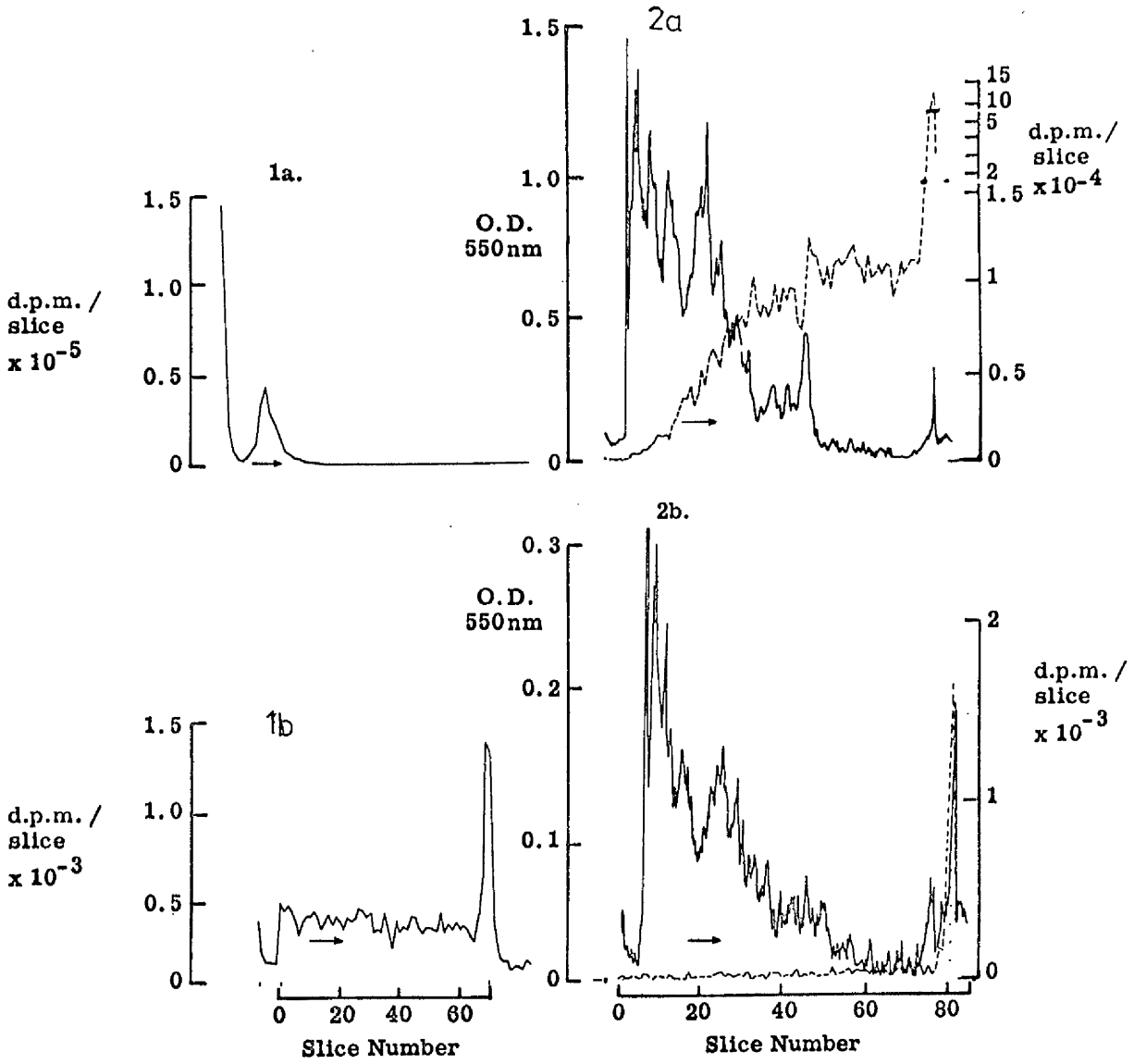

1a.
2a
1b
2b.
d.p.m. / slice x 10^-5
O.D. 550nm
d.p.m./ slice x10^-4
d.p.m. / slice x 10^-3
d.p.m. / slice x 10^-3
Slice Number
Slice Number

Figure H

The Effect of Dexamethasone on the L-[^{3}H]-Tryptophan Labelling of the bulk Non-Histone Chromosomal Proteins.

The bulk NHCP were labelled with L-[^{3}H]-tryptophan (as described page 43). The concentration of dexamethasone used was 5×10^{-6}M. The top row (1) shows the pattern of stained protein; the photograph of the gel is at the bottom. The labelling pattern, (2) middle row, is shown as the amount of radioactivity per slice against slice number. The arrow denotes the direction of electrophoresis.

(a) Control, (b) 1 hour after addition of hormone, (c) 24 hours after addition of hormone.

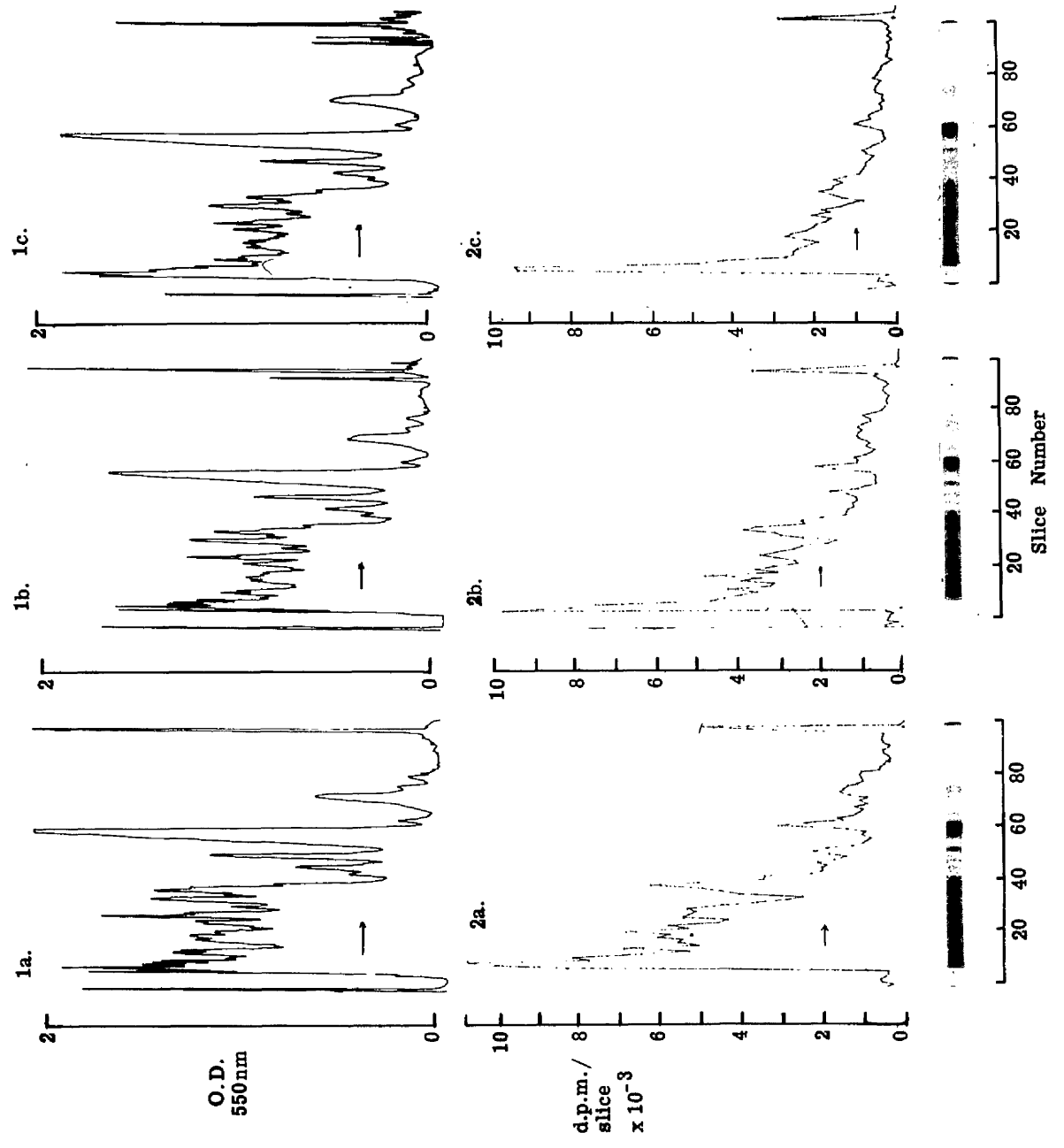

1a.
1b.
1c.
2a.
2b.
2c.
O.D.
550nm
d.p.m./
slice
x 10⁻³
Slice Number

Figure I

The Effect of Dexamethasone on the L-[^{3}H]-Tryptophan Labelling of the 0.35 M NaCl Soluble Proteins.

The 0.35 M NaCl soluble proteins were labelled with L-[^{3}H]-tryptophan (as described page 43). The concentration of dexamethasone used was 5×10^{-6}M. The top row (1) shows the pattern of stained protein; the photograph of the gel is at the bottom. The labelling pattern, (2) middle row, is shown as the amount of radioactivity per slice against slice number. The arrow denotes the direction of electrophoresis.

(a) Control, (b) 1 hour after addition of hormone, (c) 24 hours after addition of hormone.

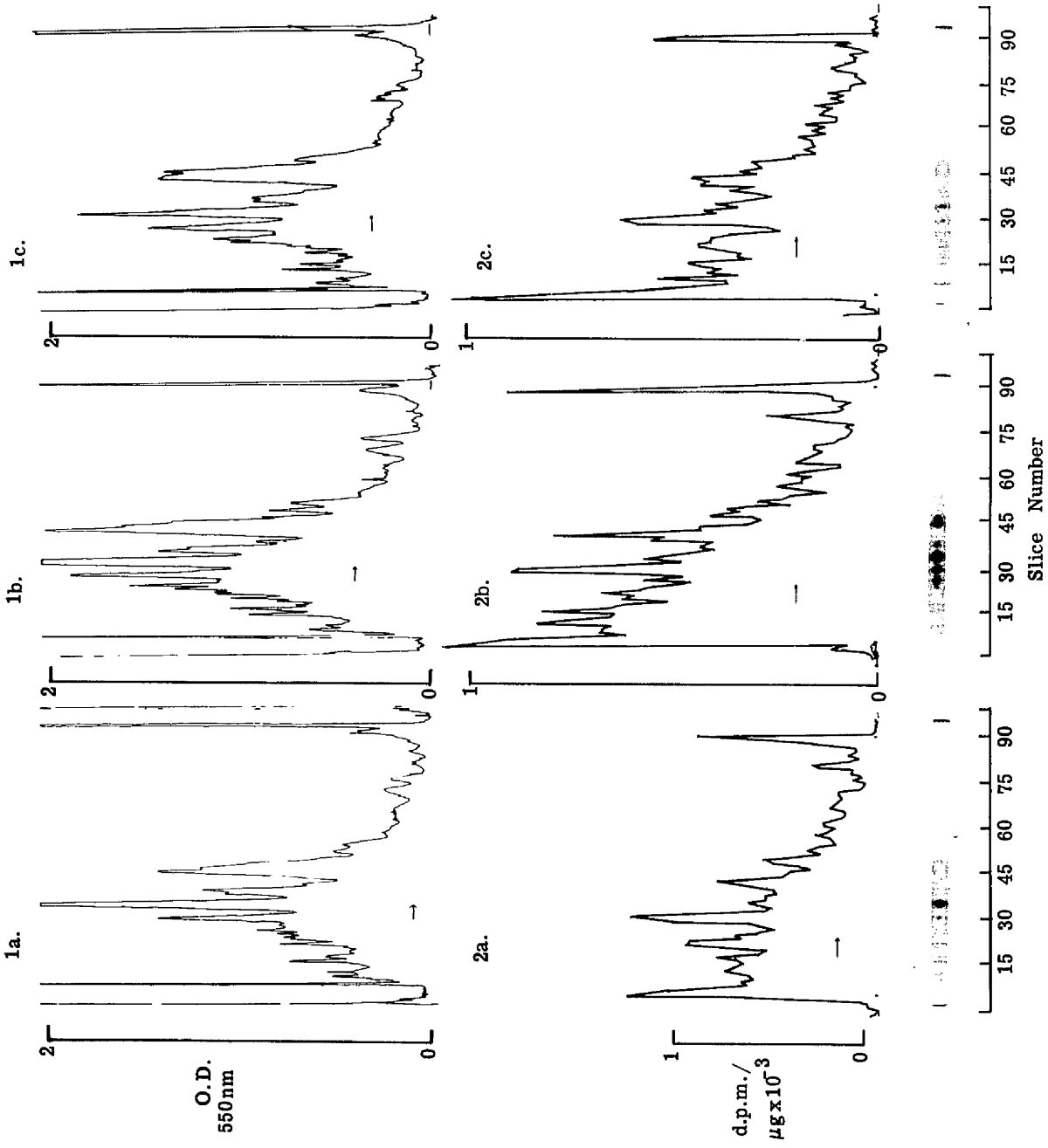
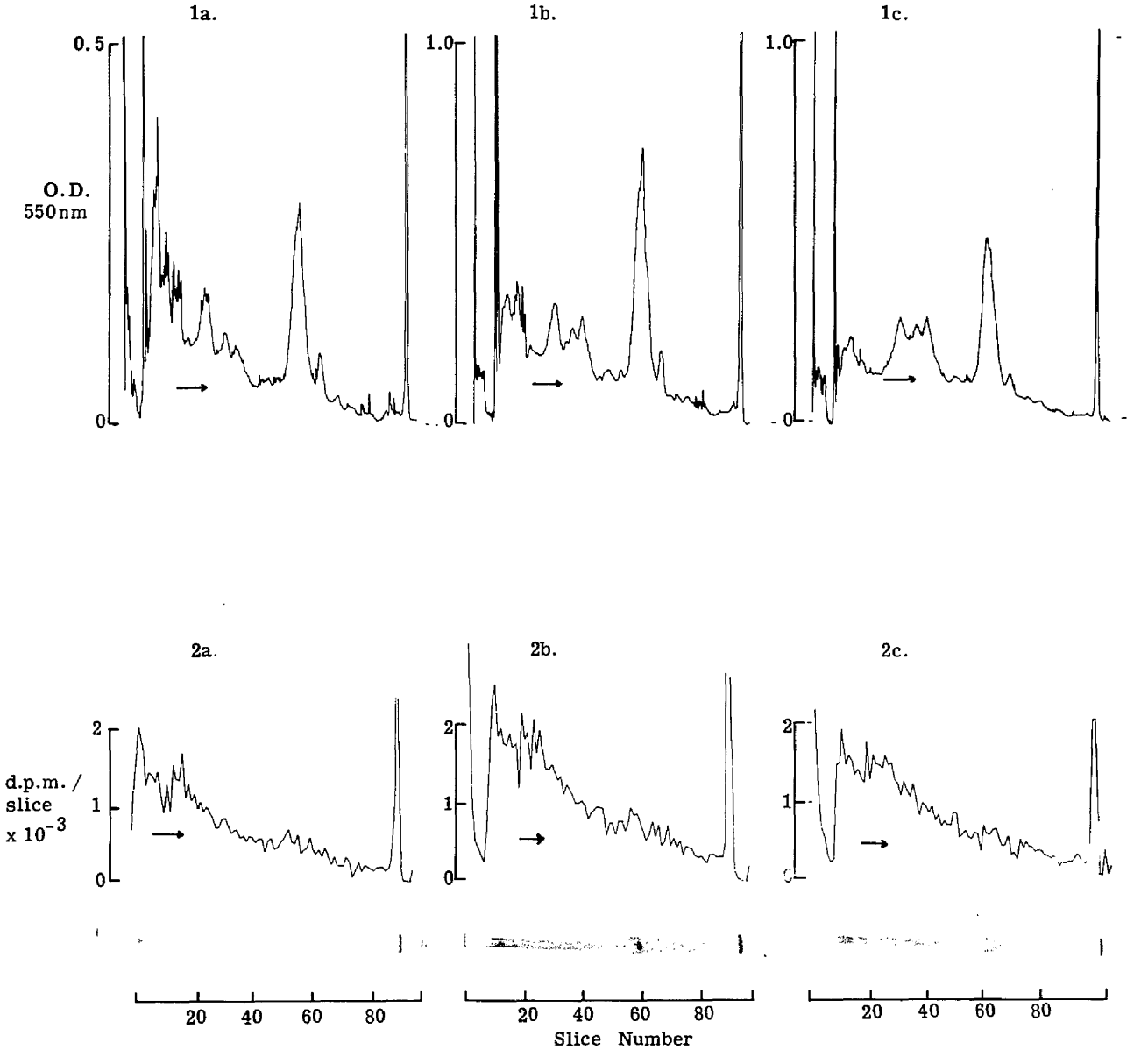

1a.
1b.
1c.
2a.
2b.
2c.
O.D.
550nm
d.p.m./
μg x10⁻³
Slice Number
15 30 45 60 75 90

Figure J

The Effect of Dexamethasone on the L-[^{3}H]-Tryptophan Labelling of the Proteins Tightly Bound to DNA.

The proteins tightly bound to DNA were labelled with L-[^{3}H]-tryptophan (as described page 43). The concentration of dexamethasone used was 5×10^{-6}M. The top row (1) shows the pattern of stained protein; the photograph of the gel is at the bottom. The labelling pattern, (2) middle row, is shown as the amount of radioactivity per slice against slice number. The arrow denotes the direction of electrophoresis.

(a) Control, (b) 1 hour after addition of hormone, (c) 24 hours after addition of hormone.


1a.
1b.
1c.
0.5
1.0
1.0
O.D.
550nm
0
0
0
2a.
2b.
2c.
2
2
2
d.p.m. /
slice
x 10^-3
1
1
1
0
0
0
20 40 60 80
20 40 60 80
20 40 60 80
Slice Number

R.M. of 0.61 is due to DNAase and/or RNAase.

The staining pattern of the proteins tightly bound to DNA was observed to differ with the particular precursor used, an extra band appearing when [^{32}P]-orthophosphate was used (Figure J compared with Figure N). This band corresponds to a protein of low molecular weight (R.M., 0.98), possibly being one of the RNAase bands (R.M., 0.99)used in the isolation procedure.

3.6 Comparison of Different Nuclear Protein Fractions and the Effect of Dexamethasone on the Turnover and the Phosphorylation of the Nuclear Proteins.

Table D shows that in HTC cells the rate of L-[^{3}H]-tryotophan labelling was similar in all fractions of NHCP while the phosphorylation rate was greatest in bulk NHCP, successively less in tightly-bound NHCP and 0.35 M NaCl soluble NHCP and least in the histones which were only phosphorylated to 12 % of the extent to which the bulk NHCP were phosphorylated. The proteins tightly bound to DNA have been investigated by a number of workers. The work of Pederson & Bhorjee (1975) showed that in HeLa cells, these proteins have a higher rate of synthesis than the NHCP or histones (L-[^{3}H]-leucine as label) while they are phosphorylated at a much lower level than the NHCP or histones. In subsequent experiments, a possible reason for the discrepancy between the results presented and those of Pederson & Bhorjee (1975) came to light. (See section 3-10 on the phosphorylation of the proteins tightly bound to DNA).

The turnover rate of the NHCP was found to vary following dexamethasone stimulation. Figure K(i) shows that between 1 to 4 hours after adding hormone, there is a decrease in the turnover of the L-[^{3}H]-tryptophan labelled NHCP which returns to the control level by 12 to 24 hours. Figure K(ii) shows an increased turnover of [^{32}P]-orthophosphate labelled NHCP between 1 to 4 hours, returning to the control level at 8 hours and possibly decreasing from 12 to 24 hours. The increase in phosphorylation could arise from the fact that the turnover of the NHCP

Table D

The Specific Activity of Various Nuclear Protein Fractions and the Effect of Dexamethasone on the Specific Activities.

i The Chromosomal proteins were labelled with 250 μCi of L-[^{3}H]-tryptophan (column 1) and 1.5 mCi of [^{32}P]-orthophosphate (column 2) respectively for 1 hour (described on page 43). The results are shown as the mean ± standard error (number of samples in brackets).

ii Row 1 is the result of differing times of treatment with 5×10^{-6}M dexamethasone on the pulse labelling with 1.5 mCi of [^{32}P]-orthophosphate (described on page 43) of the 0.35 M NaCl soluble proteins (column 1) and the proteins tightly bound to the DNA (column 2).

The results are shown as the mean ± standard error (number of samples in brackets).

Table D

i

Protein Fraction	L-[^{3}H]-Tryptophan Incorporation (d.p.m./µg).	[^{32}P]-Orthophosphate Incorporation (c.p.m./µg).
NHCP	740±60(22)	530±50(24)
0.35 M NaCl Soluble Proteins	840±70(6)	130±10(9)
Proteins Tightly Bound to DNA	990±150(4)	340±10(3)
Histones	-	64±3(6)

ii

Time of Hormone (Hours)	0.35M NaCl Soluble Proteins. L-[^{3}H]-Tryptophan Incorporation. (d.p.m./µg).	Proteins Tightly Bound to DNA. L-[^{3}H]-Tryptophan Incorporation. (d.p.m./µg).
0	910±25(2)	1380
1	740±15(2)	760
24	880±230(2)	1040
	[^{32}P]-Orthophosphate Incorporation. (c.p.m./µg).	[^{32}P]-Orthophosphate Incorporation. (c.p.m./µg).
0	110	340
1	130	320
4	90	-
8	115	-
12	120	-
24	155	360

Figure K

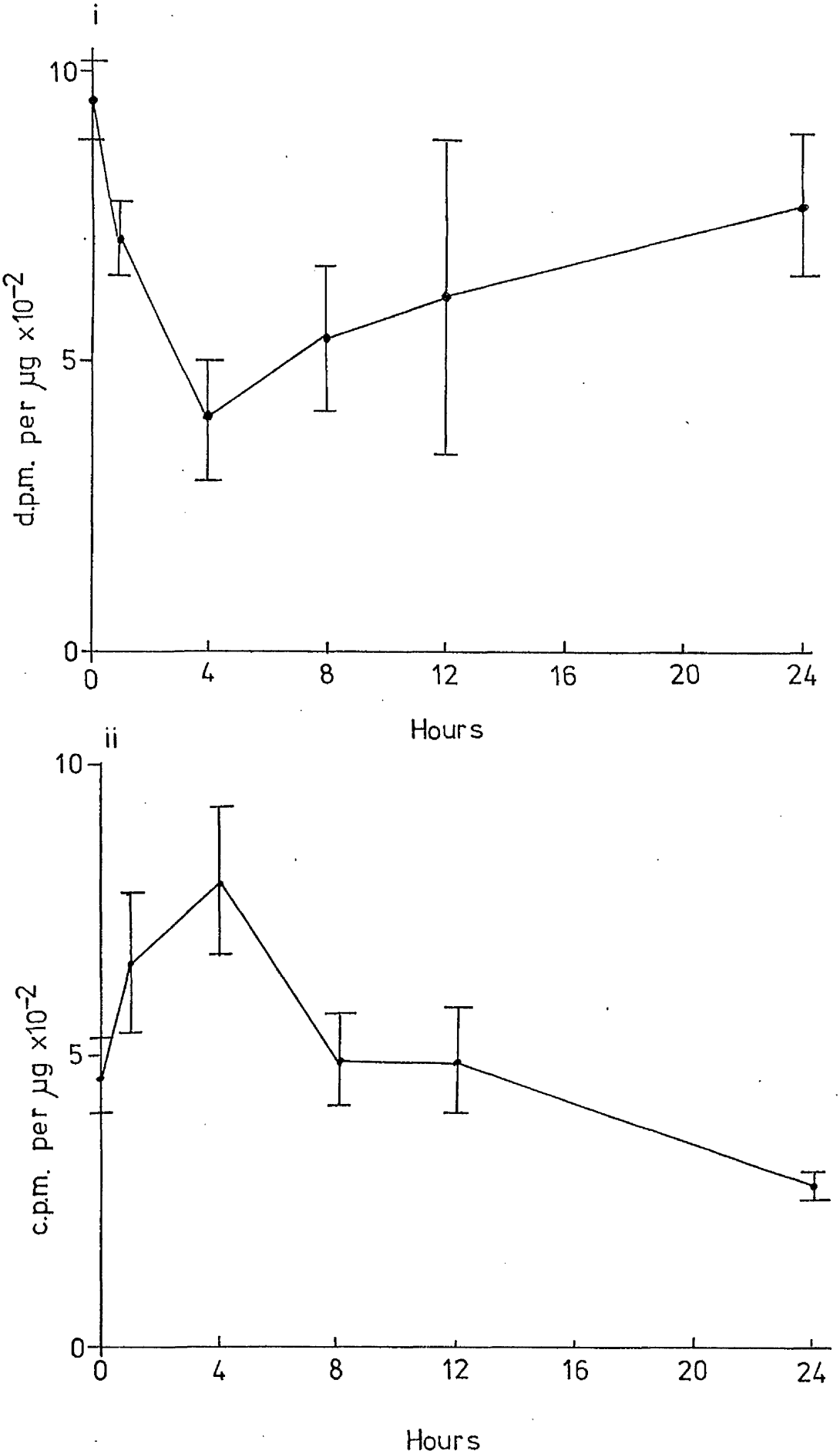
The Effect of Dexamethasone on the Turnover and Phosphorylation of the Non-Histone Chromosomal Proteins.

The time course of the alteration in the turnover (L-[^{3}H]-tryptophan) and phosphorylation ([^{32}P]-orthophosphate) (labelling as described page 43) in response to Dexamethasone (5×10^{-6}M). Each time point is shown as the mean $\pm$ standard error (4 samples per point).

(i) Labelling with L-[^{3}H]-tryptophan. The results are plotted as d.p.m./µg protein versus time.

(ii) Labelling with [^{32}P]-orthophosphate. The results are plotted as c.p.m./µg protein versus time.

Figure K


i
10
5
0
d.p.m. per µg x10⁻²
0
4
8
12
16
20
24
Hours
ii
10
5
0
c.p.m. per µg x10⁻²
0
4
8
12
16
20
24
Hours

decreases while the phosphorylation rate remains constant. Interestingly, the phosphorylation of the proteins tightly bound to the DNA and of the 0.35 M NaCl soluble proteins was unaffected by hormone (Table Dii) but the tryptophan labelling of these fractions exhibited the same trends as the bulk NHCP (Table Dii and Figure Ki).

3.7 The Effect of Dexamethasone on the Labelling with L-[^{3}H]-Tryptophan on the Bulk Non-Histone Chromosomal Proteins, 0.35 M NaCl Soluble Proteins and the Proteins Tightly Bound to DNA.

i. The tritiated amino acid labelling and the staining pattern of the bulk NHCP can be seen not to vary with hormone treatment (Figure H).

ii. The tritiated amino acid labelling and the staining pattern of the 0.35 M NaCl soluble proteins were also found to be unaltered by hormone treatment (Figure I).

iii. The staining pattern of the proteins tightly bound to DNA was found not to vary after hormone treatment (Figure J). The labelling pattern did not show individual peaks but the overall pattern can be seen to be reproducible and no differences were observed.

The turnover of the NHCP was also examined for hormone-dependent changes by double-labelling techniques (results not shown). The control cells were labelled with L-[^{14}C]-tryptophan and the hormone-treated cells were labelled with L-[^{3}H]-tryptophan. The control and hormone-treated NHCP were mixed before co-electrophoresis. After electrophoresis the gels were sliced and the ratio of tritium to radioactive carbon was calculated for each slice. This ratio remained constant in each of the fractions with sufficient radioactive material to allow accurate measurement. In the low molecular weight region of the gel, the low counts resulted in a ratio which was erratic and unrepeatable. This result substantiates the above results that hormone effects were not observed on individual NHCP turnover rates when the proteins were separated on one dimensional polyacrylamide gels.

NHCP prepared by the methods of Wilson & Spelsberg (1973) and Levy et al. (1972) gave very similar profiles of visible and radioactively labelled proteins. In both cases, hormone effects were not observed at this level of resolution.

3.8 The Effect of Dexamethasone on the Phosphorylation of the Bulk Non-Histone Chromosomal Proteins.

It can be seen when [^{32}P]-orthophosphate labelled NHCP are separated on one dimensional polyacrylamide gels, that treatment with dexamethasone results in the reduced labelling of one of the peaks (R.M.,0.19) in the labelling pattern of the NHCP (Figure L). A protein with a relative mobility of 0.19 would have an apparent molecular weight of 81,000. This alteration can be observed after 1 hour and does not alter with respect to time of hormone treatment. The general pattern of phosphorylation is reproducible and while the relative heights of the various peaks show small variations between different treatments and experiments the reduction of this peak is the only consistently observed hormone induced change at this level of resolution. The change is also observed in NHCP isolated by the method of Levy et al. (1972).

3.9 The Effects of Dexamethasone on the Phosphorylation of the 0.35 M NaCl Soluble Proteins.

The low specific activities of the 0.35 M NaCl soluble proteins resulted in a labelling pattern without any definite peaks (Figure M). The counts present in each slice were so low that a general pattern of labelling could not be seen. Some labelled material which did not stain with Coomassie Brilliant Blue R was found to migrate ahead of the bromophenol blue tracker dye. The low specific activity made analyses of any effects of the hormone impossible.

3.10 The Effect of Dexamethasone on the Phosphorylation of the Proteins Tightly Bound to DNA.

The labelling pattern of the proteins tightly bound to DNA (Figure N) can be seen to be reproducible but no differences were noted after hormone

Figure L

The Effect of Dexamethasone on the Phosphorylation of the Bulk Non-Histone Chromosomal Proteins.

The NHCP were labelled with [^{32}P]-orthophosphate (described on page 43). The concentration of dexamethasone used was 5×10^{-6}M. The three radioactivity profiles are shown as c.p.m./slice against slice number and are superimposed above each other. The arrow denotes the direction of electrophoresis.

– – – – –Control

–·–·–·–1 hour after addition of hormone

————24 hours after addition of hormone

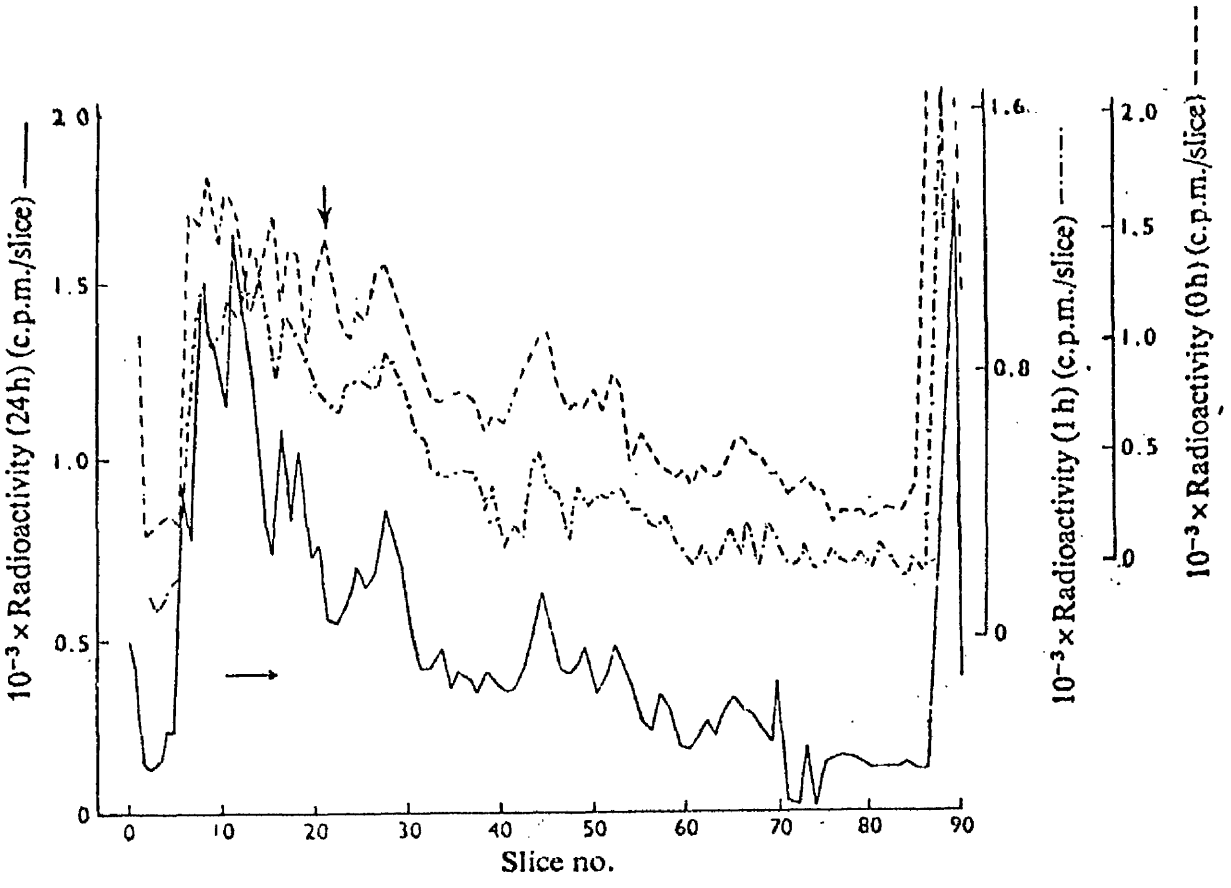

10⁻³ × Radioactivity (24 h) (c.p.m./slice) ——
10⁻³ × Radioactivity (1 h) (c.p.m./slice) -·-·-
10⁻³ × Radioactivity (0 h) (c.p.m./slice) - - - -
Slice no.
0
10
20
30
40
50
60
70
80
90
0.5
1.0
1.5
2.0
0.8
1.6

Figure M

The Effect of Dexamethasone on the [^{32}P]-Orthophosphate labelling of the 0.35 M NaCl Soluble Proteins.

The 0.35 M NaCl soluble proteins were labelled with [^{32}P]-orthophosphate (described on page 43). The concentration of dexamethasone used was 5×10^{-6}M. The top row (1) shows the pattern of stained protein; the photograph of the gel is at the bottom. The labelling pattern, (2) middle row, is shown as the amount of radioactivity per slice against slice number. The arrow denotes the direction of electrophoresis.

(a) Control, (b) 1 hour after addition of hormone, (c) 24 hours after addition of hormone.

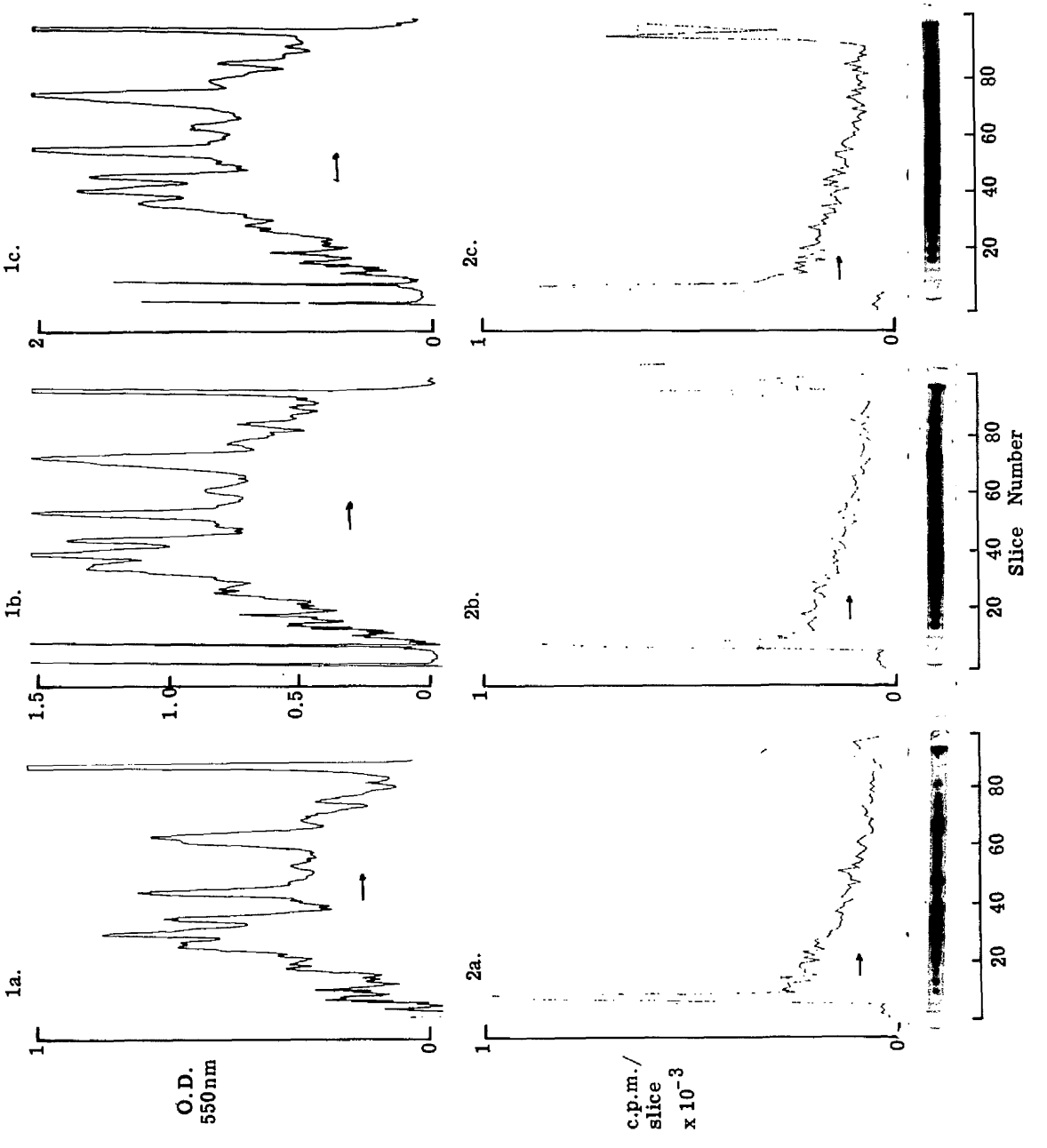
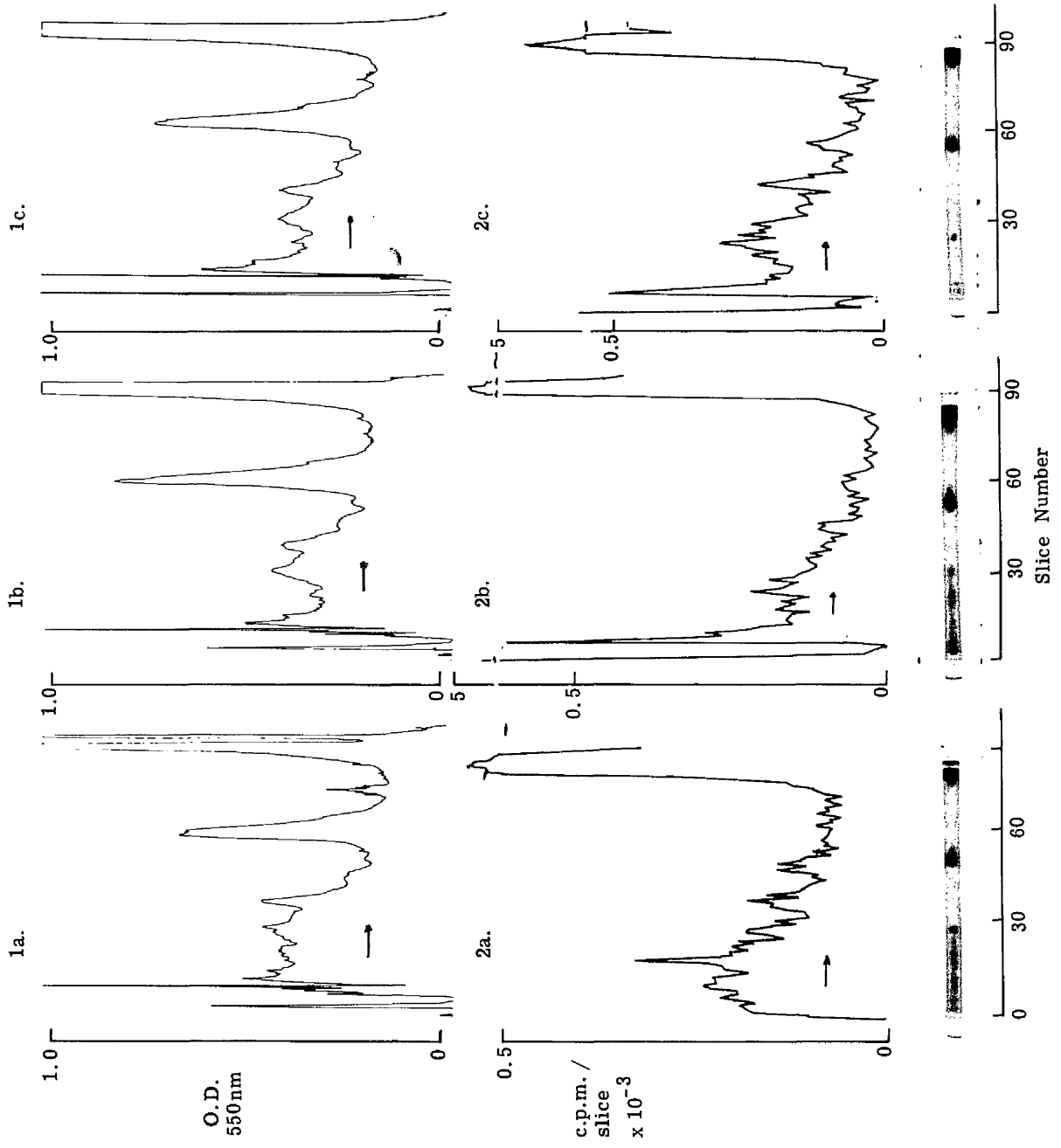

1a.
1b.
1c.
2a.
2b.
2c.
O.D.
550nm
c.p.m./
slice
x 10⁻³
Slice Number
20
40
60
80

Figure N

The Effect of Dexamethasone on the $[^{32}P]$-Orthophosphate labelling of the Proteins Tightly Bound to the DNA.

The proteins tightly bound to DNA were labelled with $[^{32}P]$-orthophosphate (described on page 43). The concentration of dexamethasone used was $5x10^{-6}$M. The top row (1) shows the pattern of stained protein; the photograph of the gel is at the bottom. The labelling pattern, (2) middle row, is shown as the amount of radioactivity per slice against slice number. The arrow denotes the direction of electrophoresis.

(a) Control, (b) 1 hour after addition of hormone, (c) 24 hours after addition of hormone.


1a.
1b.
1c.
2a.
2b.
2c.
O.D.
550nm
c.p.m./
slice
x 10⁻³
1.0
0
0.5
5
~5
30
60
90
Slice Number

treatment.

In all these experiments, contaminating RNA was degraded with RNAase to remove the high background of phosphate in RNA. Without the RNAase treatment, the [^{32}P] profile due to phosphorylated proteins was superimposed on a [^{32}P] profile due to RNA which was assumed to be similar to the pattern of labelling obtained with [^{3}H]-uridine (Figure G2a).

It has already been mentioned that the HTC cells' tightly binding proteins did not have the very low rate of phosphorylation reported by Pederson & Bhorjee (1975) (see Table D). The gel analysis of these proteins may explain this discrepancy (Figure N). Even after extensive RNAase digesting there is a considerable amount of [^{32}P] label which migrates with the tracker dye in the position of RNA and DNA degradation products. It seems highly probable that oligonucleotides of nucleic acids are contributing significantly to the specific activity of the proteins tightly bound to DNA. Pederson & Bhorjee (1975) avoided this problem by incubating for 30 min in 10 % trichloroacetic acid at 90^{o}C to remove DNA and RNA. However, Knowler & Spelsberg (unpublished observations 1976), have found that some chromatin proteins tightly bound to DNA are solubilised by hot trichloroacetic acid, so the question remains unresolved.

3.11 The Effect of Dexamethasone on the Two Dimensional Polyacrylamide Gel Electrophoresis Pattern of the Non-Histone Chromosomal Proteins.

Recent advances in the techniques of fractionation of NHCP have utilised a two dimensional system employing isoelectric focussing as well as SDS-urea polyacrylamide gel electrophoresis fractionation.

Figures O and P show the electrophoresis patterns of NHCP isolated by the procedures of Wilson & Spelsberg (1973) and of Levy et al. (1972) respectively and fractionated on the two dimensional system of Suria & Liew (1974b).

Figure O shows a comparison of the effects of hormone on the NHCP

Figure 0

Two Dimensional Polyacrylamide Gel Electrophoresis of the Phosphorylated Non-Histone Chromosomal Proteins. (Isolated by the Method of Wilson & Spelsberg (1973)).

The HTC cells were labelled with [^{32}P]-orthophosphate (described on page 43). The NHCP were separated by the method of Suria & Liew (1974b) (described on page 51) and the [^{32}P]-labelled material was detected by autoradiography (described on page 54).

The top row shows the autoradiographs of the corresponding gels in the bottom row.

(a) Control.

(b) 1 hour after addition of Dexamethasone ($5x10^{-6}$M).

(c) 24 hours after addition of Dexamethasone ($5x10^{-6}$M).

The arrow shows the difference in the staining pattern.

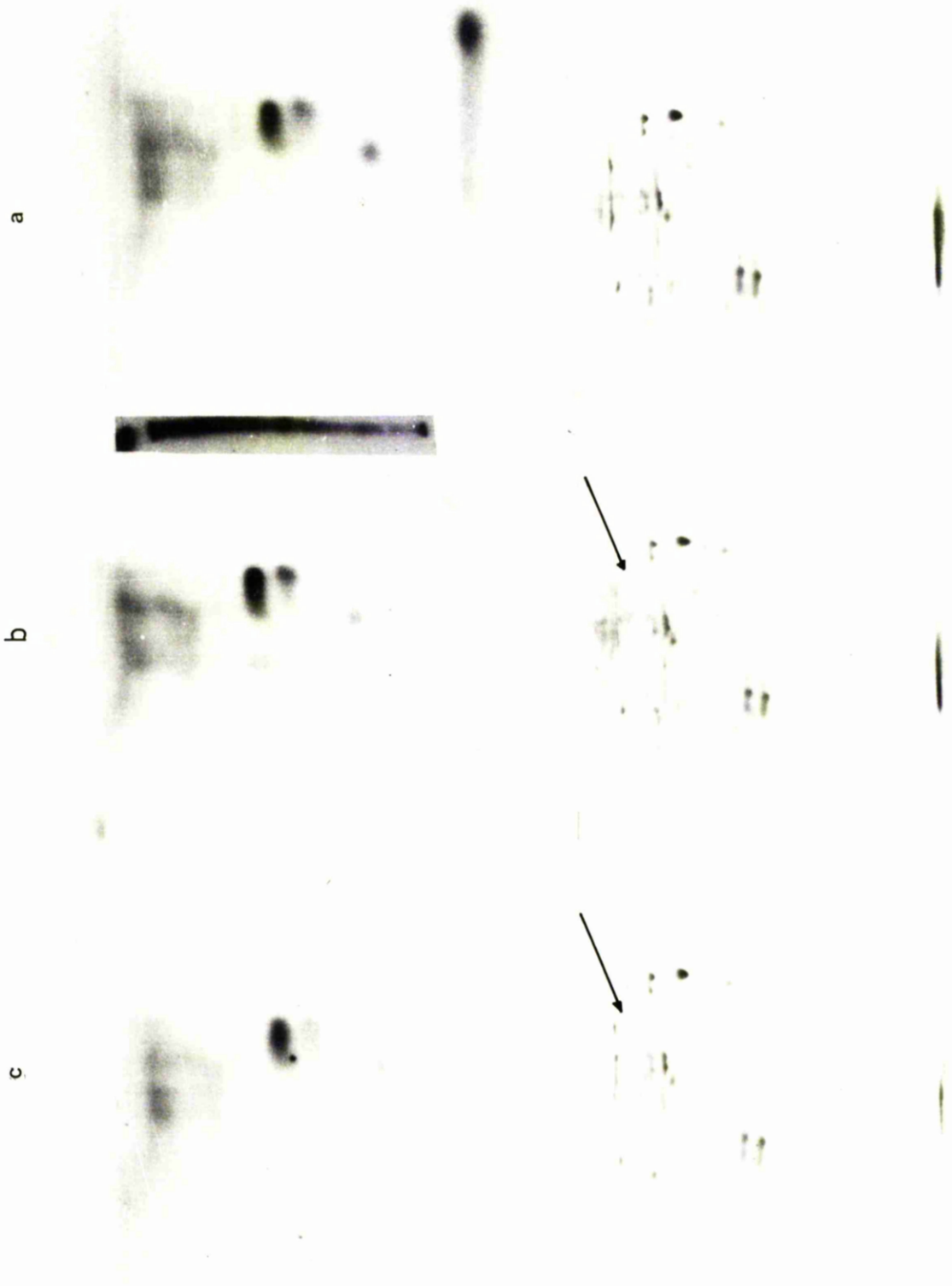
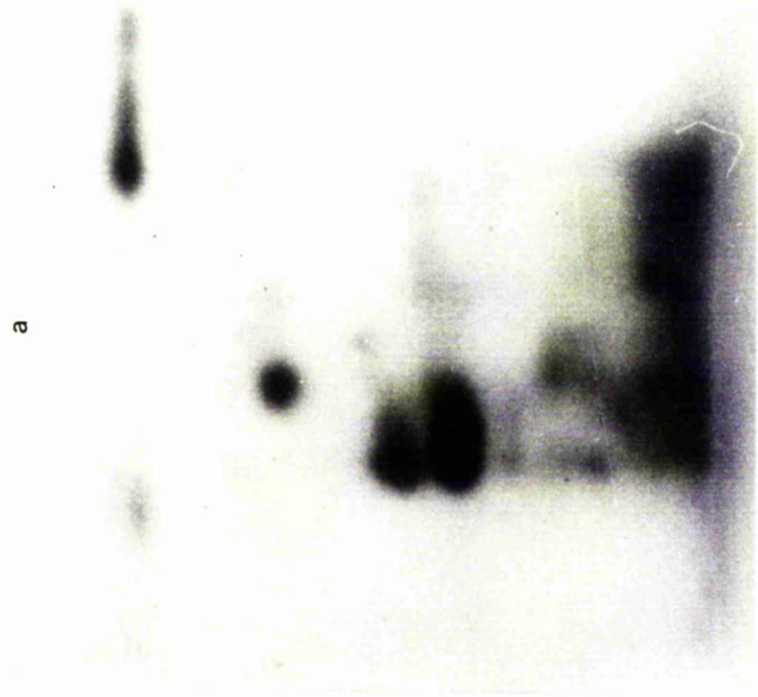

a
b
c

Figure P

Two Dimensional Polyacrylamide Gel Electrophoresis of the Phosphorylated Non-Histone Chromosomal Proteins Isolated by the Method of Levy et al. (1972).

The HTC cells were labelled with [^{32}P]-orthophosphate (described on page 43). The NHCP were separated by the method of Suria & Liew (1974b) (described on page 51) and the [^{32}P]-orthophosphate was detected by autoradiography (described on page 54).

The autoradiograph (a) and the stained gel (b) are shown side by side.



a

b

isolated by the procedure of Wilson & Spelsberg (1973). The pattern of staining shows a slight alteration at the position R.M., 0.12 and pI, 5.4 in that a new spot appears in the two hormone-treated samples. The autoradiographs show that many of the spots correspond to phosphorylated proteins but the high molecular weight phosphoproteins are not resolved and the change in the stainable proteins cannot therefore be seen. An R.M. of 0.12 corresponds to a molecular weight of 86,000.

It seems highly unlikely that hormone treatment for so short a time as 1 hour could result in the synthesis of a new protein in sufficient quantity to produce a stainable spot. However a modification of an existing protein could occur during 1 hour and might result in a change in isoelectric point and therefore its migration on isoelectric focussing gels.

Figure P shows the separation of NHCP from cells that were not treated with hormone isolated by the method of Levy et al. (1972) to see if any differences due to the method of isolation could be observed. It has been shown by Sonnenbichler & Nobis (1970) and Spelsberg et al. (1973) that acid treatment can effect the NHCP and cause differences in their immunological properties and circular dichroism. The acid extraction procedure was also found by Sonnenbichler & Nobis (1970) to result in histones becoming bound to DNA, thus becoming insoluble so that they appeared in the NHCP fraction. The differences between the NHCP isolated by the methods of Levy et al. (1972) and Wilson & Spelsberg (1973) are slight with a few extra spots above pH 8.0 and less proteins appearing in the high molecular weight range in the NHCP isolated by the technique of Levy et al. (1972).

The electrophoretic system was found to be reproducible from experiment to experiment.

3.12 The Effect of Dexamethasone on the Two Dimensional Polyacrylamide Gel Electrophoresis Pattern of L-[^{35}S]-Methionine Labelled Non-Histone Chromosomal Proteins.

Immediately after the completion of the above experiments, a method was published by O'Farrell (1975) in which total Escherichia coli proteins were labelled with either [^{14}C]-amino acids or [^{35}S]-sulphate and the isolated proteins were detected by autoradiography. To increase the resolution further the labelled proteins were detected by scintillation autoradiography by the method of Bonner & Laskey (1974). The combined systems seemed ideal to pursue the above results.

HTC cells were labelled with L-[^{35}S]-methionine, as described in the Methods section 2.7, and the NHCP were isolated by the method of Wilson & Spelsberg (1973). The chromosomal proteins' specific activities were as follows:-

Sample	d.p.m./µg (mean ± standard error (number of samples in brackets)).
Control	10,000 ± 300 (2)
Hormone-Treated	17,300 ± 2,800 (2)

Figure Q shows the scintillation autoradiograph of the NHCP from control and hormone-treated cells. The second dimension is 5 % polyacrylamide which was found to be better than 7.5 % polyacrylamide in this system. The protein pattern of Figure Q is similar to that of Figure O and P, particularly in the region between R.M. 0.19 to 0.43 and the pH range 5.25 to 5.80 (coordinates with reference to the system used in section 3.11). The alteration seen previously using the system of Suria & Liew (1974b), (R.M.,0.12; pI, 5.4, section 3.11) due to hormone, was therefore unfortunately not observed.

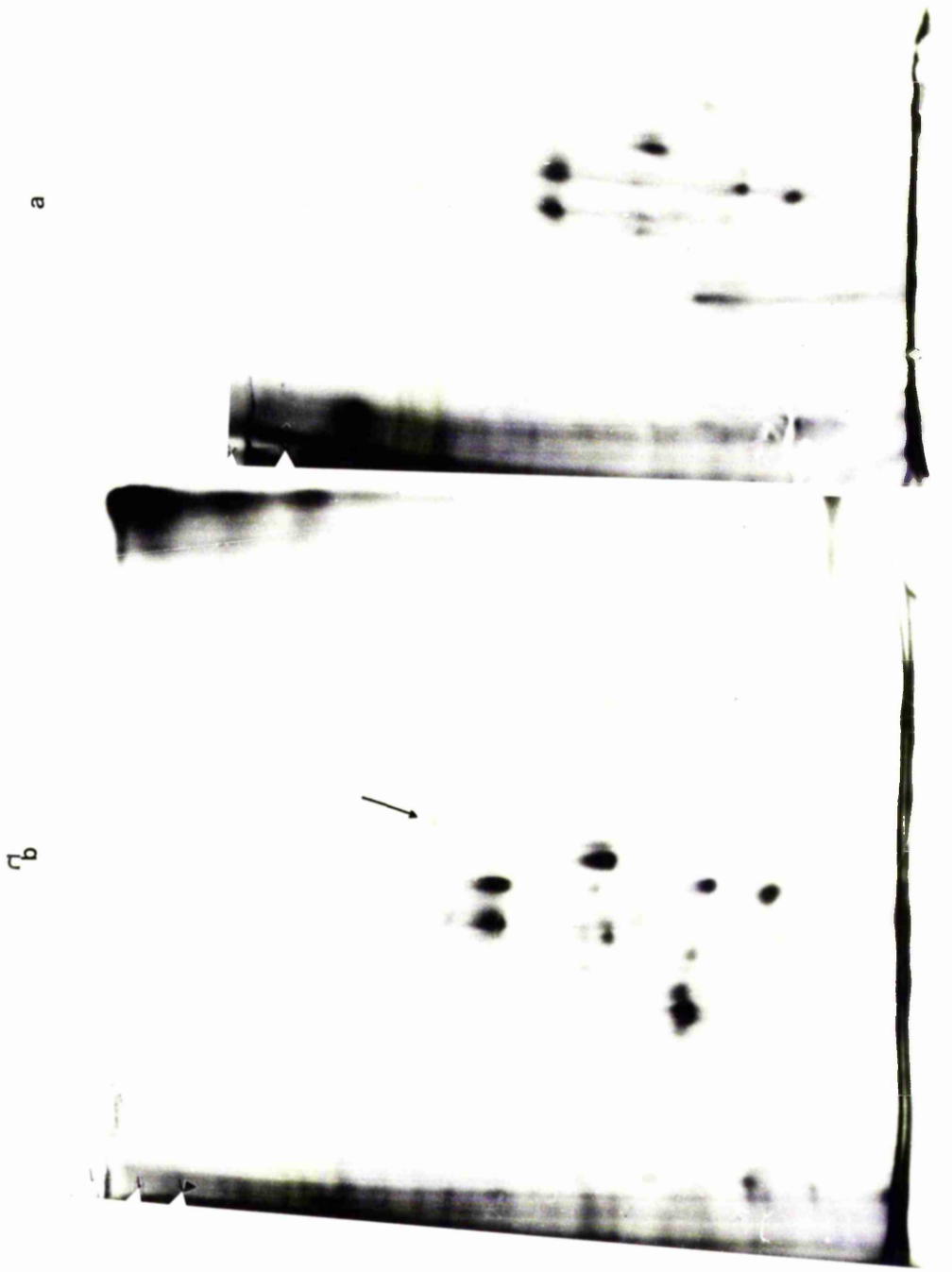
However the hormone-treated preparation, Figure Q, does show a new spot with a R.M., 0.4 and a pI, 5.7. A R.M. of 0.4 is equivalent to a molecular weight of approximately 80,000.

This electrophoretic system was found to be reproducible since this result was repeated in an identical experiment.

Figure Q

The Effect of Dexamethasone on the L-[^{35}S]-Methionine Labelling of the Non-Histone Chromosomal Proteins.

The L-[^{35}S]-Methionine labelled NHCP were separated by two dimensional polyacrylamide gel electrophoresis by the method of O'Farrell (1975) (described on page 51). Figure Q shows the scintillation autoradiographs of the NHCP isolated from cells untreated (a) and treated with 5×10^{-6}M dexamethasone (b). The arrow shows the difference between the patterns,


a
b

Discussion.

4. The HTC Cell as a Study system.

Research into chromosomal proteins over the past decade has produced a wealth of evidence indicating that at least some of the NHCP exert a controlling influence on the expression of the genome. There is a limit however to the amount of information that can be obtained from a study of the changes in the NHCP spectrum observable in differentiating and stimulated systems, unless the changes can be related to the expression of specific genes.

The HTC cell system has many advantages over more complex differentiated systems. In the HTC cell, corticosteroids induce only a small number of changes and there would be reason to hope that any differences in the spectrum of NHCP brought about by corticosteroids could be directly related to the induction of these changes. Unfortunately there are practical difficulties to this argument. It would be difficult to prove the relationship conclusively, but more importantly in a system in which one is looking for the controlling elements for a few genes, the changes may be so small as to be detectable by techniques which are currently available.

Some insight may be gained into the validity of looking for NHCP changes in the HTC cell by analogy with the Lac repressor of E.coli. Lin & Riggs (1975) have presented a calculation of the likely level of a given eukaryote repressor based on known levels of the Lac repressor in E.coli. In E.coli, there are 5 to 10 molecules of the repressor per cell (Gilbert & Müller-Hill, 1966). The Lac repressor has an affinity for both the operator DNA and the non-operator DNA sequences with a calculated ratio in vivo of 1 to 50. Lin & Riggs (1975) assumed that this situation would apply in eukaryotes and, since eukaryotes have a much greater amount of DNA, there would be a lower concentration of operator regions. They calculate that a regulator molecule would need to exist at a level of 1,500 to 15,000 molecules per nucleus. Assuming an average protein molecular weight of 80000 and 14×10^{-12} g DNA

per nucleus (Pederson, 1974a) and taking the ratio of NHCP to DNA as 0.6, one would expect such a regulator to exist as 0.02-0.002% of the NHCP.

Most models of eukaryote control envisage a multiplicity of controlling sites in the eukaryote genome and the fact that the number of steroid binding sites in the nucleus is estimated at several thousand support this concept. NHCP that complex with the steroid-receptor have been found in various systems (Spelsberg et al., 1976; Mainwaring et al., 1976) and have been termed acceptors. Different acceptor site species have been found with differing steroid-receptor affinities. Spelsberg et al. (1976) have estimated that there are 10,000 to 30,000 functional progesterone-receptor binding sites in the chick oviduct nucleus while the number of nuclear acceptor sites in HTC cells, which are polyploid, have recently been estimated at 35,000 per haploid genome (Simons et al., 1976). A recalculation of the level of the regulator NHCP in the bulk NHCP, using these figures, indicates that a single regulator might make up as much as 0.08-0.1 % of the NHCP. This figure seems high but hormones bring about profound changes in target cells and if these depend on a single regulatory protein, then this amount is perhaps not too high.

The present techniques of analyses of the NHCP have a certain limit of protein detection. The limit of detection of Coomassie Brilliant Blue R stainable protein band on one dimensional polyacrylamide gel electrophoresis is 0.4 µg (Bertolini et al., 1976) but O'Farrell (1975) estimated that 0.01 µg of stainable protein could be detected on a two dimensional polyacrylamide gel electrophoresis system. The alteration of 0.002-0.1 % would not be detectable on one dimensional polyacrylamide gel electrophoresis systems. In the two dimensional method of Suria & Liew (1974b), 250µg of protein is taken for electrophoresis, i.e. 0.002-0.02 % would comprise 0.005-0.05µg. An alteration of 0.002-0.02 % could therefore only be detected at the uppermost level of regulator stimulation

while an alteration of 0.08-0.1 % could be detected easily on a two dimensional electrophoresis system.

The incorporation of L-[^{3}H]-tryptophan into NHCP would increase the power of detection in the experimental system. An average loading of 200µg of HTC cell proteins containing approximately 15,000 d.p.m. means that 0.002-0.02% of the protein would constitute 3-30 d.p.m. This would probably still be outside the resolving power of a one dimensional gel but if the change involved as much as 0.08-0.1 % of the protein it would be possible to detect the resultant change of 120-150 d.p.m. in a single peak.

The use of L-[^{35}S]-methionine for a long period of labelling enables detection of small quantities of protein. O'Farrell (1975) estimated that 1 c.p.m. above background could be detected after 20 days of exposure if the new protein was in a region of the gel separate from the rest. Scintillation autoradiography should enhance the detection of the radioactivity, Bonner & Laskey (1974) calculated that scintillation autoradiography would be ten times more sensitive than conventional autoradiography.

The experiments described in this thesis, where the NHCP were labelled with L-[^{35}S]-methionine, indicate that after allowing for experimental losses, scintillation autoradiography would have been conducted on approximately 1.2×10^{5} d.p.m.

An alteration of 0.002-0.02 % would be equivalent to 2.3-22.5 d.p.m., assuming equal distribution of the label throughout the NHCP. This should be detectable as determined by O'Farrell (1975). If, as mentioned above, the regulator were associated with the steroid-receptor sites, the value could be as high as 90-112 d.p.m. which would be easily detectable.

Combining the methods of separation of O'Farrell (1975) and the detection of products by Bonner & Laskey (1974) thus makes the detection of a regulator NHCP quite feasible. Therefore the HTC cell system of

induction represents a potentially useful system in which to relate changes in chromosomal proteins with the induction of new cytoplasmic enzymes or structural proteins.

Non-Histone Chromosomal Protein Synthesis.

The turnover studies illustrated in Figure K show that dexamethasone has an effect on the turnover of the NHCP in a non-specific manner. These hormonal effects on NHCP synthesis and phosphorylation show different kinetics to hormone-induced enzyme synthesis. The induction of TAT by 5×10^{-6}M dexamethasone is above control level by 1 hour and the level reaches a maximum after 8 hours. Conversely, the changes in NHCP were found to alter within 1 hour of hormone treatment and the magnitude of the alteration did not change between 1 and 24 hours.

The results of the L-[^{35}S]-methionine labelling over several cell generations, in the presence and absence of hormone, indicate that some specific protein may be synthesised in response to hormone (Figure Q). However, caution is necessary since the two dimensional separation system is such that a protein modification may sufficiently alter a protein's isoelectric point so that it could migrate to a different position in the isoelectric focussing gel. Thus the result in Figure Q may represent a modification of a protein and not synthesis.

This sort of explanation seems even more plausible for the results with the two dimensional system of Suria & Liew (1974b) in which a new stainable spot of protein appears 1 hour after hormone treatment. It seems unlikely that in so short a period of time a new protein could accumulate in sufficient amounts to be detectable by staining but an existing protein could conceivably be modified.

Yet another possibility exists, namely that a protein not previously bound to DNA could become bound under the influence of dexamethasone i.e. becomes a NHCP. A precedent for this exists in the work of Benjamin & Goodman (1974) on the effect of ecdysone on the salivary gland of Scaira coprophila. They showed that ecdysone stimulates

the association of a previously synthesised protein with the chromosomes. Thus, the additional spot in Figure Q could be an extra chromosomal protein that was stimulated to complex with the HTC cells' chromosomes after dexamethasone treatment. Indeed the hormone-receptor would be just such a protein.

An apparent hormone-induced alteration of the NHCP could result from a contamination of isolated chromatin by other hormone-induced proteins. Pederson (1974a) investigated cortisol action on rat liver nucleoproteins. He found the labelling of both the acidic proteins and the heterogeneous nuclear RNA complement of ribonucleoprotein particles were increased by cortisol treatment. In his hands, however, the labelling of the acidic chromosomal proteins did not alter. Conversely, Shelton & Allfrey (1970) claimed to detect a corticosteroid-induced synthesis of a chromatin protein with a molecular weight of 40,000. Pederson (1974b), however, has shown that a protein of molecular weight of 40,000 forms a major constituent of ribonucleoprotein particles and has suggested (Pederson, 1974a) that the results of Shelton & Allfrey (1970) arise from contamination of the chromatin.

It has been reported by Dingman & Sporn (1964) that the solution 0.08 M NaCl, 0.02 M EDTA, pH 6.3, used in the preparation of the chromatin from HTC cells would remove any final traces of cytoplasmic and nucleoplasmic proteins and any loosely bound RNA. The solution should therefore overcome the problem of contamination of the chromatin by free ribonucleoprotein particles. However, my studies using [^{3}H]-uridine to label chromatin RNA indicated that a considerable quantity of nascent RNA remains complexed to the chromatin and it seems likely that protein associated with this nascent RNA would also form part of the chromatin. These arguments do not detract from the value of the observed changes because the proteins of these particles may be the elements controlling the transport of mRNA to the cytoplasm.

Non-Histone Chromosomal Protein Modification.

As outlined in the introduction to this thesis, modification of NHCP can take many forms. Some preliminary analyses were made of the glycosylation of the HTC cells' NHCP by using the staining techniques of Fairbanks et al. (1971) and Wood & Sarinana (1975) and by analysing the products after the incorporation of L-[^{3}H]-fucose. The results (not shown) were that L-[^{3}H]-fucose could be incorporated into the isolated NHCP, although the amount detected by one dimensional electrophoresis was insufficient to determine which, if any, of the NHCP were glycoproteins. Both of the glycoprotein staining techniques gave negative results but they were designed to monitor glycoproteins with a high proportion of carbohydrate.

Another modification investigated was acetylation. NHCP were labelled with [^{3}H]-acetate for 1 hour, isolated and the specific activity of the incorporated tritium determined. It was found that dexamethasone treatment of the cells caused a variation in the specific activity which was very similar to that seen after labelling with L-[^{3}H]-tryptophan, i.e. a reduction after 1 hour of hormone treatment and a return to almost control level by 24 hours of hormone treatment in both the NHCP and the 0.35 M NaCl soluble proteins. Due to the difficulty of distinguishing between acetylation and the label incorporated as amino acids synthesised in part from [^{3}H]-acetate, acetylation studies were not continued.

The only protein modification that produced positive results was phosphorylation. A small but reproducible change was found in the bulk NHCP, separated on one dimensional electrophoresis gels, in that the degree of [^{32}P]-orthophosphate incorporation into one peak was significantly reduced in response to dexamethasone. As previously discussed, there was no alteration in either the staining or labelling (L-[^{3}H]-tryptophan) pattern of the NHCP indicating that there was no dramatic change in the protein species isolated. The decrease in phosphorylation was between 12 and 22%. In the two dimensional electrophoresis system (Suria & Liew, 1974b), an extra stainable spot was found to occur but due to the fact that the new spot was located in the region of the gel where the [^{32}P]-

orthophosphate labelled material caused an overall blackening of the autoradiograph, i.e. at the higher molecular weight (protein) range, it was impossible to tell whether the new spot was phosphorylated or not. It is therefore not known whether there is any relationship between the alteration of phosphorylation on the one dimensional system and the appearance of the spot on the two dimensional system. It would be desirable to extend the study of the phosphorylation of the HTC cells' NHCP on the more sensitive system of O'Farrell (1975).

Assuming that the dexamethasone induced regulator is a single phosphorylated protein, one can calculate the likelihood of detection as before i.e. assuming a change of either 0.002-0.02 % or 0.08-0.1 %. The change in the NHCP on a one dimensional gel system in which 200µg are electrophoresed with a specific activity of 550 c.p.m./µg (Table D), would be an extra 2-22 c.p.m. in the first instance and 88-110 c.p.m. in the second case. The only change that would be detectable on a one dimensional system would be the latter. Using the two dimensional system with the [^{32}P]-orthophosphate labelled material detectable by autoradiography, even the lower estimate of 2-22 c.p.m. above background should be detectable, as determined by O'Farrell (1975). These calculations assume that the regulator would be well separated from the other proteins.

Many laboratories have tried to fractionate the NHCP and this has proved a difficult problem. The work in this thesis has centred on three fractions; bulk NHCP, a 0.35 M NaCl extract and a fraction of proteins tightly bound to DNA.

1). Bulk NHCP.

This fraction constitutes the bulk of the chromosomal non-histone proteins and has been prepared both by dissociation from chromatin and as a residue after extraction and digestion of the histones and the DNA. Both preparations are similar, differing only in their content of basic proteins and high molecular weight proteins (Figures O and P). Most of the work in the literature to date on NHCP has been a study of this fraction and it is

in this fraction that most of the changes have been found, namely:-

i. A non-specific alteration in the turnover (L-[^{3}H]-tryptophan labelling) and phosphorylation.

ii. A diminished peak of phosphorylation on a one dimensional electrophoresis system of a protein of molecular weight of approximately 80,000.

iii. A new stainable spot on a two dimensional fractionation.

iv. A new L-[^{35}S]-methionine labelled spot on a two dimensional fractionation.

2) 0.35 M NaCl Soluble proteins.

This fraction is obtained during the preparation of chromatin and its identity as chromosomal protein is controversial. The 0.35 M NaCl extract is supposed to remove contaminating extra-chromosomal proteins. The work of Johns & Forrester (1969) showed that cytoplasmic and nucleoplasmic proteins could associate with chromatin in 0.14 M NaCl but they were extractable in 0.3 M NaCl. However there is a danger in this extraction. It seems highly likely that proteins controlling gene expression, like the Lac repressor, exist in equilibrium between being bound to the operator and either not bound or weakly bound to non-specific sites. Indeed the receptor proteins which transport steroid hormones to the nucleus and help them bind to the chromatin are an example. It has been found by Beato et al. (1973) and Higgins et al. (1973) that 0.35-0.4 M NaCl would give the maximal release of glucocorticoid-receptor from chromatin.

It seems likely, therefore, that the 0.35 M NaCl solubilised proteins, while undoubtably containing contaminants, may also contain important controlling proteins. Fujitnai & Holoubek (1973) demonstrated a similarity in the banding pattern between the NMCP and the 0.35 M NaCl soluble proteins on polyacrylamide gel electrophoresis. This finding supports the idea of the possible distribution of the chromosomal proteins between the two fractions.

The removal of the saline-soluble proteins has been shown to cause functional alterations in the chromatin. The differences between quiescent WI 38 cells' chromatin and chromatin isolated from cells stimulated to proliferate has been shown to be removed following saline extraction (Nicolini et al., 1975). The differences included chromatin template activity, circular dichroism spectra and the binding of ethidium bromide.

The bulk NHCP and the 0.35 M NaCl soluble proteins from HTC cell chromatin gave staining patterns on one dimensional polyacylamide gel electro-phoresis that included bands common to both but the NHCP tended to have more protein species in the higher molecular weight range. After labelling the two fractions (NHCP and 0.35 M NaCl soluble proteins) with precusors, L-[^{3}H]-tryptophan labelling was similar while labelling with [^{32}P]-orthophosphate showed differences. The 0.35 M NaCl soluble proteins were not phosphorylated to the same level as the cytoplasmic proteins (result not shown). This indicates that the 0.35 M NaCl soluble proteins are not identical with either the cytoplasmic proteins or the NHCP, although some cross contamination between fractions is likely and some proteins are likely to be common to both fractions.

In the light of the above observations, it was decided to perform the 0.35 M NaCl extraction in the preparation of "clean" chromatin and to retain it for subsequent analysis. Some turnover differences due to dexamethasone were detected in this fraction but no specific hormone-induced change was seen. However, this does not detract from the possible importance of this group of proteins.

3) Proteins tightly bound to DNA.

Notwithstanding the above argument, there is also reason to believe that some controlling proteins may reside in the fraction most tightly bound to the DNA. Tissue differences in the NHCP appear to reside principally in the proteins tightly bound to DNA (Wakabayaski et al., (1974); Chiu et al., 1975b). Furthermore, recent work on an

acceptor protein in chick oviduct chromatin which complexes with the progesterone-receptor, reveals that the acceptor is tightly bound being released by 7M but not by 5M guanidine HCl (Spelsberg et al., 1976). Conversely, work by Pederson & Bhorjee (1975) demonstrated that the proteins tightly bound to DNA were associated with the template-inactive region of the genome.

The work in this thesis with the fraction which remains bound to DNA in 6M urea, 0.4 M guanidine HCl, 0.1 M sodium phosphate, pH 7.0, 0.1 % ($^{v}/v$) β-mercaptoethanol has been somewhat discouraging. These proteins appear to have such a low turnover that it is very difficult to label them sufficiently for analysis and it seems unlikely that such apparently stable proteins could be associated with something as transient as a hormone-induced enzyme synthesis.

The role of the hormone-receptor complex in gene activation.

The major unresolved question on the molecular mechanism of steroid hormone action concerns the events between the time of the nuclear binding of the hormone-receptor complex to the chromosome and the resultant stimulation of protein synthesis. Recently, it has been shown that the induction of conalbumin and ovalbumin in chick oviduct by oestrogen (Palmiter et al., 1976) shows a similarity to the induction of "puffs" in the salivary gland polytene chromosomes in insects by ecdysone (Ashburner, 1974). Palmiter et al. (1976) showed the chromosomal binding by the oestrogen-receptor complex to be saturated within 15 min of oestrogen administration. Before the oestrogenic induction of conalbumin there was a period of only 30 min before the accumulation of conalbumin mRNA whereas there was a lag of 3 hours before the induction of ovalbumin. This is analogous to the early and late "puffs" of the polytene chromosome. Each worker has put forward a different hypothesis to account for the time lag between binding of the hormone-receptor complex and appearance of the specific mRNA.

Palmiter et al. (1976) propose that the hormone-receptor complex

interacts firstly with non-hormone regulated DNA regions of the genome, after which the hormone-receptor complex moves to a region adjacent to the specific hormone activated gene before translocating to the initiation site of the gene and promoting its transcription. The lag period is assumed to be associated with the events from binding through translocation to the initiation of transcription. Those proteins induced with no lag period are assumed not to require an intermediate and the translocation process is almost immediate.

Ashburner (1974) has assumed, after protein synthesis inhibitor studies, that a protein intermediate is required for the production of the late "puffs", whereas Palmiter et al. (1976), also using protein synthesis inhibitors, have deduced that the intermediate, if any, is non-protein in nature. The protein intermediate is the essential difference between Ashburner's (1974) model and that of Palmiter et al. (1976) model.

A recent publication by Schwartz et al. (1976) has shown, in vitro, that the hormone-receptor complex can stimulate the number of initiation sites for RNA polymerase in chromatin isolated from hormonally withdrawn chick oviducts. As the work monitored only initiation, these stimulated genes could be of proteins that arise without any lag period but it does conform with the theory that some hormonally-induced products do not require an intermediate to be either synthesised or stimulated to complex with the chromatin.

The role of the nuclear proteins in the chick oviduct has been investigated by a number of workers. It has been found by Tsai et al. (1976), by reconstitution experiments with chromatin isolated from chick oviduct, that proteins of the bulk NHCP are responsible for directing the synthesis of the oestrogen induced ovalbumin mRNA. Spelsberg et al. (1976) have found a nuclear acceptor protein fraction isolated from chick oviduct chromatin that binds the progesterone-receptor complex to DNA. The work of Palmiter et al. (1976) also showed that during the 3 hours after oestrogen administration no alterations in the stainable pattern,

a double-label electrophoresis pattern, or the phosphorylation of the proteins, could be detected although they do acknowledge that the proteins were probably below the level of detection.

The possibility exists that a differentiated target cell has two types of controlling mechanisms at the genomic level, depending on whether the hormone induction has a lag period or not. Where there is no lag period, the chromatin may contain pre-existing protein or RNA species that allow it to respond to the hormone without the appearance of any new controlling elements. If there is a lag period before induction starts, there might be, as indicated from the work of Palmiter et al. (1976) and Ashburner (1974), a requirement for the synthesis of an intermediate. The candidate for the intermediate could either be a protein or a nuclear RNA species.

The merits of the HTC cell as a system in which to study the role of NHCP in Hormone Action.

It has been demonstrated by various types of experimentation that the NHCP contain controlling agents of transcription. Whether the hormone-receptor complex has to be included as a true regulator still has to be determined. Furthermore, the differences between the regulation of induced proteins with or without lag periods require further analysis.

The majority of NHCP are obviously structural components and enzymes. The regulators are probably only detectable using the most sensitive methods presently available and under the most favourable conditions. The alterations found during this work could be alterations of a more gross kind, such as changes in structural or enzymic NHCP, required for the activation process by glucocorticoids. Further examination of the nuclear protein fractions by the two dimensional system of O'Farrell (1975) combined with the amplification technique of scintillation autoradiography of Bonner & Laskey (1974) would be worthwhile, particularly if the analysis could be done on a smaller

number of proteins produced by preliminary fractionation. It would also be worthwhile to look for specific proteins of the kind recently described by Spelsberg *et al.* (1976) in the HTC cell system. They have found, not only an acceptor protein for the progesterone-receptor complex but also a tissue-specific group of proteins which contain elements which can repress acceptor activity. Mainwaring *et al.* (1976) and Stefanowicz *et al.* (1976) have also described acceptor protein fractions in androgen-sensitive tissues and the *in vitro* advantages of the HTC system should make this a good candidate for looking for glucocorticoid acceptor proteins.

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