CHARACTERIZATION AND BIOSYNTHESIS OF HISTONES IN CULTURED CELLS OF *ACER PSEUDOPLATANUS* L.

by

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This thesis is my own work unless otherwise acknowledged and has at no time been submitted for another degree.

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I certify that this statement is correct.

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CONTENTS

ABBREVIATIONS

BPB	bromophenol blue
Bisacrylamide	N, N'-methylenebisacrylamide
cAMP	cyclic AMP (cyclic adenosine monophosphate)
CHO	Chinese hamster's ovary
conc.	concentration(s)
cpm	counts per minute
2,4-D	2,4-dichlorophenoxyacetic acid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNP	deoxyribonucleoprotein
EB	ethidium bromide
EDTA	ethylenediaminotetra-acetic acid
HTC	hepatoma tissue culture
^{3}H-dT	^{3}H-thymidine
^{3}H-U	^{3}H-uridine
^{3}H-lys	^{3}H-lysine
^{3}H-trp	^{3}H-tryptophan
NHP	nonhistone protein
PCA	perchloric acid
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate = sodium lauryl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylenediamine
Tris	2-amino-2-hydroxylmethyl-propan-1,3-diol

ABSTRACT

A new technique has been developed for the isolation of nuclei from suspension culture cells of Acer pseudoplatanus L. This technique involves the use of a glycerol-containing grinding medium at -20 °C. The whole isolation process is simple and consistently produces about 20% nuclear yield with reasonable purity.

Histone extraction from this nuclear fraction by the H_2SO_4-EtOH method is superior to other methods examined. The extracted Acer histones exhibit a typical histone pattern on polyacrylamide gels, and the major fractions are identified as H1, H2A, H2B, H3 and H4 (in sequence from the anode to the cathode end on the gel); their molecular weights are respectively 24,500, 13,500, 13,300, 12,800 and 11,000. Both the H3 and H4 histones of Acer cells are identical with those of calf thymus in terms of their mobilities on acid-urea and SDS gels. Identification of the Acer histone fractions has been assisted by a newly developed differential staining method which stains the 5 major histone fractions of calf thymus in 5 different colours.

The total extracted Acer histone fraction contains 22% of basic amino acids, and the ratio of lysine to arginine is 2.6 which is higher than that for calf thymus and is probably due to the low content of arginine in Acer H2A.

The Acer histones have been shown to be synthesized in the cytoplasm and then transported into the nucleus. The synthesis occurs throughout the cell cycle but reachs its maximum rate while DNA is being synthesized. Histone samples obtained from both asynchronous and synchronous cultures at intervals during the progress of their growth show uniform electrophoretic patterns on the gels, suggesting that the Acer histones are generally homogeneous and that any modification of

the histones probably affects only a very small proportion of the total histones. The possible existence of such modified histone derivatives and their functions remains to be investigated. Directions along which the present studies could be developed are discussed.

CHAPTER 1

The Components of Chromatin and Their Possible Functions

1. The chromatin
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4. Gene expression — functions of unmodified histones and non-histone proteins
5. The relationship between chromosomal structure and function
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1. The chromatin

In eukaryotes, the primary genetic material, the deoxyribonucleic acid (DNA), is largely contained in the nucleus. The length of this DNA is enomous relative to the size of cell nucleus and it is therefore thought that the DNA molecules must be contracted and packed into some kind of structure rather than 'naked'. Both cytologists and biochemists have produced evidence for the presence of deoxyribonucleoprotein (DNP) in the nucleus. It is this DNP which is known as chromatin. The composition of the DNP varies with the cell type and its cycle stage. Metaphase chromosomes contain 13-17% DNA, 8-15% RNA and 68-79% protein, whereas chromatin isolated from the interphase cell contains less RNA (3-4%), proportionately more DNA (25%) and about 70% protein (Comings, 1972).

When chromatin of an interphase cell is observed by light microscopy following the application of special staining techniques (Comings, 1972), or is examined in the electron microscope (Littau et al., 1964; Huxley and Zubay, 1961), it appears to be differentiated into two distinguishable forms. The dense clumps of compact fibrils constitute condensed (or hetero-) chromatin; the diffuse regions of loosely extended filaments constitute diffuse (extended or eu-) chromatin. As the cell proceeds to metaphase, the structure of the chromatin becomes more condensed than interphase heterochromatin. The chromatin is organized to form chromosomes, through which the genetic materials are equally distributed into the daughter cells.

The macromolecules which are associated with DNA in chromatin are histone proteins, nuclear acidic proteins and a variety of ribonucleic acids (RNAs). The nuclear acidic proteins include enzymes such as DNA-dependent RNA polymerase, and non-histone protein (NHP) considered

to be involved in maintaining intranuclear structure. Native chromatin contains, relative to histones and DNA, only small amounts of NHP and RNA. Although heterochromatin and euchromatin do not differ greatly in their histone to DNA ratios (Comings, 1967; Pallotta et al., 1970), much higher proportions of acidic proteins (Alfert, 1958; Himes, 1967; Himes et al., 1969) and phosphoproteins (Kleinsmith et al., 1966) are found in euchromatin than in heterochromatin. In general, chromatin contains approximately equal amounts of histones and DNA by weight. By contrast, the content of NHP and RNA molecules varies considerably according to the cell type and tissue source; metabolically inactive cells contain less, while active cells contain more.

2. General properties of histones

Histones are basic nuclear proteins with a high content of the basic amino acids lysine and arginine. They are bound to the DNA in the nucleus of most eukaryotic somatic cells. According to their amino acid compositions, the five common histone fractions are : very lysine-rich histone (H1), slightly lysine-rich histone (H2B), arginine- and lysine-rich histone (H2A), arginine-rich histone (H3) and glycine- and arginine-rich histone (H4). The major properties of these fractions are well characterized in animal tissues. Table 1 illustrates some properties of each individual histone fraction as well as the nomenclature systems used for histone classification.

The amino acid sequences of histones have been studied by several researchers. The complete sequences of histones H4 and H3 have been determined in various tissues, and the results demonstrated the sequences to be remarkably conserved through evolution (DeLange et al., 1969a, 1969b; Ogawa et al., 1969; Sautière et al., 1971a, 1971b). When the sequence of H4 from pea seedling was compared with that of calf thymus

Table 1. Nomenclature of histones and some properties of histone fractions.

Main histone fractions	very lysine-rich	arginine- and lysine-rich	slightly lysine-rich	arginine-rich	glycine- and arginine-rich
Nomenclature commonly used	VLR F1 I	AL F2a2 IIb1	SLR F2b IIb2	AR F3 III	GAR F2a1 IV
Nomenclature used in this text *1	H1	H2A	H2B	H3	H4
Molecular weight *2	19,500 21,000	12,500	14,000	14,000	11,000
Approximate number of amino acid residues	212	129	125	135	102
Moles of lysine (%) *3	26.8	10.2	16.0	10.0	10.8
Moles of arginine (%)	1.8	9.4	6.4	13.3	13.7
Lysine to arginine ratio	15.0	1.1	2.5	0.8	0.8
Basic to acidic ratio	4.6	1.4	1.9	1.6	2.5

*1. It was also the nomenclature system accepted by the participants of the Ciba Foundation Symposium in 1974.

2. data of Panyim and Chalkley (1971).

3. data of amino acid compositions are those of Johns (1971).

(DeLange et al., 1969a) only two conservative amino acid substitutions were found : an isoleucine substituted for a valine at position 60 and an arginine for a lysine at residue 77. Similary, the sequence of the H3 histone from chicken erythrocytes is highly homologous to the calf thymus H3 with one cysteine replaced by a serine residue and only one or two other substitutions (DeLange et al., 1972, 1973; Brandt and Van Holt, 1972). In carp testis H3, one cysteine residue is replaced by a serine (Hooper et al., 1973). The sequence is identical to calf thymus H3 at all other positions. Histones H2A, H2B, and H1 are less conservative than H3 and H4. A comparative study of the amino acid sequence of H2A in four animal species (calf, rat, trout and sea urchin) showed most of the sequences to be conserved; the differences were located principally in the NH_2-terminal and COOH-terminal part of the molecules (Sautière et al., 1975). Histone H2B has little sequence homology to H4 (Iwai et al., 1970); however, in both sequences, there is clustering of basic residues and the centre of the molecule is largely hydrophobic. The variation of histone H2B in mammals was found to be located in the hydrophobic region (Franklin and Zweidler, 1977). Histone H1 is the most variable fraction among all the histones. It shows species-specific and tissue-specific differences (Kinkade, 1969; Bustin and Cole, 1968). Histone H1 from most sources was also found to be composed of subfractions (Kinkade and Cole, 1966a, 1966b), some of which result from side-chain phosphorylation (Balhorn et al., 1972; DeLange and Smith, 1971), some from sequence changes (Rall and Cole, 1971). The basic residues of histone H1 are unevenly distributed. The COOH-terminal region, which consists largely of lysyl, alanyl and prolyl residues, is the most basic region, whereas a less basic region is located near to the NH_2-terminal. In general, histone proteins contain two well

defined segments, (i) basic segments (basic regions) which are the sites that interact with DNA through electrostatic ionic bonds to form the primary structure of histone-DNA complex; (ii) hydrophobic segments (apolar regions) which favour histone-histone interaction, through hydrophobic bonds to form quaternary structure (Bartley and Chalkley, 1972).

In addition to the five common histone fractions, there are also unique species of histones present in some tissues. Histone H5 (F2c, V), a lysine- and serine-rich histone, was found to be erythrocyte-specific (Hnilica, 1964; Tsai and Hnicica, 1975). Histone H6 (T), a lysine-rich histone, was found in rainbow trout testis (Wigle and Dixon, 1971). Three different histone fractions were found in rat testis (Branson et al., 1975). These tissue-specific histones do not appear to replace any of the common histones, but rather occur as an additional fraction in particular cells.

3. The molecular structure of chromatin

Since histones are closely associated with DNA in chromatin, they have been thought of in connection with gene control and the stabilization of the fine structure of chromatin. During the past few years, the molecular basis of chromatin structure has been intensively studied, and the model of 'beads on a string' is now generally accepted. This concept arose from the finding that at least 50% of the DNA in chromatin is resistant to nuclease (Clark and Felsenfeld, 1971; Hewish and Burgoyne, 1973). When native chromatin was examined in the electron microscope, it appeared to show that over large regions of the sample there were globular particles (ν bodies) strung together like a row of beads (Olins and Olins, 1973, 1974). These particles were not changed

in size, histone content and the property of resistance to nuclease; the structure of chromatin is therefore proposed to be based on a repeating unit (Kornberg, 1974; Noll, 1974; Baldwin et al., 1975; Barrett, 1976; Woodcock et al., 1976a).

The isolated ν body (or nucleosome) contains about 100,000 daltons of protein together with a unit length of DNA, and is a globular structure 80-100 Å in diameter (Shaw et al., 1974). The proteins have been shown to be histones. Two molecules of H3 associate with 2 molecules of H4 to form a tetramer, and 2 molecules of H2A associate with 2 molecules of H2B to form an oligomer or a short polymer, while H1 is present as a monomer (Kornberg and Thomas, 1974; Thomas and Kornberg, 1975a, 1975b). These histone proteins except H1 interact with each other through the apolar segments to form an octameric protein core, and the basic segments interact electrostatically with DNA which is supercoiled on the outside of the unit (Baldwin et al., 1975).

The length of DNA per nucleosome varies from species to species. In higher eukaryotes, the length of DNA per nucleosome is very close to 200 base-pairs (Noll, 1974; Van Holde et al., 1974; Kornberg, 1974; Barrett, 1976). Recently, Chambon and his coworkers (Compton et al., 1976) have found that in higher eukaryotes, the nucleosomes of genetically dormant cells contain more DNA, while those of cells from actively dividing tissues contain less. In comparison with those in higher eukaryotes, the value for yeast is about 165 base-pairs (Thomas and Furber, 1976), for Aspergillus nidulans 150 base-pairs (Morris, 1976a; Goff, 1976), and for Neurospora crassa 170 base-pairs (Noll, 1976). However, in all the chromatin studied so far, it appears that the repeating unit of the portion most resistant to micrococcal nuclease always has 140 base-pairs. This is the length of DNA involved in core particles,

which do not vary among species or from tissue to tissue (Lohr et al., 1977). This core particle consists of an octameric protein core together with 140 base-pairs of DNA, and is termed the monomer. The variation in repeat length of DNA is probably attributable to the spacer (linker) region between core particles (Noll, 1976; Morris, 1976a, 1976b).

X-ray diffration studies have shown that H1 is not required for the formation of the monomer (Murray et al., 1970; Richards and Pardon, 1970). Histone H1 contains more than 25% lysine. Structurally, it consists of an apolar-rich central region, a short N-terminal end and a very long, basic C-terminal end. H1 molecules interact with one another through the apolar region, while the basic region is the main site at which they interact with chromatin-DNA (Chapman et al., 1976). It has been shown that H1 interacts with DNA at the linker regions of adjacent monomers (Lindigkeit et al., 1974; Barrett, 1976; Thomas and Kornberg, 1974; Morris, 1976a). If the size of the DNA linker between nucleosomes is related to the number of positive charges in the histones, this intercore linker region could vary because different kinds of H1 are present in different species or tissues (Morris, 1976b; Noll, 1976).

To summarize : chromatin is composed of a series of threads (or nucleofilaments); each thread is a piece of nucleohistone containing monomers (beads) strung together by spacers; within the monomer the DNA is supercoiled over a protein core containing 2 molecules of each of histones H2A, H2B, H3 and H4. In the spacer region histone H1 together with DNA plays a role as an intercore-linker. The length of DNA within this 'model' is contracted 7 fold.

The size and distribution of nucleosomes are found to be similar in extended and condensed chromatin (Bostock and Christie, 1976). This agrees with the biochemical finding that the histone-to-DNA ratio is similar in euchromatin and heterochromatin. The heterochromatin presumably

is the result of nucleofilaments that are condensed into a supercoil or solenoidal structure (Finch and Klug, 1976). Histone H1 is found to be required for the stabilization of this solenoidal structure, and it is therefore assumed that histone H1 plays an important role in maintaining the secondary configuration of chromatin.

4. Gene expression - functions of unmodified histones and non-histone proteins

The genetic information of the living cells is expressed by the mechanism of transcription and translation. The DNA-coded information is transcribed into RNA, and then translated to synthesize specific proteins. The property of the specific protein is determined by the information carried by the RNA molecule, which is transcribed from differential regions of DNA according to the cell type, cell-cycle stage and the phase of development. It is mainly due to the presence of such differential gene activity that individual living organisms can express their specific characters.

When the DNA contents in eukaryotes are compared with those in prokaryotes it is found that eukaryotes contain a dramatically larger amount, and this amount is not correlated with the evolutional complexity. For example , Euglena has almost as much DNA as man, and the lowly liverwort has 18 times our complement of DNA. Therefore, it is likely that some of the DNA in the chromatin must be present as excess (Comings, 1972), and each individual cell achieves in its own way the suppression of the transcription of such unused DNA.

In 1950, Stedman and Stedman first suggested that differential gene expression of eukaryotes may be regulated by histones through their masking the chromatin DNA. Huang and Bonner (1962) indicated that DNA

which is fully complexed with histone is inactive in supporting DNA-dependent RNA synthesis. By adding the histone fraction to the DNA *in vitro* or removing it from isolated nuclei, they concluded that histones suppress the template activity of DNA in native chromatin. A similar experiment was performed on calf thymus lymphocytes by Allfrey *et al.* (1963). They came to the same conclusion about the inhibitory activity of histone, and supported the view that the function of the histones in chromatin is to restrict those genes designated to be inactive. 80% of the total DNA in the native chromatin of pea embryo lacks template activity (Huang and Bonner, 1962); similarly 90-95% of the DNA in calf thymus lymphocyte is not transcribable (Paul and Gilmour, 1966a). These restrictions of template availability are found to be tissue or organ-specific (Paul and Gilmour, 1966b; Bonner *et al.*, 1968; Smith *et al.*, 1969; Spelsberg and Hnilica, 1970; Barrett *et al.*, 1974). When the histones were added to the pure DNA, it suppressed all ~~its~~ this template activity, ~~while~~ and if the histones were added back to dehistoned chromatin, the template activity of this reconstituted chromatin was similar to the native chromatin. These results suggest that histones are essential for restriction of DNA quantitatively, but there is no evidence that their repression is tissue-specific. It is likely that the other macro-molecules remaining in the dehistoned chromatin are actually participating the direction of specificity (Gilmour and Paul, 1969; Spelsberg *et al.*, 1971). Both non-histone proteins (Gilmour and Paul, 1970; Spelsberg and Hnilica, 1971) and RNA molecules (Huang and Huang, 1969) have been reported to be necessary for the expression of organ or tissue-specific restriction. In general, histones because of their small number, common features of structure, and relatively limited heterogeneity, are considered to be primarily structural proteins, although the possibility that they may have special or general regulatory functions can not be ruled out.

In contrast to the histone proteins, there are many more varieties of non-histone proteins in the chromatin. The species and quantity of non-histone proteins in chromatin differ considerably with species, with tissue, and with cell cycle stage. Changes of non-histone proteins have been observed during cell development and proliferation (Bhorjee and Pederson, 1972; Gregor et al., 1974; Newman et al., 1976). Recently, Yoshida and Sasaki (1977) have reported on an investigation of chromatin template activity during germination of wheat embryos. They found that two species of NHP decreased in parallel with the increase of template activity, while no change was observed in the number of histone components. Park et al. (1976) studied the in vitro transcriptional activity of chromatin from HeLa cells, and found that S-phase chromosomal proteins enhanced the specific transcription of histone genes on G1-phase chromatin. In a fractionation and reconstitution experiment, they obtained evidence that S-phase chromatin contains a non-histone protein which has the ability to render the histone genes available for transcription. The difference of gene expression in different cycle stages is due to the absence of the NHP in G1-phase chromatin rather than the presence of some inhibitor molecule in G1-phase. Therefore, it is likely that the general transcriptional control mechanism is accomplished by histone-non-histone protein interaction.

In studies on erythrocyte and reticulocyte chromatin, Gadski and Chae (1976) dissociated the chromatin in 2M NaCl-5M urea, and analyzed the resultant reconstituted chromatin on acid-urea polyacrylamide gels to determine which histones had bound to the DNA, as well as on SDS-polyacrylamide gels to determine which of the NHPs had bound. They found that NHPs possess a wide range of binding characteristics. Those which bind to free DNA are generally of low molecular weight and do not show species specificity, while others which require histones for complete

reassociation, must bind to the chromatin complex, and may function to direct the specificity. It is hoped that a wide range survey of this kind will give clues as to which of the NHPs are responsible for controlling the specificity of transcription, and how they accomplish this function.

5. Relationship between chromosomal structure and function

There are a number of observations which attest that the physical state of the chromatin correlates closely with its biosynthetic activity. The giant chromosomes of the salivary glands and other tissues of Dipteran insects possess certain segments which undergo a characteristic 'puffing' at certain stages during development (Beermann, 1959). The 'puffs' represent swellings of those chromosomal regions which are the sites of most intensive RNA synthesis (Pelling, 1964). Another remarkable instance is the 'lampbrush' chromosome. Such chromosomes are characterized by the presence of DNA-containing lateral loops. They occur in the oocytes of a wide variety of animals, and in the spermatocytes of certain insects. Autoradiographic experiments using ^{3}H-uridine have shown that the loops are sites of intensive RNA synthesis (Gall and Callan, 1962). Both 'puffing' and 'lampbrush' formation involve a conformational change, from a condensed structure to an extended structure, and it is the extended regions which are the sites of intensive RNA synthesis. A similar situation is also found in interphase chromatin of mammalian cells. Following the incorporation of ^{3}H-uridine, the autoradiographic results showed that heavy grains were localized over euchromatin, and very few grains were localized over heterochromatin (Littau et al., 1964). Since both euchromatin and heterochromatin are based on the same chromatin subunits but just differ in the second level conformation, it seems that the structural modification is correlated with the biological function of chromatin.

6. Histone modifications and their possible functions

Although the amino acid sequences of the histones are extremely conserved, all histone proteins are subjected to modification by side-chain substitutions. These substitutions result from phosphorylation, acetylation, methylation, thiolation and ribosylation. Substitutions appear to be on the conserved regions of the histones and at specific sites. Different kinds of substitution can occur in the same histone, and the same kind of substitution can happen at different sites; the substitution can be single or multiple. For example, residue 20 of histone H4 is the only lysine N-methylated in calf thymus (both the monomethyl and dimethyl derivatives are present) but this residue is not methylated in pea seedling histone H4 (DeLange et al., 1969a, 1969b). Lysine 16 is the major site of N-acetylation in both the pea and calf histone H4 (DeLange et al., 1968, 1969a, 1969b), but other sites are also acetylated (DeLange et al., 1969b; Wangh et al., 1972). In trout testis histone H4 as many as four lysines may be acetylated (Candido and Dixon, 1971). Residue 1 of H4 may be O-phosphorylated in the trout material (Sung and Dixon, 1970) and both histidines (residues 18 and 75) may be N-phosphorylated (Smith et al., 1973).

Any side-chain modification of histones may change the structure of chromatin in those regions where these derivatives are formed. Some changes affect a large proportion of the histone molecules whereas others affect a small proportion. These changes may consequently influence the process of gene derepression and repression, the binding of enzymes and other non-histone proteins, and the initiation of RNA synthesis.

Methylation of histones mainly occurs on the residues of basic amino acids, and preferentially appears on arginine-rich histones. The reaction is an irreversible process which takes place after histone synthesis is completed (Tidwell et al., 1968). This modification does

not correlate with an increase in DNA template availability for RNA synthesis nor with the initiation of histone, non-histone proteins or DNA synthesis. It is suggested to play a role in the condensation of chromatin (Gershey et al., 1969; Tidwell et al., 1968).

The modification of histones by the binding of ADP-ribose is less well known. However, Burzio and Koide (1970, 1971) have shown that the complex resulting from ribosylation of histones inhibits DNA synthesis in rat liver but does not affect the transcription of RNA from DNA.

There are two types of histone acetylations. The NH_2-terminal acetylation occurs at serine (residue 1) of histones H4 and H2A (Phillips, 1963, 1968). This reaction is coupled with histone synthesis and takes place in the cytoplasm. 'Internal' acetylation occurs at lysine residues within polypeptide chains of arginine-rich histones (DeLange et al., 1969a, 1970; Gershey et al., 1968; Vidali et al., 1968) after the newly-synthesized histones have entered the cell nucleus (Liew et al., 1970). The acetyl groups in the liver histones of rats have a half-life of about 2 hours, while the corresponding half-lives of the parent histones are about 100-200 hours (Byvoet and Morris, 1971). Acetylation results in a decrease in the net positive charge on the basic proteins. This would be expected to alter the affinity of histones for DNA. Consequently, the fine structure of the chromatin would be changed, and presumably this would influence RNA synthesis. Evidence in favour of this is that the 'extended' chromatin is shown to be the most active site of acetylation (Allfrey and Mirsky, 1963; Allfrey, 1964), and of RNA synthesis (Gallwitz and Sekeris, 1969).

The change of thiol groups to disulphide bridges via oxidation is one of the mechanisms which can alter the physical state of histone H3 and its interaction with other protein molecules. In vitro DNA-dependent RNA synthesis is found to be more repressed by the oxidized

(disulphide) state of H3 than the reduced (thiol) state (Hilton and Stocken, 1966). In the first cell cycle in fertilized Echinus eggs, the proportion of thiol to thiol + disulphide in acid-soluble proteins increases during the DNA synthetic phase, and then falls as the cells enter mitosis (Ord and Stocken, 1970). This is in accord with the observations that histone H3 occurs mainly in the reduced monomeric form in interphase chromosomes, but that in metaphase chromosomes the thiol groups are oxidized and histone H3 is either polymerized or complexed with non-histone proteins through interpolypeptide disulphide bonds (Sadgopal and Bonner, 1970). Calf thymus appears to have two moles of cysteinyl residues per histone H3 molecule, and can be oxidized to form polymers, while pea bud contains only one cysteinyl residue and can only form histone H3 dimers (Fambrough and Bonner, 1968).

Histone phosphorylation has been studied in a variety of tissues, in mammalian culture cells, and in naturally synchronous cultures of Physarum polycephalum . Phosphorylation has been found to occur on serine and threonine residues and to take place at a variety of reaction sites, which fall structurally into three distinct classes.

Class 1 comprises the phosphorylation sites on the hydroxy groups of the N-terminal acetyl-seryl residues of histones H2A and H4. This phosphorylation has been found in regenerating rat liver (Sung et al., 1971), and in synchronized cultures of Chinese hamster ovary (CHO) cells (Gurley and Walters, 1973).

Class 2 appears to be phosphorylated on α-seryl residue of homologous peptide sequences from histones H2B, H3 and H6 of trout testis, all of which are phosphorylated to a relatively low level. The homologous peptide of this class includes a common sequence of -arg-lys-ser-. It is particularly interesting that this phosphorylation is accompanied by either methylation or acetylation. The lysine adjacent to serine is

methylated on histone H3 (DeLange et al., 1972) and H6 (Huntley and Dixon, 1972), and it is acetylated on H2B (Candido and Dixon, 1972).

Since phosphorylation of class 1 and class 2 clearly occur in different sequences, it is reasonable to assume that different protein phosphokinases are involved in each class of phosphorylation. However, Louie et al. (1973) from the observation that H2A and H4 of class 1 are phosphorylated at different rates, have argued for the presence of different protein kinases for individual histones.

Class 3 concerns the phosphorylation of histone H1. This includes interphase phosphorylation and metaphase phosphorylation. The interphase phosphorylation occurs in non-dividing cells, and is limited to 1-15% of the total H1 molecules. In rat liver, the phosphorylation sites have been found to be serine residues 38 and 106 (Langan et al., 1971). The phosphokinase involved in interphase phosphorylation is cyclic AMP (cAMP) dependent, and is responsive to those hormones that can increase the intracellular concentration of cAMP, such as adrenaline and glucagon (Lake, 1973a). This interphase phosphorylation of H1 occurs most rapidly in S phase, and is suggested to be related to the deposition of the newly synthesized H1 onto DNA (Balhorn et al., 1973; Louie and Dixon, 1973).

The most significant phosphorylation of histones is however the metaphase phosphorylation of H1. This phosphorylation only appears in dividing cells, and is correlated with the rate of cell division. In several tissues, including dividing trout testis spermatogonia and spermatocytes (Louie and Dixon, 1972a, 1972b, 1973), regenerating liver, hepatoma tissue culture (HTC) cells (Chalkley et al., 1973) as well as synchronized HeLa S-3 cells (Marks et al., 1973) and CHO cells (Lake and Salzman, 1972; Lake et al., 1972), up to 85% of histone H1 molecules are phosphorylated at 1-4 sites. This extensive phosphorylation of H1

is cAMP independent (Lake, 1973a), and the substitution occurs in the C-terminal fragment (residues 108-212) of trout histone H1 (Sung and Dixon, 1970; Bustin and Cole, 1969).

The phosphorylation starts in synchronized mammalian cells in culture just before S phase and remains high throughout until it finally falls sharply after mitosis, whereas the phosphorylation in Physarum occurs in late G2 and falls after mitosis (Bradbury, 1974a). On the whole, the phosphate content of H1 is positively correlated with the cell replication rate, and the maximum content is at the time of chromosome condensation. This gross amount of the phosphorylation of H1 in metaphase is suggested to play a role in either the initiation (Lake, 1973a, 1973b) or the maintenance (Bradbury et al., 1973, 1974a) of chromosome condensation. This hypothesis is supported by the observation that heterologous preparations of histone H1 phosphokinase can accelerate the naturally synchronous culture of Physarum polycephalum to enter the mitosis 40 min earlier than the control did. Bradbury et al. (1974b) therefore further suggested that the histone H1 phosphokinase is the 'mitotic trigger'.

This concept of mitotic mechanism is, however, criticized by several researcheres. Gorovsky and Keevert (1974, 1975) indicated that H1 is undetectable in the micronuclei in Tetrahymena pyriformis but they proceed normally through mitotic division. The macronuclei which undergo amitotic division, however, contains a large amount of histone H1, and the histone is extensively phosphorylated. Furthermore, Tanphaichitr et al. (1976) showed that metaphase HTC cells can proceed through normal chromosomal decondensation into G1 under the condition where histone H1 is hyperphosphorylated in the presence of $ZnCl_2$. Such observations strongly argue against the suggestion that phosphorylation of H1 is an important step in the initiation of mitosis. The precise function of

this massive phosphorylation of histone H1 thus still remains to be determined.

In general, phosphorylation tends to reduce the net positive charge of histone molecules. This might be expected to change the comformation of the protein or to lessen its binding affinity for DNA. Therefore, phosphorylation could act (i) to 'uncover' specific genes which are repressed by histone; (ii) to modify the structure of chromatin in preparation for DNA synthesis and cell division; or (iii) it may aid in the removal of the histones from DNA to facilitate replacement by another type of protein; and the function of the histones could consequently be switched from 'negative' to 'positive' effect in terms of gene regulations.

7. The nature of the present investigation

The main object of the present work was to characterize the histones from cultured cells of Acer pseudoplatanus L. and to study their biosynthesis throughout the cell-cycle. Although histone biosynthesis has been extensively studied since the early 1960s, most of the reports are concerned with animal systems. The derived 'general' concepts cannot be accepted as applying to all eukaryotes unless they are also shown to apply in work with higher plant material.

As far as the choice of material for such a study is concerned, not only do cultured cells provide material suitable for standard analysis and extraction procedures, but also the growth of cultured cells can be controlled to yield uniform cell populations at high cell densities and in which almost the whole of the cell population is cycling. In animal systems, many researchers have carried out their studies on mammalian cultured cells and such studies have brought out some important information as described earlier. Hitherto, very few studies have been performed

using plant culture cells. To my knowledge, only suspension culture cells of tobacco (Chalkley and Maurer, 1965; Flamm and Birnstiel, 1964) and of carrot (Gregor et al., 1974) have been used for studying chromosomal proteins. Furthermore, neither of these studies has dealt with the relationship between histone synthesis and cell-cycle stage.

Suspension cells of Acer pseudoplatanus L. can grow in a defined nutrient medium with high dividing percentages (Stuart and Street, 1969), and the culture can be synchronized by nitrate starvation and re-growth (King et al., 1973, 1974; Gould and Street, 1975). Presumably, this suspension culture, especially when it is synchronized, would be an ideal system for studying the precise relationship between the synthesis of histones and that of DNA.

Previously in this laboratory, A. Gould had used this culture system to carry out some preliminary investigations on histone synthesis. However, the present study differs significantly from that initiated by Gould (1975) in that (i) we defined histones differently (the 'histones' as extracted by Gould did not exclude basic proteins from sources other than nuclei); (ii) the histones extracted by Gould's procedure were not characterized by separation on polyacrylamide gels. Hence the extraction procedures for cultured cells of Acer pseudoplatanus L. needed to be critically established and the search for histone extraction methods was the initial stage of the present study. These extraction and characterization methods for histones were established using asynchronous cultures. The methods were then applied to the cultured cells from both synchronous and asynchronous cultures. These experiments aimed to survey the variations of individual histone fractions at different cycle stages of the cells : the cell cycle and the growth cycle for synchronous and asynchronous cultures respectively.

Radioisotope labelling techniques were applied to the synchronous

cultures to study the relationships between histone and DNA synthesis, and a similar approach was also performed using the asynchronous cultures to study the effect of hydroxyurea on the synthesis of acid-soluble and acid-insoluble proteins. A double labelling technique concomitant with a 'pulse' and 'pulse-chase' experiment was carried out to investigate the site of histone biosynthesis. The considerable technical problems encountered in this research programme are included and discussed.

CHAPTER 2

Materials and Methods

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(b) Specific activity of histone synthesized

(D) Measurement of radioactivities

(a) Reagents

(b) Methods

1. Culture techniques

(A) Origin of culture

Acer pseudoplatanus L. strain AM was used as plant material. It was selected from the stock suspension cultures of sycamore by A. Gould in March 1972 (Gould, 1975). The original callus from which the stock cultures are derived was initiated from stem cambium of Acer pseudoplatanus L. by Lamport (1964) and was supplied by Dr. D. H. Northcote (Biochemistry Department, University of Cambridge).

(B) Cleaning of culture vessels

250 ml conical flasks were washed in a 'Hydrojet' washer (Heinicke, Hollywood, Florida) using BHC 2008 detergent (Savilles Hydrological Corporation), and were rinsed in tap water, and then in two changes of glass distilled water. They were finally dried in an oven at 100-120 °C.

1-litre and 10-litre culture bottles, and the 10 litre synchronous culture vessel, were soaked in chromic acid overnight, rinsed with tap water thoroughly, and finally rinsed with glass-distilled water and dried as above.

(C) Culture medium

Heller's medium as modified by Stuart and Street (1969) was used throughout the work reported here. The medium was prepared from concentrated stock solutions to give the final concentrations. All chemicals used were of analytical reagent (A.R.) grade. The distilled water used was double distilled. The composition of the modified Heller's medium is shown in Table 2.

(D) Maintenance of stock culture

3 litres of Heller's medium lacking urea were prepared every 6 weeks. 57 ml aliquots were dispensed into 250 ml conical

Table 2. Composition of Heller's medium

Component	Final concentration ($mg\ l^{-1}$)	Stock concentration / Final concentration in medium
KCl	750	10
$MgSO_4.7H_2O$	250	10
$NaNO_3$	600	10
$NaH_2PO_4.2H_2O$	130	10
$CaCl_2.6H_2O$	110	10
$ZnSO_4.7H_2O$	1.0	1000
H_3BO_3	1.0	1000
$MnSO_4.4H_2O$	0.1	1000
$CuSO_4.5H_2O$	0.03	1000
KI	0.01	1000
$FeCl_3.6H_2O$	1.0	1000
thiamine.HCl	1.0	1000
pantothenic acid	2.5	1000
choline chloride	0.5	1000
meso-Inositol	100	100
cysteine HCl	10	added as solid
Kinetin	1.25	100
2,4-D	1.0	100
sucrose	2.0×10^4	added as solid
urea	200	commercially prepared sterile 40% solution (added to medium after autoclaving)
pH adjusted to 5.2 before autoclaving		

flasks, and sterilized at 121 °C for 15 min. Just before subculturing, 3 ml of diluted urea (40% filter-sterilized solution was diluted 100X with sterilized distilled water) was added to the medium, and this was inoculated with 10 ml of a 14-day old suspension culture. Flasks were then sealed with 2 layers of sterilized aluminium foil, and were incubated on a horizontal rotary shaker in a temperature-controlled room at 25 °C. The culture room was illuminated continuously with white fluorescent light. The stock culture was maintained by subculturing in this manner every 14 days.

(E) Large-scale asynchronous cultures

A 1 litre culture bottle and a 10 litre culture bottle containing respectively 500 ml and 5 litres of Heller's medium were sterilized at 121 °C for 35 min. Sterilized urea was added aseptically to the cool medium and the 500 ml culture was initiated by inoculation with a 14-day old stock culture (60 ml). After 14 days' incubation on the shaker the 500 ml culture was used to initiate the 5 litre culture (in the 10 litre bottle). This was then set on a rotary spinner (Short et al., 1969), and spun continuously in the culture room for the required incubation period.

(F) Synchronous cultures

Cell division synchrony was initiated by inoculating stationary phase cells of Acer pseudoplatanus L. at low density ($2\text{-}5 \times 10^4$ cells ml^{-1}) into 9 litres of medium in a large-scale batch culture apparatus with automatic sampling assembly. The system has been described by Wilson, King and Street (1971), and two photographs are reproduced here from that publication (Plate 1 and Plate 2). However, in the present work, a 10 litre culture

Plate 1. The basic, batch culture unit used for synchronous suspensions of *Acer pseudoplatanus* L.

Key:

A = aerator

AO = air outlet

C = Flat-flange lid clip

CW = cotton wool filter

F = miniature air line filter

GC = glass, temperature control coil

IP = inoculation port

S = magnetic bar stirrer

SR = sample receiver

SWL = sterile water line

T = thermometer

TCW = temperature control water line

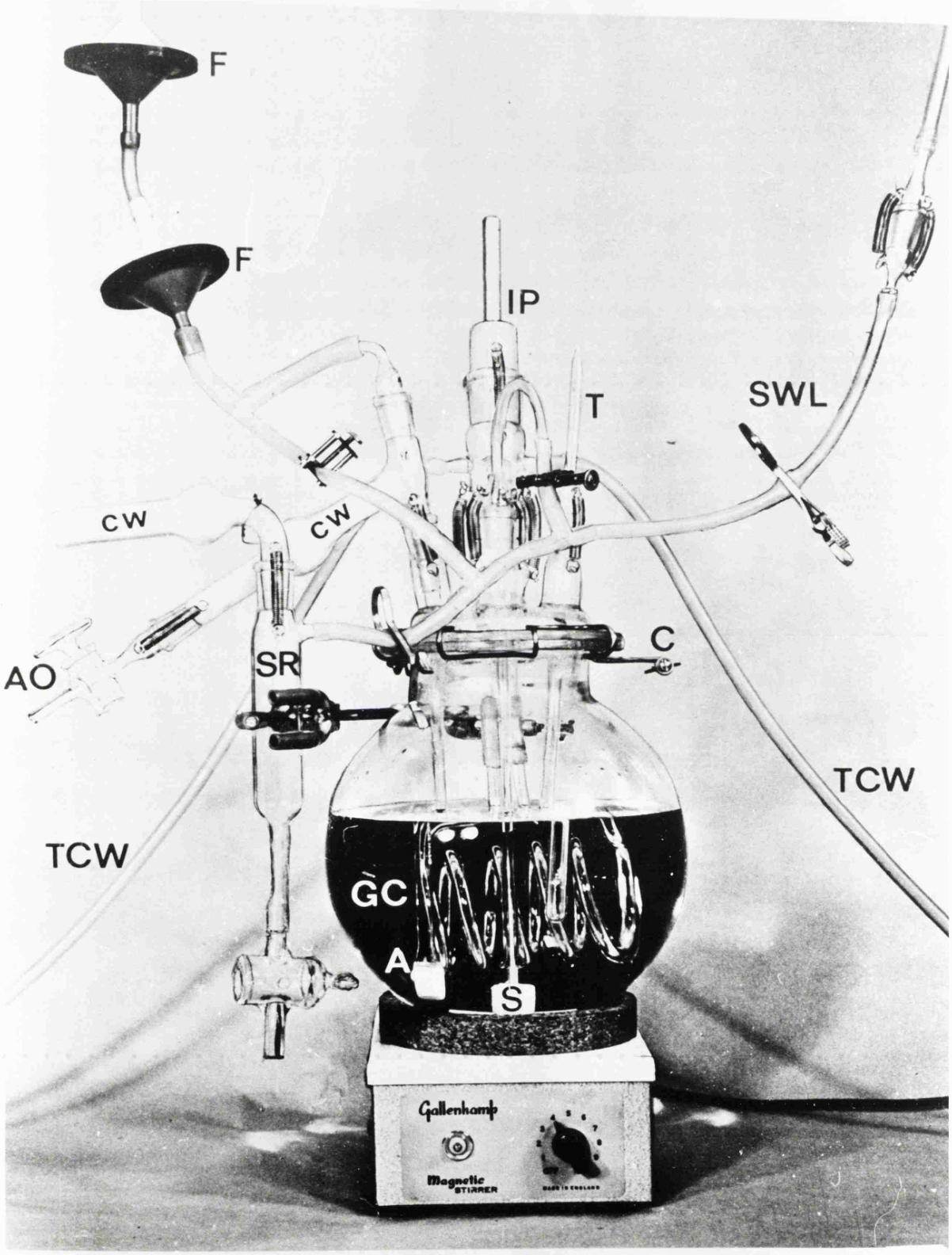

F
F
IP
T
SWL
CW
CW
AO
SR
C
TCW
TCW
GC
A
S
Gallenkamp
Magnetic
STIRRER

Plate 1

Plate 2. The automatic sampling assembly

A. View of complete assembly

Key:

AP = air pump

ES = empty solenoid valve

LD = latching device

NV = needle valve for automatic sample delivery

SO = sample outlet line from needle valve

SR = sample receiver (manual sampling)

SSL = sterile saline supply line

SVD = sample volume detector electrode

TCW = temperature control unit

TT = turntable

WS = wash solenoid valve

B. Sample volume detector

E = electrodes

TC = teflon cones

C. Exploded view of stainless-steel valve unit

FC = flexible cable

R 1, 2, 3, 5 = silicon rubber 'O' rings

SO = sample outlet

SSL = sterile saline line

W1 = stainless steel washer

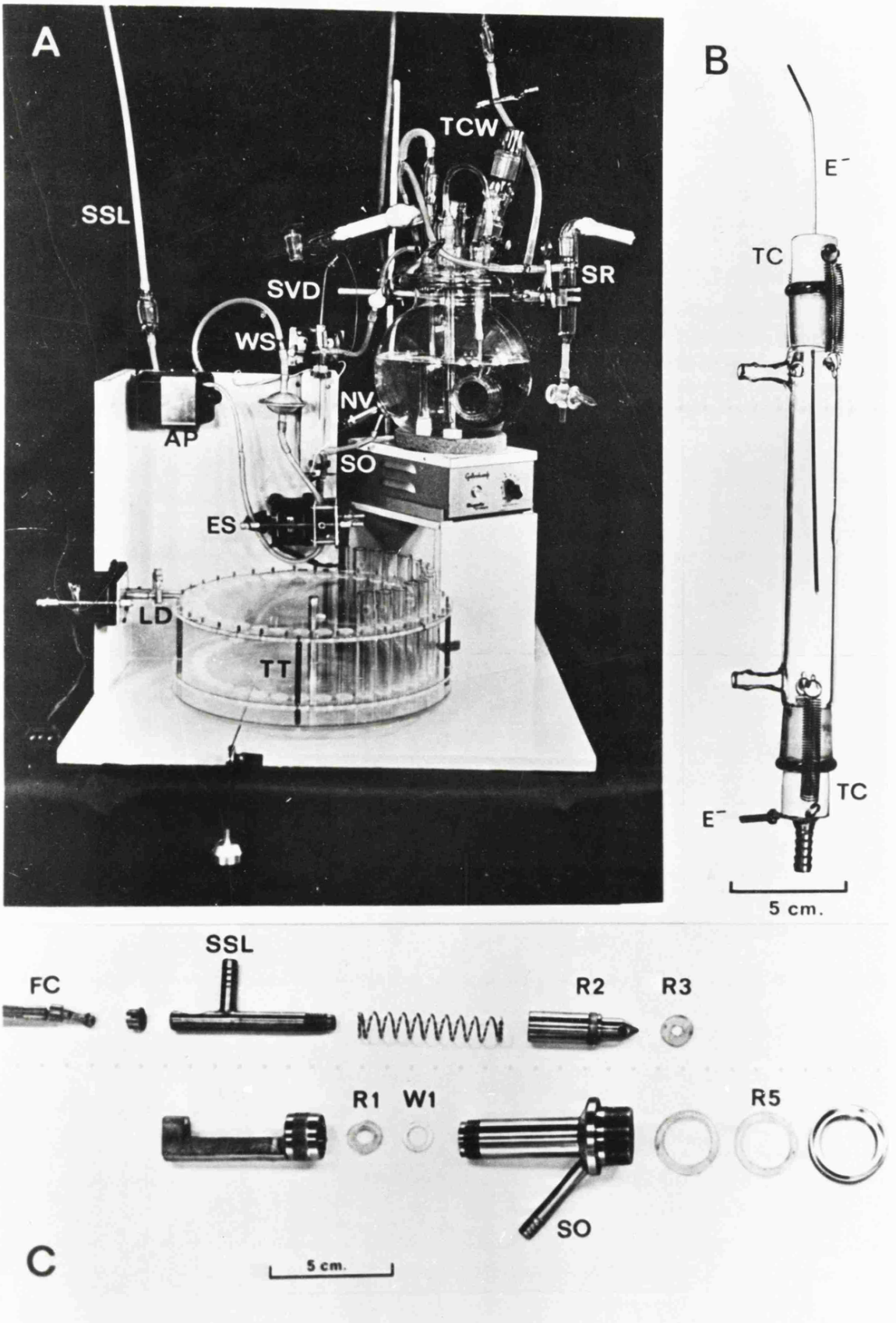

A
SSL
TCW
SVD
SR
WS
NV
AP
SO
ES
LD
TT
B
E⁻
TC
TC
E⁻
5 cm.
SSL
FC
R2
R3
R1
W1
R5
SO
C
5 cm.

Plate 2

vessel was used instead of the standard 5 litre vessel. This was necessary to provide the large quantity of culture material required for the analytical work to be carried out.

The 10 litre batch culture vessel containing 9 litres of Heller's medium (lacking urea) was sterilized at 121 °C for 45 min. 4.5 ml of urea (40% filter-sterilized solution, Oxoid Ltd.) was added aseptically to the cool sterilized medium via the inoculation port of the culture vessel, and 3 flasks of 28-day old stock cultures (60 ml each) were then introduced into the culture vessel through the same inoculation port. These inoculations were carried out in a sterile cabinet and rigorous aseptic condition was maintained at all times.

To estimate the cell number in the culture, samples of less than 10 ml were collected automatically into a test tube containing 5 ml of 10% chromium trioxide. Samples were collected at predetermined time intervals at selected periods during the programme of growth, and were macerated and counted, as described later.

Samples of over 20 ml were collected manually through the sample receiver. The quantity taken in this way varied from experiment to experiment depending upon the cell concentration and the experimental purpose for which the sample was required. In most cases 2-5 ml samples were removed from these large samples for cell number estimation.

2. Parameters for culture analysis

(A) Cell number estimation

The cell counting technique was based upon that of King, Cox, Fowler and Street (1974). The method was as follows:

(a) Sample preparation

(i) 2-5 ml of stock culture was added to an equal volume of 10% (w/v) chromium trioxide solution contained in a thick-walled sample bottle. The bottle was heated in a water bath at 70°C (maceration) for 5-20 min depending on the physiological state of the cells. After heating, the sample bottle was closed with a screw cap and shaken vigorously on a flask-shaker (Baird and Tatlock, Ltd.) for 2-15 min depending upon the fragility of the cells.

(ii) From a synchronous culture samples of 5-9 ml were collected automatically into 5 ml of 10%(w/v) chromium trioxide solution(contained in tubes calibrated to 15 ml). By making the volume of the mixture up to the 15 ml mark, the sample volume was calculated. Samples were macerated and shaken as described above. For certain samples, 1-2 ml of 50% (w/v) chromium trioxide had to be added to improve cell separation.

(b) Cell counting

The treated sample was diluted and pipetted into the wells of a special counting slide. The volume of the counting slide was such that the area of each viewing field in a Watson Microsystem 70 Microscope (W. Watson and Sons, Ltd., England) at magnification X100 corresponded to 0.8 µl. Estimation of cell number per ml was made from cell counts of 100 random fields. The standard error of the mean was also calculated.

(B) Measurement of DNA content by microdensitometry

Cells for mitotic index and DNA content measurement were fixed in 50% (v/v) formic acid to disperse starch grains and to soften the cell walls. Usually, samples were fixed at 4°C for 2-3 weeks, and all samples from the same culture were processed

for Feulgen staining at the same time.

Cells were removed from fixative, washed 3 times with distilled water (2-3 min per change), and then submitted to Feulgen staining (Hillary, 1939, 1940). The cells were next hydrolysed for exactly 12 min in 1 N HCl at 60 °C, and then spun down and resuspended in basic fuchsin solution (Magenta basic fuchsin crystals, technical C1 42500. Cat. No. 26120, BDH Chemicals Ltd.) and allowed to stain for 2 hours. The cells were washed 3 times with freshly prepared sulphur dioxide water, with five minute changes, and finally suspended in 25% (v/v) aqueous acetic acid prior to preparation of squashes. Permanent slides were made and mounted with Gurr's XAM neutral mounting medium (Searle Diagnostic, High Wycombe, Bucks; Conger and Fairchild, 1953).

Densitometric measurements of Feulgen stained nuclei were done on a Vickers M85 scanning microdensitometer with a 100X Microplan oil immersion objective. A wavelength of 565 nm was used.

(C) Autoradiographic technique

^{3}H-thymidine (^{3}H-dT) labelled, ^{3}H-lysine or ^{3}H-arginine labelled cells were fixed in 50% (v/v) formic acid, and were hydrolysed and stained in basic fuchsin solution as described above. Stained cells were then squashed on slides which had been cleaned and coated with a layer of gelatin. The sample-bearing slides were then passed down an alcohol dilution series from absolute alcohol to 95% alcohol to 30% alcohol, and finally to double distilled water. There was a 15 min interval between successive transfers. Specimen slides were transferred to a dark room, and mounted with Kodak Fine Grain Autoradiographic Stripping Plate AR 10 under a Kodak safelamp. Slides were set in a rack and allowed

to dry overnight in the dark room. The whole slide rack was then retained in a box with a package of silica gel. The box was wrapped in 5 black plastic bags, and stored in a cold room for exposure to proceed.

The extent of exposure was checked every 2 days, after 7 days in the cold room, and developing a single slide each time until the testing slide showed clear dots on the film. Slides were developed with Kodak D-19 developer for 7.5 min at 16 °C, and fixed with Unifix for 5 min. After the slides had been washed with tap water, distilled water and then dried, they were examined under oil immersion using a microscope to score the percentage of nuclei with sliver grains (for ^{3}H-dT labelled samples) or to observe the distribution of the silver grains within the cells (for ^{3}H-lysine or ^{3}H-arginine labelled samples).

3. Isolation of nuclei

The procedure described below is that developed as described in Chapter 3.

(A) Reagents

(a) Grinding medium - 20 mM Tris-HCl, pH 7.8, 1 mM Mg acetate, 2 mM $CaCl_2$ and 70% (v/v) glycerol - stored in a deep-freeze at -18 °C.

(b) Dilution buffer - 20 mM Tris-HCl, pH 7.8, 1 mM Mg acetate, 2 mM $CaCl_2$ and 0.2% (v/v) Triton X-100 - stored in a refrigerator at 4 °C.

(c) Washing medium - 3 parts of grinding medium plus 1 part of dilution buffer - stored in a refrigerator at 4 °C.

(B) Preservation of cultured cells before isolation of nuclei

The suspension culture of Acer pseudoplatanus L. was filtered

through nylon cloth, and the cells were washed twice with large quantities of cold distilled water, drained under vacuum, and weighed to obtain the fresh weight. The cells were then suspended in chilled grinding medium at a concentration of 0.25 g cells ml^{-1}. The suspension was stored in a deep-freeze at -18 °C until required for the isolation of nuclei.

(C) Isolation of nuclei

The above suspension of cells was poured into a glass tube (2.5 cm dia.) immersed in an ethanol-dry ice bath, and stirred gently with a thermometer (BC 1704) until the reading reached -20 °C. The cells were then homogenized with a glass Potter-Elvehjem homogenizer fitted with a loose-fitting teflon pestle driven by a mechanical stirrer. Two up and down movements were applied to each suspension. One-third volume of the cold dilution buffer was added to the ruptured suspension, and it was then filtered through 4 layers of Miracloth (Calbiochem). The Miracloth was saturated with cold washing medium before the filtration was started. The filtrate was then distributed into pre-chilled centrifuge tubes and centrifuged at 1,500 Xg for 30 min at 0 °C in an MSE High Speed 18 Centrifuge. The supernatant was discarded and the nuclei were scraped free from the underlying starch grains, suspended in washing medium, and centrifuged at 4,500 Xg for 15 min. This centrifugation was repeated twice. The resulting crude nuclear pellet was suspended in 10 ml of washing medium, layered onto 30 ml of grinding medium, and purified by centrifugation through the grinding medium at 11,000 Xg for 30 min.

4. Extraction of histones

(A) Extraction with $CaCl_2$ and precipitation with TCA ($CaCl_2$-TCA method)

This was by the method of Mohberg and Rusch (1969). The purified nuclear pellet, containing less than 500 µg DNA, was suspended in 12 ml of pre-warmed 1 M $CaCl_2$ with a Potter-Elvehjem homogenizer, incubated at 85 °C for 10 min and then chilled in ice (the heating process is to inhibit protease activity). The viscous mixture was stirred slowly on a magnetic stirrer overnight in a cold room. It was then spun at 25,000 rpm for 20 min in a Beckman LB-2 Ultracentrifuge, using a 60 Ti rotor. The supernatant was removed and stored on ice. The pellet was resuspended in 3 ml of cold 1 M $CaCl_2$ and centrifuged again for 20 min. The two $CaCl_2$ soluble fractions were bulked, and 5 ml of 100% (w/v) trichloroacetic acid (TCA) was added (to a final concentration of 25% (w/v) TCA). After 2 hours at 0 °C the histones were pelleted by centrifugation for 30 min at 25,000 rpm in a Beckman LB-2 Ultracentrifuge using a 60 Ti rotor. The supernatant was discarded, and the pellet was scraped from the wall of the tube into 5 ml of cold 25% TCA and centrifuged for a further 20 min. The tube was drained and the fibrous pellet was suspended in 500 µl of 0.02 N H_2SO_4. The slurry was transferred into 1 cm diameter Visking tubing and dialysed overnight in a cold room, against 0.02 N H_2SO_4 (500X volume, changed 2-3 times). The contents of the dialysis tubing were then spun at 5,000 rpm for 5 min in an MSE Bench Centrifuge to remove the insoluble fraction. The supernatant (histone fraction) was stored in small specimen tubes in a deep-freeze until required for analysis.

(B) Extraction with H_2SO_4 and precipitation with ethanol (H_2SO_4-EtOH method)

This was by the method of Fambrough and Bonner (1966) with some modifications. The purified nuclear pellet, containing less than 500 µg DNA, was suspended in 4 ml of 0.4 N H_2SO_4 with a glass Potter-Elvehjem homogenizer, and stirred on a magnetic stirrer in an ice bath for 1 hour. It was then spun at 25,000 rpm for 30 min in a Beckman LB-2 Ultracentrifuge, using a 60 Ti rotor. The supernatant was pipetted out and put aside in ice, and the pellet was washed with 2 ml of 0.4 N H_2SO_4 and centrifuged again. The two supernatants were bulked and 4 volumes of absolute ethanol added and the histone allowed to precipitate at -18 °C for at least 24 hours. This was followed by centrifugation at 25,000 rpm for 30 min. The precipitate (histone sulphate) was scraped from the wall of the tube into 3 ml of ethanol, and again centrifuged. The histone sample was drained of ethanol, and was dried in a desiccator.

In order to determine the ratio of DNA to histones, a known portion of nuclear fraction was withdrawn prior to the last centrifugation of the nuclear preparation. The DNA was extracted and determined as described later in the Methods. If the histone sample was obtained by the H_2SO_4-EtOH method, it was first dissolved in 0.02 N H_2SO_4 and the content was then determined as described later.

5. Extraction of DNA and Protein

This was by the method of Short, Brown and Street (1969). A sample of ruptured suspension or nuclear fraction was suspended in 0.3 N KOH, and incubated at 37 °C overnight. After cooling, the mixture was neutralized with 0.1 volume of 3 N HCl and 10 min later

an equal volume of cold 0.5 N perchloric acid (PCA) was added. The solution was mixed vigorously and cooled in ice. 20 min later the sample was spun down in a MSE Super-Minor Centrifuge. The supernatant was discarded and the pellet was washed 3 times with cold 0.25 N PCA, and finally suspended in 1 ml of 0.5 N PCA, incubated at 70 °C for 20 min, and then cooled in ice. The hydrolyzed DNA was obtained in the supernatant after centrifugation to remove insoluble material. The pellet was extracted once more and the two supernatants combined, and this was referred to as extractable DNA.

The remaining pellet after DNA extraction was suspended in 1 N NaOH, extracted twice by boiling in a water bath for 15 min, chilled in ice and then centrifuged. The combined supernatants after centrifugation was referred to as extractable protein.

6. Determination of DNA content

(A) By the diphenylamine reagent

This was by the method of Burton (1956) with some modifications.

(a) Preparation of standard DNA solution

A stock solution of DNA containing 300 µg per ml was prepared as follows. 7.5 mg of calf thymus DNA was dissolved in 20 ml of 0.5 N PCA. This was heated at 70 °C for 20 min, chilled in ice and made up to 25 ml with 0.5 N PCA in a volumetric flask. The stock solution was then distributed into small vials and kept in a refrigerator. One vial of this stock solution was used each time to prepare a dilution series containing 15, 30, 50, 75 and 100 µg DNA ml^{-1}.

(b) Preparation of diphenylamine reagent

Diphenylamine (1.5 g) was dissolved in 100 ml of glacial

acetic acid, and 1.5 ml of concentrated sulphuric acid was then added, and the resulting solution kept in a refrigerator until required for use. The reagent was completed just before use by adding 1/200 volume of 1.6% (w/v) aqueous acetaldehyde. The stock of 1.6% aqueous acetaldehyde wasalso stored in the refrigerator.

(c) Colour reaction and measurement

To 1 ml of DNA standard solution or DNA-containing sample was added 2 ml of diphenylamine reagent, and mixed thoroughly. The sample tubes were sealed with parafilm (NESO) and incubated at 30 °C for 16 hours. The samples were then cooled to room temperature, and submitted to spectrophotometry at 590 nm and 700 nm using a Unicam SP 800 Spectrophotometer. A standard curve was drawn relating the DNA content to $E_{590} - E_{700}$ (Short et al., 1969), from which the DNA content of the sample was determined. A typical standard curve is shown in Fig. 1.

(B) By absorbance at 260 nm and 280 nm

When the DNA content of a particular sample was estimated to be higher than 100 $\mu g\ ml^{-1}$, a small volume of the sample was withdrawn, diluted with 0.5 N PCA and the absorbance at 260 nm and 280 nm measured with an SP 800A Ultraviolet Spectrophotometer (Humphrey and Kellarman, 1955). By using 0.5 N PCA as a blank and the optical densities of standard calf thymus DNA solutions, a standard curve was drawn, from which the DNA content of the sample was estimated. A typical standard curve is shown in Fig. 2. Those samples with high DNA content were also determined by the diphenylamine reagent, after proper dilution.

Figure 1

Standard curve for DNA estimation by the diphenylamine method.

A series of calf thymus DNA standard solutions were prepared in 0.5 N PCA, hydrolyzed and reacted with diphenylamine reagent (Burton, 1956), and the absorbance was measured at 590 nm and 700 nm using a Unicam SP 800 Spectrophotometer.

Figure 2

Standard curve for DNA estimation by UV spectrophotometry.

Calf thymus DNA standard solutions were prepared in 0.5 N PCA and the absorbance at 260 nm and 280 nm was measured against 0.5 N PCA, using an SP 800A Ultraviolet Spectrophotometer.

Fig. 1

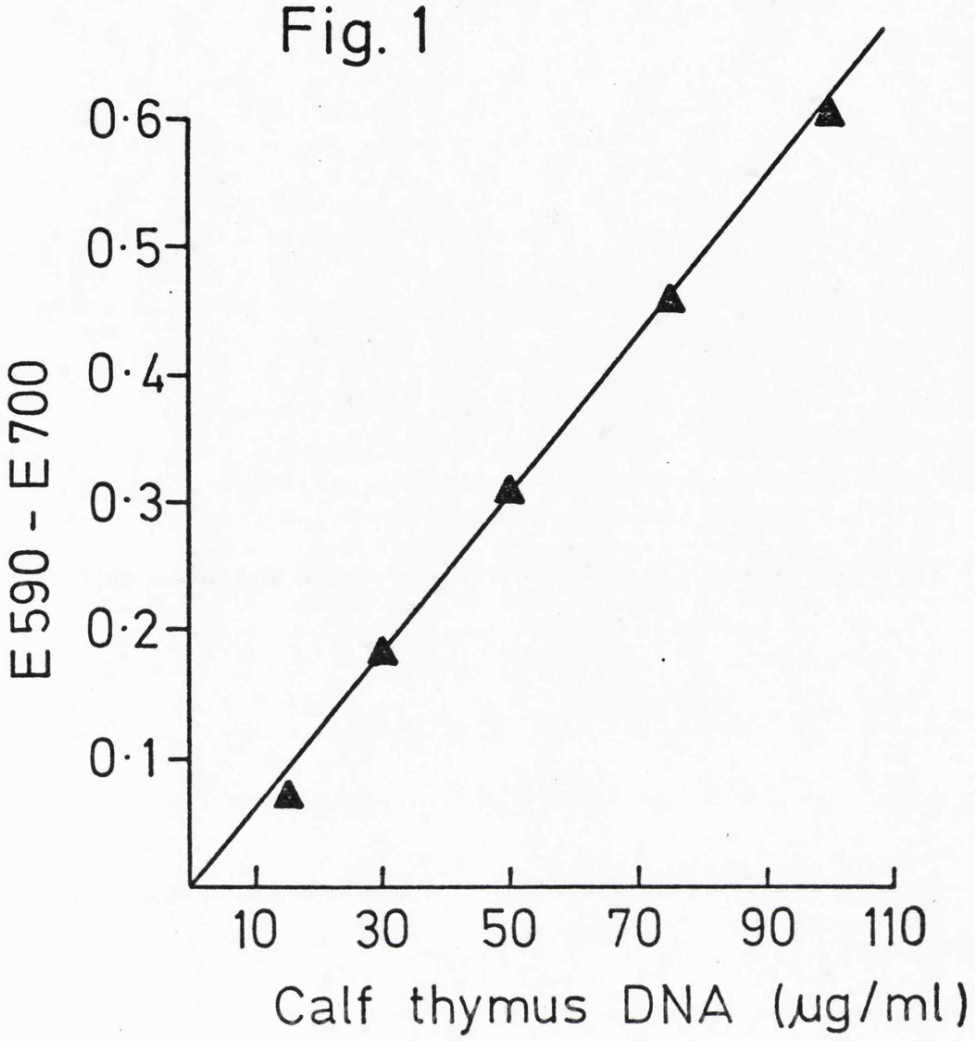
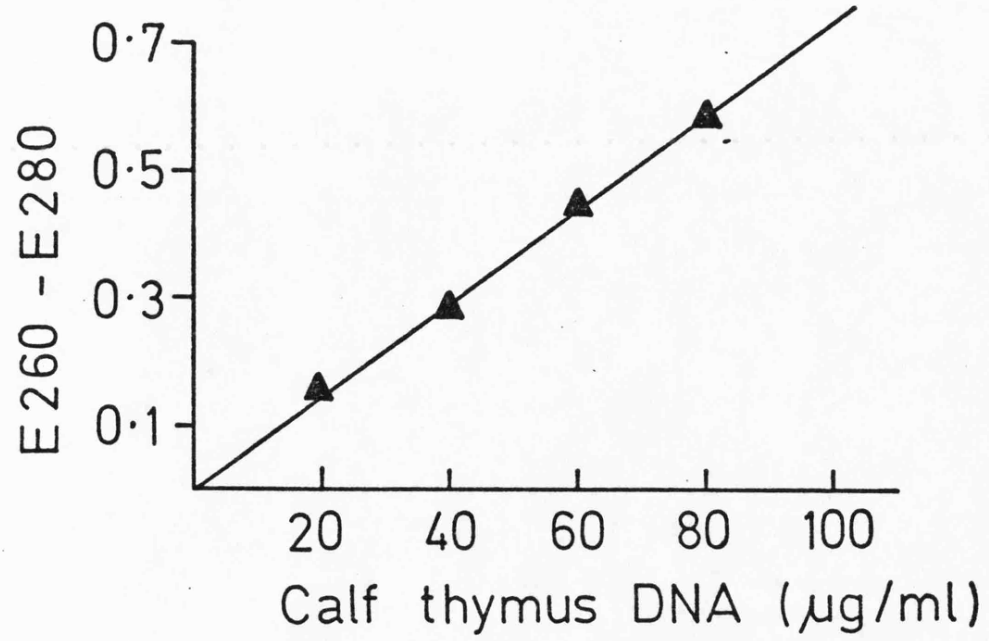

0·6
0·5
0·4
0·3
0·2
0·1
E590 - E700
10
30
50
70
90
110
Calf thymus DNA (µg/ml)

Fig. 2


0·7
0·5
0·3
0·1
E260 - E280
20
40
60
80
100
Calf thymus DNA (µg/ml)

7. Determination of protein content

(A) By the 'Folin and Ciocalteu Phenol' reagent

This was by the method of Lowry et al. (1951) with some modifications.

(a) Preparation of protein standard solution

Calf thymus histone (25 mg) was dissolved in 25 ml of 0.02 N H_2SO_4 in a volumetric flask to give a concentration of 1.0 mg ml^{-1}. The stock solution was distributed into small vials and stored in a refrigerator. The stock solution was added 0.02 N H_2SO_4, to prepare a series of standards. A standard solution of bovine serum albumin was also prepared as described above, but using 1 N NaOH instead of 0.02 N H_2SO_4 as the solvent.

(b) Preparation of alkaline copper reagent

The alkaline copper reagent was prepared from the following aqueous solutions :

(i) 2.68% (w/v) NaK tartrate - 4 H_2O — stored in a refrigerator

(ii) 1% (w/v) $CuSO_4$. 5 H_2O

(iii) 2% Na_2CO_3-0.1 N NaOH (4g NaOH plus 20 g anhydrous Na_2CO_3 made to 1 litre with distilled water). Immediately before use, 2 ml of solution (i) was mixed with 2 ml of solution (ii), and then 100 ml of the solution (iii) was added.

(c) 'Folin and Ciocalteu Phenol' reagent (Phenol reagent, BDH)

This was diluted with an equal volume of distilled water just before use.

(d) Colour reaction procedure

To samples of 20-50 µg of protein in a volume of 0.2 ml was added 5 ml of alkaline copper reagent. The mixture was

shaken vigorously and left to stand at room temperature for 30 min. 0.5 ml of diluted Phenol reagent was then added, mixed immediately on a Whirlimixer, and set aside for 1 hour for colour development. The samples were read at 750 nm with a Unicam SP 800 Spectrophotometer.

Calf thymus histone was used as a standard when histone samples were analyzed, while bovine serum albumin was used as a standard when total protein and acidic protein contents were determined. Typical standard curves are shown in Fig. 3.

(B) By absorbance at 230 nm (- for histones only)

A concentration series of calf thymus histone was prepared in 0.02 N H_2SO_4 and measured at 230 nm with a Unicam SP 800A Ultraviolet Spectrophotometer. The histone samples were dissolved in 0.02 N H_2SO_4 and measured as described. A standard curve was drawn from the optical densities of calf thymus histones (Fig. 4), from which the protein content of the histone samples was estimated. This method is more accurate than the Lowry method provided that histone content is higher than 100 $\mu g\ ml^{-1}$.

8. Amino acid analysis

Amino acid analysis was performed according to the method of Spackman et al. (1958). The histone sample was dissolved in 0.02 N H_2SO_4, centrifuged to remove the insoluble fraction, and the clean solution was dried in an Edwards freeze dryer. The dried histone sample was then dissolved in 0.5 ml of 6 N HCl, and transferred into a glass tube (1.2 cm inner diameter). The solution was flushed with nitrogen gas for 2 min before the tube was sealed. The protein was hydrolyzed by heating the N_2-filled tube in an oven at 110 °C for 24 hours. The tube was then cooled to room temperature and opened at the top end. The hydrolyzate was dried in a desiccator under vacuum,

Figure 3

Standard curve for protein estimation by the method of Lowry *et al.*

Bovine serum albumin standard solutions were prepared in 1 N NaOH, and calf thymus histone standard solutions were prepared in 0.02 N H_2SO_4. The protein contents were then determined by the method of Lowry *et al.*(1951).

Figure 4

Standard curve for histone estimation by UV spectrophotometry.

Standard calf thymus histone solutions were prepared in 0.02 N H_2SO_4, and the absorbance at 230 nm was measured against 0.02 N H_2SO_4, using an SP 800A Ultraviolet Spectrophotometer.

Fig. 3

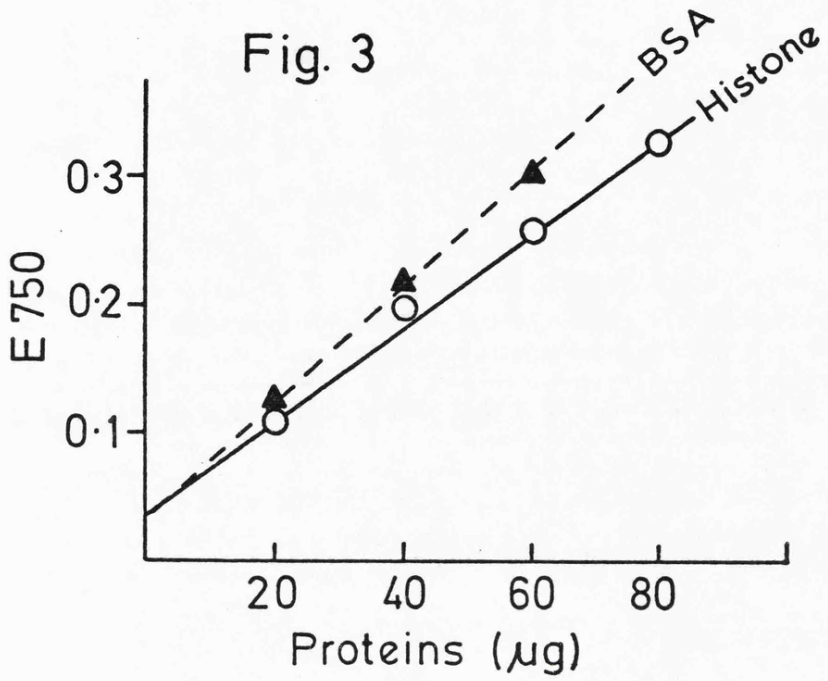
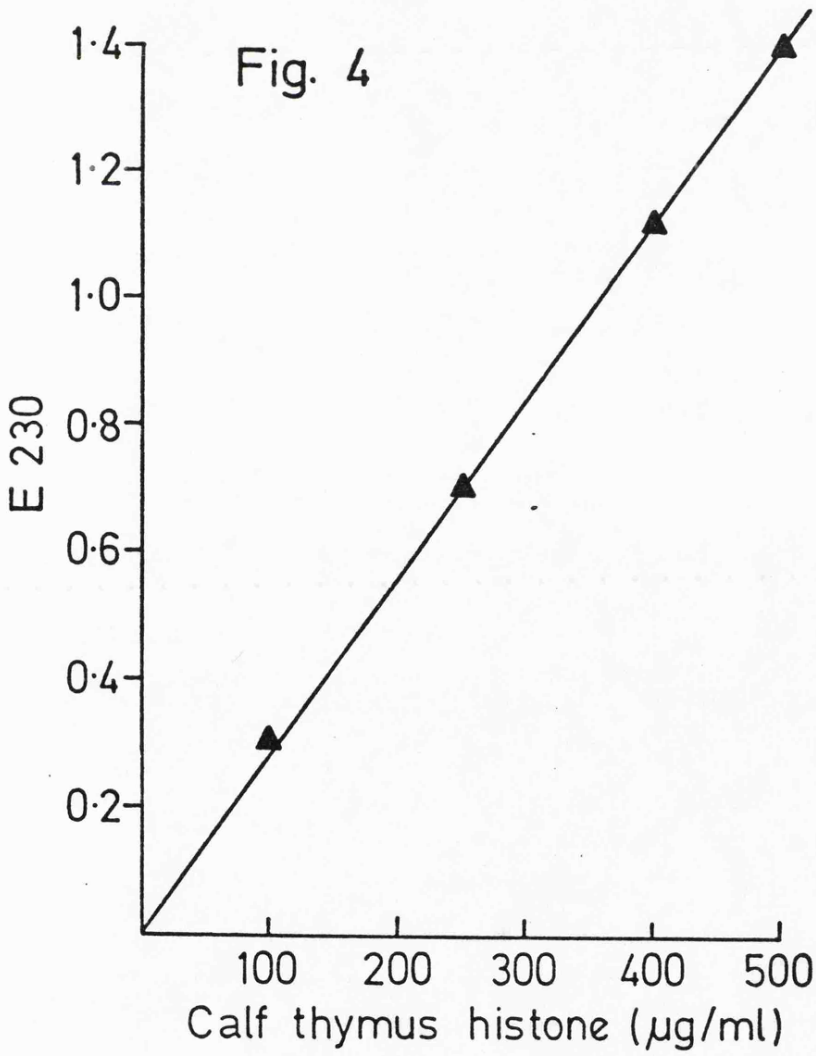

BSA
Histone
E 750
0·3
0·2
0·1
20
40
60
80
Proteins (µg)

Fig. 4


E 230
1·4
1·2
1·0
0·8
0·6
0·4
0·2
100
200
300
400
500
Calf thymus histone (µg/ml)

dissolved in 1.5 ml of lithium citrate buffer at pH 3.0, mixed with 0.1 μmole of the non-protein amino acid norleucine as an external standard, and analyzed with a Technicon Automatic Analyzer. A standard amino acid mixture purchased from Sigma Chemical Company was run for calibration purposes, and 1.5 mg of calf thymus histone (commercial product, Sigma) was hydrolyzed and analyzed for comparison. The automatic analyzer was kindly operated by Mr. M. Pratt.

The area occupied by each sample amino acid on the chart was calculated by a standard method, and was converted into the equivalent μmole by dividing by the occupied area of the corresponding standard amino acid. Each amino acid was then presented as a percentage of the total amino acid in the protein.

9. Electrophoretic techniques

(A) Chemicals

Acrylamide (BDH)

N,N'-methylenebisacrylamide (Bisacrylamide, BDH)

N,N,N',N'-tetramethylenediamine (TEMED, Sigma)

Ammonium persulphate (BDH)

(B) Acid-urea poylacrylamide gel electrophoresis

This was by the method of Panyim and Chalkley (1969) with some modifications. The gels were prepared from the following solutions :

Solution A - 60% (w/v) acrylamide and 0.4% (w/v) bisacrylamide in distilled water; - stored in a brown bottle and in the dark.

Solution B - 43.2% (v/v) glacial acetic acid and 4% (v/v) TEMED in distilled water; - stored in a brown bottle and in the dark.

Solution C - 0.2% (w/v) ammonium persulphate in 4.5 M urea. This was freshly prepared from crystalline ammonium persulphate and a stock solution of 7.5 M urea.

9 cm gels were prepared in 11 cm long glass tubes (0.55 cm inner diameter). The clean gel tubes were sealed with parafilm at their lower ends and set vertically. Gel solution for 8 tubes was prepared by mixing 4 ml of solution A, 2 ml of solution B and 10 ml of solution C. The mixture was well stirred, degassed and immediately pipetted into the gel tubes to give 9 cm of gel length. They were then overlaid with 1 cm of isobutanol (Neville, 1971), and allowed to polymerize at room temperature. Polymerization took about 30 min but the gels were left for another 1½ hours to be sure that the polymerization was complete. The gels contained 15% (w/v) acrylamide, 0.1% (w/v) bisacrylamide and 2.5 M urea.

The gel surfaces were washed with distilled water and rinsed with running buffer and pre-electrophoresed. This is to remove basic ions required for polymerization and at the same time to equilibrate the gels with running buffer. 0.9 N acetic acid at pH 2.65 was used as running buffer. A drop of α-naphthyl red (Koch-Light Lab.) solution was applied to one gel tube to act as a running indicator. Electrophoresis was carried out in a Shandon 8-place electrophoresis apparatus with 250 ml running buffer in each compartment, and run from anode to cathode with a constant current of 2 mA per gel. After running for 2.5 hours at room temperature the indicator dye had travelled two-thirds of the total gel length. The pre-electrophoresis was then terminated and the histone samples were applied to the gels. Histone samples (up to 100 µl) were suspended in 0.02 N H_2SO_4 and 5 M urea, mixed

with a drop of methyl-green solution, and layered onto the gels using a 'Finpipette'. Sample loading was performed without removal of the buffer. Electrophoresis was then continued for 3 hours, or until the indicator lines had migrated to approximately 5 mm from the lower ends of the tubes. If separation of histone H1 subfractions was the aim, the duration of electrophoresis was extended to 8 hours. After electrophoresis the tubes were immersed in iced water and the gels removed by gentle rimming with a fine hypodermic needle. The gels were then stained and destained as described below.

(C) SDS-polyacrylamide gel electrophoresis

This was by the method of Laemmli (1970) with some modifications. The gels were prepared from the following aqueous solutions;

Solution A - 60% (w/v) acrylamide - stored in a brown bottle and in the dark.

Solution B - 1.6% (w/v) bisacrylamide - stored in a brown bottle and in the dark.

Solution C - 0.4% (w/v) sodium dodecyl sulphate (SDS, sodium lauryl sulphate) -stored in the dark.

Solution D - 1.5 M Tris-HCl buffer, pH 8.8 - stored in a refrigerator.

Solution E - 0.5 M Tris-HCl buffer, pH 6.8 - stored in a refrigerator.

8 cm separation gels were prepared in 11 cm long glass tubes. The gel solution for 9 tubes was prepared by dissolving 0.016 g of ammonium persulphate in 2 ml distilled water, and adding 2 ml of solution B and 4 ml each of solutions A and D. The mixture was degassed and 4 ml of solution C and 16 µl of TEMED (Sigma) were added and mixed well. The gel solution was then pipetted

into tubes to give 8 cm of gel length. The gels were allowed to polymerize as described in section (B). The separation gels contained 15% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 0.1% (w/v) SDS and 0.375 M Tris-HCl, pH 8.8.

After the separation gels were completely polymerized, 1 cm stacking gels were layered over them. The stacking gel solution was prepared by dissolving 0.003 g of ammonium persulphate in 1.04 ml of distilled water, and adding 0.8 ml each of solutions E and C, 0.16 ml of solution A, 0.4 ml of solution B, and 3.2 µl of TEMED. This gel solution was layered on top of the separation gels after their surfaces had been washed with distilled water. The stacking gels were allowed to polymerize under isobutyric acid for 30 min. They contained 3% (w/v) acrylamide, 0.2% (w/v) bis-acrylamide, 0.1% (w/v) SDS and 0.125 M Tris-HCl, pH 6.8.

The standard molecular weight markers used were cytochrome-c (11,700 daltons), haemoglobin (15,500 daltons) and bovine serum albumin (65,000 daltons).

If the histone sample to be examined was in acid solution, the histone was precipitated by ethanol (80% final concentration), washed once with ethanol and dried before analysis. Samples of calf thymus histone, Acer histone and the standard markers were individually dissolved in loading buffer containing 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS and 0.0625 M Tris-HCl, pH 6.8. Standard markers were applied as a mixture, while histone samples of 15 µg were applied to each gel tube. A trace of bromophenol blue (BPB) was included in the sample solution, to act as a running indicator. All samples were applied onto separate gel tubes and electrophoresed, using a running buffer of 0.1% (w/v) SDS, 1.44% (w/v) glycine and 0.3% (w/v) Tris at pH 8.3.

Electrophoresis was carried out from cathode to anode at room temperature, at 2 mA per gel, and was continued for 3.5 hours. The gels were then removed from the tubes as described previously, and stained.

(D) Staining and destaining of gels

Gels were stained, in the appropriate staining solutions, in test tubes overnight at room temperature. Destaining, in the appropriate destaining solutions, was carried out in conical flasks shaken in a water bath at 55 °C. The destaining solutions were changed frequently until the destaining process was complete. The combination of staining and destaining solutions was as follows:

(a) Staining solution - 0.1% (w/v) Amido Black (naphthalene black 12 B) in 45.5% methanol, 9.1% acetic acid.

Destaining solution - 45.5% methanol and 9.1% acetic acid.

(b) Staining solution - 0.25% (w/v) Coomassie Brilliant Blue in 45.5% methanol, 9.1% acetic acid.

Destaining solution - the same as (a).

(c) Staining solution - 0.1% (w/v) Amido Black and 0.0125% (w/v) Ponceau S in 30% ethanol and 7% acetic acid.

Destaining solution - 40% ethanol.

(d) Staining solution - 0.01% (w/v) Bromophenol Blue in distilled water at pH 3.0.

Destaining solution - 40% n-propanol.

(E) Scanning of gels

Gels after destaining were transferred to a quartz container (0.7 cm X 11 cm). The container was then filled with destaining solution, and located on a gel scanning apparatus. Scanning was

carried out in a Beckman Spectrophotometer Model 24. A wavelength of 615 nm was used for gels stained with Amido Black. A wavelength of 570 nm was used for all other staining procedures.

(F) Slicing of gels

To measure the distribution of radioactivities along the gels, the gels were frozen in an aluminium trough on dry ice, and cut into 1.0 mm slices with a gel-slicer (the MICKLE laboratory Engineering Co.). Each slice was then held in a polycarbonate capsule, and ignited in an Oxymat (Intertechnique, Plaiser, France) prior to liquid scintillation counting.

10. Experiments with radioisotopes

(A) Radiochemicals

All radiochemicals were obtained as aqueous solutions from the Radiochemical Centre, Amersham, and stored in a deep-freeze at -25 °C. They were as follows :

L-[4,5-(n)-^{3}H]lysine monohydrochloride; specific activity 18 Ci mmole^{-1}, 1 mCi ml^{-1}.

L-[U-^{14}C]lysine monohydrochloride; high specific activity, 287 mCi mmole^{-1}, 50 µCi ml^{-1}.

L-[U-^{14}C]arginine monohydrochloride; high specific activity, 324 mCi mmole^{-1}, 50 µCi ml^{-1}.

L-[5-(n)-^{3}H] tryptophan, specific activity 29 Ci mmole^{-1}, 1 mCi ml^{-1}.

L-[U-^{14}C]leucine; specific activity, 324 mCi mmole^{-1}, 50 µCi ml^{-1}.

[methyl-^{3}H]-thymidine; specific activity 45 Ci mmole^{-1}, 1 mCi ml^{-1}.

[5-^{3}H]uridine; specific activity 29 Ci mmole^{-1}, 1 mCi ml^{-1}.

The incubation conditions involved in these experiments are described in Chapter 5.

(B) Experiments on asynchronous cultures

(a) Determination of radioisotopes remaining in the culture medium

Duplicate samples were treated as follows: 2 ml suspension was pipetted into a centrifuge tube, and spun immediately at 5,000 rpm for 3 min in an MSE Bench Centrifuge. 0.1 ml of the supernatant was removed from each tube, mixed and transferred into a disposable scintillation vial. 10 ml of scintillation fluid was then added and the mixture shaken well and counted. as described later.

(b) Determination of radioisotope uptake by the cells

The packed cell volume after centrifugation of the samples described above was recorded, the supernatant removed and the cells suspended in 4 ml of a culture medium modified to contain 'cold' chemicals corresponding to the isotopes used, but at 100X their concentration. The resulting cell suspension was stirred with a Whirlimixer, and the cells then collected on a Sartorious-membranfilter (25 mm dia., 1.2 μ pore size). The filter had been pre-soaked with modified culture medium. Rapid filtration was achieved with a filter apparatus, which could filter 4 samples simultaneously. Samples were drained under vacuum and the filtrates collected in a waste receiver. After washing 5 times with 'cold' medium, the sample-bearing filters were folded into capsules, ignited in the Oxymat and the radioactivity was measured, as described later. The radioactivity obtained from each sample was converted to radioactivity per unit packed cell volume, and the average of the corrected radioactivities from duplicate samples recorded.

(c) Determination of radioisotope incorporation into macromolecules

Duplicate samples were taken from cultured suspension at each sampling time. 2 ml suspension was pipetted into 2 ml of 10% ice-cold 'cold' TCA*, left on ice for a period of time, and then centrifuged to obtain the packed cell volume. The supernatant was removed and the cells were resuspended in 4 ml of ice-cold 'cold' 5% TCA.

For determination of the incorporation into DNA, the cells were collected on Satorious membranfilters, washed 5 times with ice-cold 'cold' 5% TCA (4 ml each time). Samples were then counted as described later.

For determination of the incorporation into protein, the cells were washed twice with 4 ml of ice-cold 'cold' 5% TCA, suspended in 2 ml of the same 5% TCA, and boiled for 15 min to hydrolyze the DNA. After chilling the sample in ice, cells were collected on filters previously saturated with the ice-cold 'cold' 5% TCA, and then washed and counted as described later.

(d) Double-labelling concomitant with a pulse-chase experiment

This experiment was performed to study the location of histone biosynthesis. The procedure was as follows :

2 flasks of 7-day old stock culture were bulked together. The flask was left on a bench until the cells had settled

* The 'cold' 10% TCA contained 10%(w/v) TCA plus 'cold' compound at 200X the concentration of the isotope-labelled compound present in the incubated suspension. The 'cold' 5% TCA is half-strength 'cold' 10% TCA

(c. 5 min). Two-thirds of the medium was then removed to increase the cell density in the suspension. This concentrated culture suspension contained 3.56×10^6 cells ml^{-1}. The double-labelling mixture was prepared by mixing 40 µl of ^{3}H-tryptophan with 370 µl of ^{14}C-lysine, using a micropipette.

For the 'pulse-chase'treatment, 200 µl of the double-labelling mixture was added to 25 ml of the concentrated culture suspension, and the culture shaken in a water bath at 25 °C(the final concentration of ^{14}C-lysine was 1.31×10^{-6}M, and ^{3}H-tryptophan was 2.76×10^{-8}M). After 5 min shaking, 100 ml of fresh 'cold' culture medium (Heller's medium containing 2×10^{-5}M lysine and 2×10^{-5}M tryptophan) was added and shaking continued for a further 10 min. 5 ml of the suspension was then pipetted into 5 ml of ice-cold 'cold' culture medium, and a second 5 ml suspension was pipetted into 5 ml of ice-cold 'cold' 10% TCA (10% TCA containing 2×10^{-5}M lysine and 2×10^{-5}M tryptophan). These two samples were kept on ice and were later used for determination of total uptake and incorporation into protein. Soon after this sampling, the rest of the culture was filtered through a nylon cloth, and the cells washed 3 times with 200 ml ice-cold 'cold' culture medium, and finally suspended in 18 ml of grinding medium for nuclear isolation. These cells were fractionated into cytoplasmic and nuclear fractions as described below.

For the 'pulse' treatment, 200 µl of the double-labelled mixture was added to 25 ml of the concentrated culture suspension. After 5 min shaking at 25 °C, the incubation was

stopped by adding 100 ml of ice-cold 'cold' culture medium. Two samples (each 5 ml) were taken, and the remaining cells were filtered, washed and suspended in 18 ml of grinding medium as described above.

The fractionation of labelled cells into cytoplasmic and nuclear fractions was performed as follows: cells were homogenized with a glass Potter-Elvehjem homogenizer as described in the Methods (p. 25). To the ruptured suspension was added 6 ml of dilution buffer, and 5 ml was withdrawn for determination of total protein incorporation. The rest of the suspension was filtered through 4 layers of Miracloth, and centrifuged at 4,500 Xg for 20 min. The supernatant (cytoplasmic fraction) was pipetted into a beaker, and the pellet washed twice with washing medium and finally suspended in 5 ml of washing medium to give the nuclear fraction.

An equal volume of ice-cold 'cold' 10% TCA was added to the total nuclear fraction and to 7 ml of the cytoplasmic fraction. These, together with other samples for incorporation tests, were left overnight in a refrigerator, and were then treated as described above in (c).

The samples taken immediately after incubation for uptake determination were treated as described in (b).

(e) Effect of hydroxyurea on histone biosynthesis

The cell density of a 7-day old stock culture was increased by sedimentation and decantation, as described in (d) above, to give a suspension containing 1.36×10^6 cells ml^{-1}. This suspension was then incubated with ^{14}C-leucine at a final concentration of 0.25 $\mu Ci\ ml^{-1}$ (6.76×10^{-7} M) in the presence

of different concentrations of hydroxyurea. The incubation was carried out in 25 ml conical flasks. 25 µl of ^{14}C-leucine (50 µCi ml^{-1}) and 250 µl of hydroxyurea (at different concentrations) were pipetted down the sides of the flask to avoid mixing, and then 5 ml of concentrated culture suspension was introduced to initiate the incubation. After shaking for 20 min in a water bath at 25°C, 10 ml of ice-cold Heller's medium containing 2 X 10^{-4} M leucine was added to the suspension. Triplicate samples, each of 4 ml, were taken and added to 4 ml of ice-cold 10% TCA containing 2 X 10^{-4} M leucine. The final concentrations of hydroxyurea in the culture suspension were 0, 1 X 10^{-6}M, 1 X 10^{-5}M, 1 X 10^{-4}M, 1 X 10^{-3}M and 1 X 10^{-2}M.

Samples were left in ice for 2 hours and the packed cell volume was recorded for each sample after centrifugation. The pellets were each washed once with 4 ml of ice-cold 'cold' 5% TCA, and were then suspended in 2 ml of 'cold' 5% TCA. The suspensions were boiled for 20 min, chilled in ice for 30 min, and finally spun down in a bench centrifuge. The supernatants were discarded, and the pellets of 2 of the sample sets were separately collected on Satorious membranfilters, and washed and counted as described in (c) above. The remaining set of samples was used to determine the incorporation into non-basic (HCl-insoluble) proteins. To do this the samples were suspended individually in 2 ml of 0.25 N HCl containing 2 X 10^{-4}M leucine, and left overnight in a refrigerator. The HCl-insoluble fraction was pelleted by centrifugation, washed once with 0.25 N HCl containing 2 X 10^{-4} M leucine, and finally suspended in ice-cold 'cold'

5% TCA. This HCl-insoluble protein was then collected and counted. All radioactivities were presented as counts per minute (cpm) per unit packed cell volume.

(C) Experiments on synchronous cultures

(a) Biosynthesis of macromolecules

The culture suspension was sampled every 2-6 hours once the cell density had reached 2 X 10^5 cells ml^{-1}. About 500 ml of culture suspension was harvested manually each time. From this 20 ml was taken for the following biosynthetic tests. The biosyntheses of DNA, RNA, basic protein and non-basic protein were detected respectively by following the incorporation of ^{3}H-thymidine (^{3}H-dT), ^{3}H-uridine (^{3}H-U), ^{3}H-lysine (^{3}H-lys), and ^{3}H-tryptophan (^{3}H-trp) into their respective macromolecules. This was achieved by introducing 5 ml aliquots of suspension into separate 25 ml conical flasks containing 5 µl of ^{3}H-dT, ^{3}H-U , ^{3}H-lys, or ^{3}H-trp. The final concentration of radioisotope in suspension was 1 µCi ml^{-1}. A zero time sample (1 ml) was taken from each flask and then the flasks were shaken in a water bath at 25 °C. After 15 min shaking, duplicate samples (each 1 ml) were taken. The samples were immediately added to 1 ml of ice-cold 'cold' 10% TCA (10% TCA containing 5 X 10^{-4}M 'cold' compound corresponding to the labelling-~~isotope~~ compound involved), and the incorporation into the respective macromolecules was determined, as described in the Methods (p. 39). Since these incorporation tests were performed frequently during the growth of a synchronous culture, the samples for ^{3}H-lys and ^{3}H-trp incorporation were not heated to remove nucleic acid; (this was to save time in a very heavy experimental schedule). The remaining 2 ml of suspension was

shaken for a further 15 min and the cells were spun down and fixed in 50% formic acid. These fixed samples were stored in a refrigerator until required for autoradiographic preparation.

(b) Specific activity of histone synthesized

^{3}H-lysine (10 Ci mmole^{-1}, 1 mCi ml^{-1}) was diluted 10 times with distilled water. 100 µl of this diluted ^{3}H-lys was added to 100 ml of cell suspension taken from a synchronous culture. After shaking at 25 °C for 1 hour, cells were harvested by filtration through nylon cloth, washed 3 times with a total of 400 ml ice-cold 1 X 10^{-6} M lysine, and finally suspended in 15 ml of grinding medium. The cells were then stored in a deep-freeze until required for analysis (the actual volumes of culture and isotope solution were different on different occasions but the ratio of the two always remained the same). The histones were extracted from these labelled cells and the specific activity of the histone synthesized calculated.

(D) Measurement of radioactivities

(a) Reagents

(i) For measuring radioactivity of ^{3}H, 1.0 litre of the scintillation fluid contained dioxane (700 ml), toluene (300 ml), naphthalene (20 g) and Butyl-PBD (7 g).

(ii) For measuring radioactivity of ^{14}C, 1.0 litre of the scintillation fluid contained phenylethylamine (330 ml), methanol (220 ml), toluene (400 ml), Butyl-PBD (7 g) and distilled water (50 ml).

(iii) Washing fluid, which was required when samples were ignited in an Oxymat (Intertechnique, Plaiser, France).

The washing buffer is a mixture of methanol (660 ml) and toluene (340 ml). The Oxymat is a commercial version of the Peterson apparatus (Peterson, Wagner, Seigel and Nixon, 1969).

All radioactive samples were retained in disposable scintillation vials and counted with a Beckman LS-100 Scintillation Counter.

(b) Methods

(i) 50-200 µl of liquid sample was added to 10-20 ml of scintillation fluid, mixed well and counted.

(ii) Samples collected on filter papers were dried under an infra-red lamp, cooled and counted in 5 ml of scintillation fluid.

(iii) Gel slices were held in polycarbonate capsules (LEXAN R) and ignited in an Oxymat, using the appropriate scintillation fluid. In some experiments, liquid samples and sample-bearing filters were also ignited in an Oxymat and counted. The percentages of the recovery and the memory were checked along each set of samples. The recovery percentage is always over 92% while the memory is less than 2%.

The Beckman LS-100 Scintillation Counter gave 37.5% and 72.7% counting efficiencies respectively for ^{3}H and ^{14}C when samples were counted in the appropriate fluids.

CHAPTER 3

The Development of a Nuclear Isolation Technique

1. Introduction
2. Advantages of preserving cells in glycerol-containing medium
3. Development of a grinding medium
4. Choice of a method for cell rupture
5. Separation of nuclei from other cellular material
6. Conclusion

1. Introduction

The initial problem in the isolation of the nuclear proteins is ascertaining that they come from the nucleus, and that cytoplasmic proteins are not present in the products isolated. As far as the isolation of nuclear histone is concerned, especial care has to be exerted to prevent contamination by cytoplasmic contents, which are also a source of basic proteins. Butler, Cohn and Simson (1960), Crampton and Petermann (1959) and Leslei (1961) have indicated that microsomes of rat liver contain proteins similar in composition to the histones found in the nucleus. Electrophoresis also shows that ribosomes and chromosomes from chicken liver contain similar basic proteins (Lindsay, 1966). The yield of basic proteins from microsomes of Physarum is 6 times that of the nuclear histones (Mohberg and Rusch, 1970). Basic ribosomal proteins from five mammalian tissues were found to possess similar electrophoretic patterns, giving 24 bands in polyacrylamide gels at pH 4.5 (Low and Wool, 1967). Proteins obtained by acid extraction of mitochondria (Zahler et al., 1968; Mohberg and Rusch, 1970) have been found to interfere with electrophoretic characterization of histones. Moreover, histones and histone-like basic proteins are found widely distributed in the Chinese hamster ovary cells (Shepherd and Noland, 1968). A very pure chromatin or nuclear preparation was therefore considered essential as starting material for the isolation of an uncontaminated nuclear histone fraction.

Chromatin prepared from calf thymus by the method of Zubay and Doty (1959) contains proteins of which more than 60% are of cytoplasmic origin (John and Forrester, 1969). These cytoplasmic proteins are assumed to have been adsorbed onto the chromatin during its extraction with saline-EDTA. Although these cytoplasmic proteins

could be removed by extraction with 0.3 N NaCl (Johns and Forrester, 1969; Spelsberg and Hnilica, 1971), the studies of Wilhelm, Groves and Hnilica (1972) indicate that 0.3 N NaCl also removes variable amounts of tissue-specific chromosomal proteins. Chromatin prepared from sea urchin embryos by centrifugation through dense sucrose, as described by Bonner et al.(1968), contained cytoplasmic proteins accounting for 30% of its total non-histone protein (Hill, Poccia and Doty, 1971). The presence of cytoplasmic material in chromatin prepared by this method was also detected by an immunological technique (Ševaljević, 1974). In contrast, it was found that chromatin prepared from purified nuclei consistently contained a very low level of cytoplasmic contaminants, accounting for only 5% or less of its total non-histone protein (Bhorjee and Pederson, 1972; Wilhelm et al., 1972; Augenlicht and Baserga, 1973). A series of experiments performed by Tata, Hamilton and Cole (1972) compared the nature of the contaminants on chromatin when different preparation techniques were followed. They found that chromatin prepared from whole tissue of rat liver by the method of Marushige and Bonner (1966) contained gross contamination derived from cytoplasmic membranes. The chromatin so prepared also possessed a high level of protease activity. By contrast, chromatin prepared from nuclei, especially nuclei which had been washed with Triton X-100 to remove the nuclear membranes, was contaminated with cytoplasmic material to a much lesser extent, as judged both from the activities of marker enzymes and the phospholipid content (Tata et al., 1972). Similar results have been obtained by other workers (Phillips and Johns, 1959; Haussler et al., 1969; Harlow et al., 1972).

Previously, chromatin of Acer pseudoplatanus has been extracted from whole cultured cells. The cells were washed, suspended in various

extraction media, packed in Visking tubing (Scientific Instrument Centre, Ltd., London) and frozen in liquid nitrogen. Rupturing of the cells was achieved by pressing the frozen cells against a rapidly revolving carborundum pad (Jennings and Street, 1974). Extraction media tested were sucrose-containing media (Flamm et al., 1963; Bonner et al., 1968), hypotonic buffer (Monahan and Hall, 1973) and standard saline citrate, SSC, (Gould, 1975). It was found that although the cells were 100% ruptured, only a relatively small portion of the cellular DNA content was recovered in the crude chromatin. For example, with SSC only 6% of the DNA was recovered in the chromatin. Moreover, the basic proteins prepared from this chromatin by the acid-ethanol method contained a large quantity of material other than histones, as shown by the relatively small proportion of the extracted material which co-migrated with calf thymus histone standards when electrophoresed on polyacrylamide gels. It seems that with cultured cells of Acer, as in most other cells, the contamination of chromatin with cytoplasmic material is a major hazard when chromatin is recovered from whole cells. Such 'straight chromatin' extraction methods are in fact only to be recommended for those cell types from which it is not feasible to prepare purified nuclei.

The aim must therefore be to obtain nuclei in high yield and purity with preservation of their native structure and function. There are numerous extraction procedures described in the literature which are used for extraction of nuclei from cells or tissues of higher animals (Smuckler et al., 1976). These have been developed for particular experimental systems. Thus, a method suitable for liver may not be suitable for muscle or brain. Each tissue and species presents its own problems, and these can be overcome only by trial and error. In addition, the toughness of the cell wall and the presence of large amounts of storage material in higher plant cells are problems not generally

encountered in the isolation of nuclei from animal cells.

Because of the fundamental structural difference between plant and animal cells, references to the isolation of nuclei from higher plants are few in contrast to the great number of papers dealing with the isolation of nuclei from animal tissues. The methods that have so far been reported for the isolation of plant nuclei can be classified into 4 groups : (i) involving enzyme treatment prior to the isolation of nuclei as for onion roots (Brown, 1955), and rice suspension cells (App and Granados, 1974); (ii) Behrens' method, which involved the application of a non-aqueous grinding medium, as for wheat germ (Stern and Mirsky, 1952) and pollen of Lilium longiflorum (Sheridan, 1973); (iii) the use of sucrose-containing grinding media, as for wheat embryo (Johnston et al., 1957), cultured tobacco cells (Flamm et al., 1963) and pea stems (Birnstiel et al., 1962); and (iv) the use of gum arabic and n-octylalcohol containing grinding medium, as for tobacco leaves (Kuehl, 1964), roots of Vicia faba (Dick, 1968) and cultured tobacco and soybean cells (Matthysse and Phillips, 1969). Each method has its own advantages and disadvantages.

As far as the present investigation is concerned, three criteria must be met when a method is selected for the isolation of nuclei from Acer cells. The method should (a) include a preservation step, to retain the representative samples at their particular cell-cycle stages; (b) comprise a simple isolation procedure and (c) be able to produce a reasonable yield and purity. A general consideration of the 4 methods just mentioned is as follows : (i) the involvement of enzyme treatment apparently does not fulfill criterion (a); (ii) Behrens' method is rather time consuming and the purity of the nuclei is often unsatisfactory, i.e., it does not meet the criteria (b) and (c); (iii) the use of sucrose-containing medium can not yield intact nuclei from Acer cells and (iv)

Kuehl's procedure does not cover criterion (a). Therefore, it is necessary to develop an improved isolation method to meet the requirements.

2. Advantages of preserving cells in glycerol-containing medium

Because it was planned to study histone biosynthesis through the cell cycle, it was necessary that samples of cells collected at each particular stage of the cell cycle be preserved in a way which did not change their physical or biochemical characteristics. In general, the representative cell samples can be quickly frozen in liquid nitrogen or suspended in viscous media and kept at subzero temperatures (Smuckler et al., 1976). The disadvantage of freezing is the formation of ice-crystal artifacts, which have been found to destroy the nuclear structure prior to the isolation of the nuclei. Histones prepared from frozen calf thymus were found to be less stable than those from fresh material (Neelin and Neelin, 1960), but viscous media containing glycerol have been successfully used for preserving RNA polymerase from bacterial source (Nakamoto et al., 1964). There are also reports on preserving nuclei in glycerol-containing media. Examples are the preservation of the chick oviduct nuclei in 30% glycerol at -20 °C (McGuire and O'Malley, 1968) and rat liver nuclei in 70% glycerol at -20 °C or -196 °C (Read and Mauritzen, 1970). The structure and enzyme activity of these nuclei were found rather stable under the conditions employed. Moreover, chromatin isolated from calf thymus was found to retain its native structure in 60% glycerol at -20 °C for a long period (Garrett, 1971), and soluble nucleohistone was successfully stored in 40% glycerol at -20 °C for 3 months or more (Bonner et al., 1968).

These findings prompted attempts to preserve cultured cells of Acer pseudoplatanus L. in a glycerol-containing medium. Since a 70% (v/v) glycerol-containing medium was later developed as grinding

medium, it is thus also used as the medium for cell preservation. The representative cell samples were collected by filtration and then suspended in the grinding medium and kept at -18 °C. When the ruptured cell suspensions from such unfrozen samples were compared with those obtained after freezing in liquid nitrogen it was found that more intact nuclei were present in the unfrozen samples. The histones isolated from cells which had been preserved in grinding medium at -18 °C for five months did not differ in their characteristics on polyacrylamide gels from those of freshly harvested cells. In fact, it proved easier to rupture cells which had been preserved for a period at -18 °C.

3. Development of grinding medium

The general procedure for the isolation of nuclei is to break cells of a given sample and release their contents, with the nuclei intact and unaltered, into a suitable medium, followed by the removal of debris and contaminants. The composition of the grinding medium plays an important role in determining the character of the homogenate and the extent to which the organelles remain unaltered in structure and function. Media used for the isolation of nuclei fall into 2 main groups : those in which non-aqueous media are employed, and those based on the use of aqueous solutions of sucrose or salts. The former method, based on Behrens' procedure (1932) and its subsequent modifications (Allfrey et al., 1952; Siebert, 1963) faces the primary problem of achieving satisfactory removal of cytoplasm so that there is little or no adherence of cytoplasmic components to the nuclei (Maggio et al., 1963). When applied to wheat germ, which is rich in nuclei, considerable cytoplasmic contamination persists (Stern and Mirsky, 1952). With the aqueous media commonly used for nuclear fractionations, the primary problem is that most of plant nuclei break and disperse their contents. Aqueous media

also tend to result in loss from cell organelles of soluble components important to cell function (Antoni et al., 1962; Schneider, 1952). However, replacement of the sucrose with glycerol makes the isolation of nuclei in aqueous media superior to isolation with organic solvents. Treatment with 40% to 80% glycerol does not damage the morphological appearance of ascites tumour nuclei (Antoni et al., 1962). Further, it inhibits the activities of several enzymes, such as RNase (Dounce, 1955; Schneider, 1955) and protease (Philpot and Stanier, 1956; Phillips and Johns, 1959). The inhibition of protease is important since the degradation of histones by proteolysis has been considered to occur during nuclear preparation (Crampton, 1959) and the time when most serious degradation occurs is after the rupture of the cells and before separation of the histone from the deoxyribonucleoprotein (Stellwagen et al., 1968). The glycerol medium also preserves the extractability of nucleohistone from nuclei in a homogenate left for several hours at room temperature, thus suggesting it also inhibits DNase (Philpot and Stanier, 1956). Moreover, the method utilizing glycerol as an isolating medium requires considerably less time than Behrens' method, and it avoids freeze-drying. There is also evidence that glycerol medium, unlike other aqueous media, does not permit loss of protein or nucleic acid from the nucleus (Schneider, 1952; Dallam et al., 1953). The concentration of 70% glycerol selected for the grinding medium represents the highest concentration which could be manipulated conveniently under the conditions employed. Nuclei prepared in 70% (v/v) glycerol alone, however, showed the features typical of fixed nuclei, including condensed chromatin and nuclear shrinkage. To overcome this problem the inclusion of divalent cations was found to be necessary. The role of divalent cations in maintaining the integrity of nuclei (Johnston et al., 1957; Løvtrup-Rein and McEwen, 1966), and chromosomes (Naore et al., 1961; Somers et al., 1963) is well documented,

and it has been shown that they function, at least in part, by facilitating the interaction of DNA and histone (Johns and Forrester, 1969). Magnesium was found to help maintain the nuclear chromatin in a close-packed condition (Kabat, 1967). Calcium acts as a nuclear stabilizer, hardening the nuclei and hence preventing their further breakage. Low concentrations of divalent cations have been used to prevent clumping of rat liver nuclei (Philpot and Stanier, 1956; Wang, 1961). A deficiency of calcium and magnesium in the grinding medium has been reported to result in chromosome breakage (Steffenson, 1953, 1955). In the absence of calcium, there is disruption of plasma membranes and associated nuclear disintegration (Avis, 1972). Further, the template availability of nuclear DNA has been found to be enhanced when the nuclei are isolated in the presence of divalent cations (Waqar et al., 1971).

In addition to the stabilizing agents (calcium and magnesium), the pH of the grinding medium also has an important influence on the nature of cell fractionation. Acidic media promote loss of histone during the nuclear isolation (Dounce et al., 1966). A slightly alkaline medium, however, yields nuclei that retain functional activity, and morphological features close to the state in situ (Smuckler et al., 1976). The buffering agent Tris also acts as an ionic stabilizer.

Generally speaking, the choice of medium can only be made empirically, on the basis of experiments which test the isolated sub-cellular component for retention of its structure and function. Regarding the essential factors being discussed, a grinding medium consisting of 20 mM Tris-buffer at pH 7.8, 1 mM Mg acetate, 2 mM $CaCl_2$ and 70% (v/v) glycerol was selected for the isolation of nuclei from cultured cells of Acer pseudoplatanus L.

4. Choice of a method for cell rupture

Anyone who attempts the isolation of nuclei immediately becomes aware of the difficulty of breaking the cells whilst leaving the nuclei intact. Each plant cell is surrounded by a tough wall composed of cellulose and various other polysaccharides; a treatment vigorous enough to disrupt this wall normally also disrupts the nucleus. The cell homogenization step is well known to be the least reproducible part of any cell fractionation technique. Of the mechanical methods available, the Potter-Elvehjem homogenizer appears to cause least damage to the cellular components. Using the selected grinding medium, the cell walls of cultured Acer cells were found to be comparatively easily broken at -20 °C with a Potter-Elvehjem homogenizer, whilst the nuclear membrane was less easily ruptured under the conditions employed. To determine the extent of cell breakage the ruptured suspension was examined by mixing with an equal volume of ethidium bromide (EB) and observing under UV light alone or simultaneously with low intensity tungsten light. By using EB as a fluorescent probe, nuclei fluoresce a bright orange-yellow colour (Hoffmann, 1973; Lawrence and Daune, 1976). Figure 5 shows the appearance of intact cells before homogenization. Samples were added EB and photographed in bright tungsten light (Fig. 5a), and in low intensity tungsten light plus UV light (Fig. 5b). The strong fluorescing spots shown in Fig. 5b indicate the location of nuclei; these sometimes do not appear distinctly in an ordinary phase microscope image (Fig. 5a). This fluorescent probe is particularly effective for the examination of nuclei within cells in a clump, as seen in Fig. 5c. Figure 6 shows the appearance of a ruptured suspension after homogenization with 2 complete passess in a Potter-Elvehjem homogenizer. The disrupted cells are not broken into small pieces, but rather appear to be structurally distorted

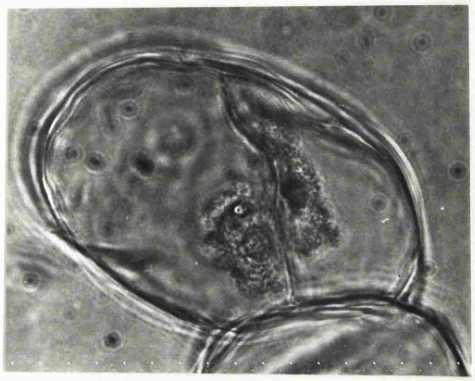
Figure 5

Phase contrast micrographs of cultured cells of *Acer pseudoplatanus* L. in grinding medium before homogenization

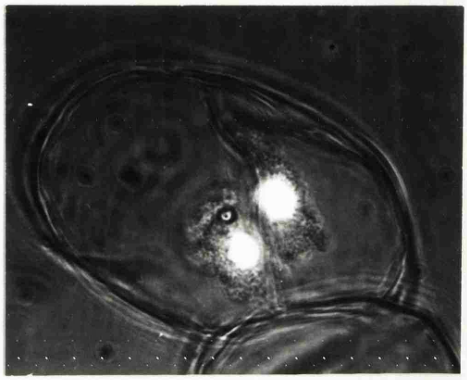
To the cell suspension was added an equal volume of ethidium bromide solution prior to photography (a) in tungsten light (X700), (b) in UV light together with low intensity tungsten light (X700), (c) in UV light (X470).

Fig. 5

a.



b.



c.

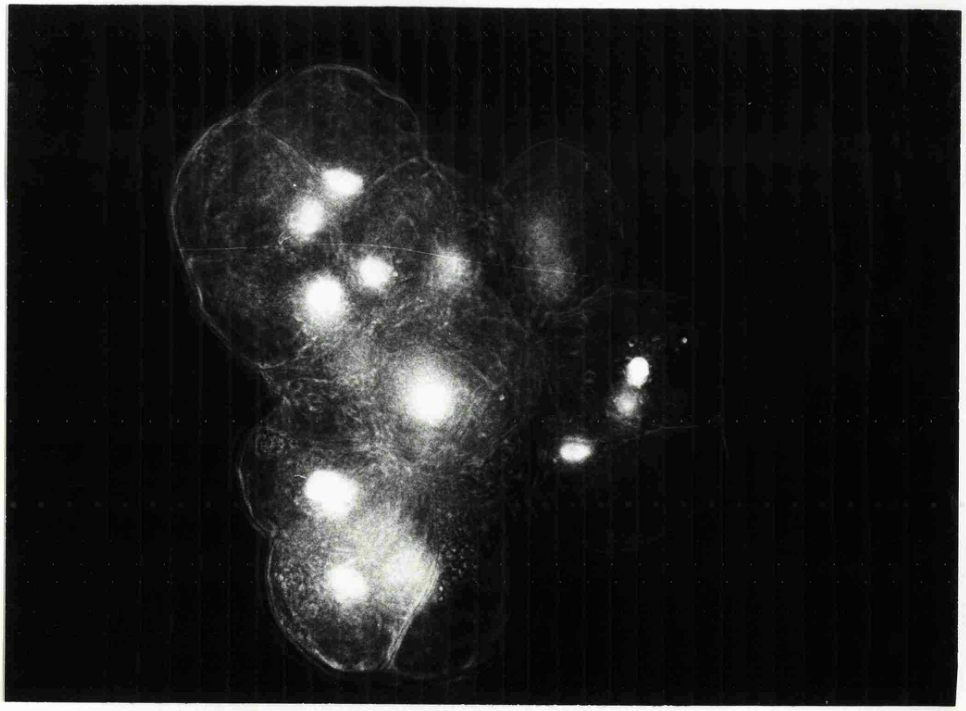


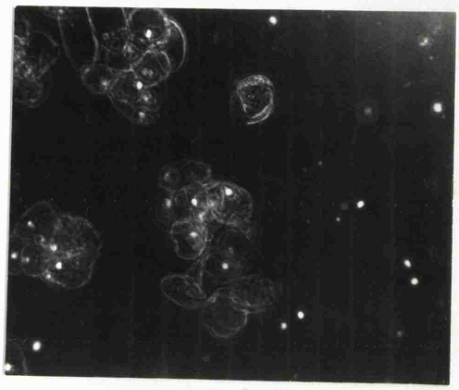
Figure 6

Phase contrast micrographs of the ruptured suspension of *Acer pseudoplatanus* L.

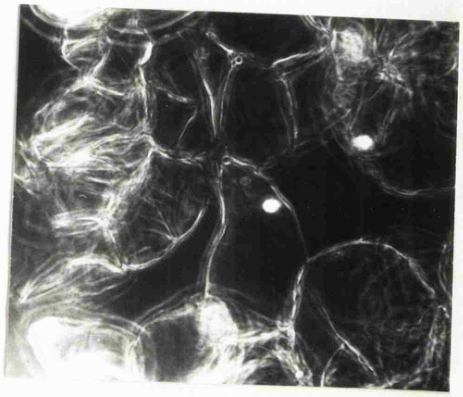
The cell suspension was homogenized with 2 complete strokes in a glass Potter-Elvehjem homogenizer. To the ruptured suspension was added an equal volume of ethidium bromide solution prior to photography (a) in UV light (X100), (b) in UV light (X280), (c) in tungsten light (X470).

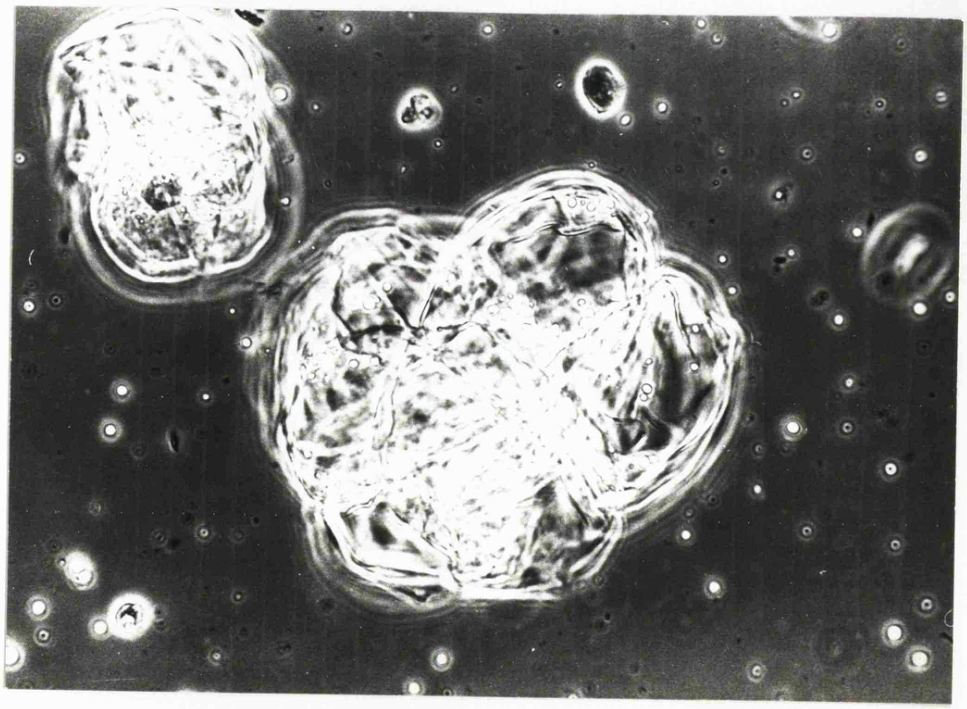
Fig. 6

a.



b.





c.

and freed of their nuclei. The intact nuclei are not bound with cytoplasmic slurry but float freely outside the cells. Fig. 6a shows the ruptured suspension at low magnification. Figs. 6b and 6c show, at higher magnification, ruptured cells and the intact free nuclei. Fig. 6c is an ordinary phase contrast view whilst Figs. 6a and 6b have used EB as a fluorescent probe. The application of 2 complete strokes to the cell suspension only breaks about 30% of the cells. However, more prolonged homogenization does not produce higher yield of intact nuclei. Table 3 shows that samples submitted to a four-stroke treatment yielded less intact nuclei than the same sample treated with only one stroke, but that the highest yield occurred with two strokes.

Table 3. Effect of number of strokes in a glass Potter-Elvehjem homogenizer on release of intact nuclei from cultured cells of Acer pseudoplatanus L.

Strokes applied	1	2	4
Intact nuclei*	266	320	249

* The average number of intact nuclei in ten counts. Each count represents the number of nuclei in 20 fields under a phase contrast microscope. All standard errors are less than 10%.

Although nuclear membranes are moderately resistant to rupture in the grinding medium at -20 °C, the prolongation of the homogenization time means that those nuclei which are liberated early in the procedure are themselves exposed to shearing forces. In other words, increase in homogenization time does actually rupture more cells, but at the same time there is more disruption of the nuclei already released.

The cell concentration was also found to affect the yield of nuclei. Too low a cell concentration reduces nuclear yield and requires extension of the rupture process. Similarly, at too high a density yield is reduced; 15% yield was recorded for 1 g cells in 2 ml grinding medium 20% for 1 g cells in 4 ml grinding medium. In the standard procedure the latter concentration was adopted.

5. Separation of nuclei from other cellular material

After homogenization the ruptured suspension contains a mixture of whole cells, broken cells, intact nuclei, nuclear fragments and cytoplasmic contents. The high concentration of glycerol in the suspension impeded the separation of the nuclei from other contaminants by centrifugation. To overcome this problem, a dilution buffer was added to the homogenate, thereby reducing the glycerol concentration and at the same time introducing Triton X-100. Triton X-100 has frequently been used to remove cytoplasmic remnants, to destroy cell membranes, and to strip off outer nuclear membranes (Hymer, 1963; Løvtrup-Rein, 1966; Roodyn, 1972; Smuckler et al., 1976). To separate the nuclei from the cell debris, the diluted ruptured suspension was filtered through 4 layers of Miracloth. This effectively removed whole cells, broken cells and other large pieces of cell debris. The filtrate contained intact nuclei, starch grains and cytoplasmic fragments.

To remove cytoplasmic contamination such a filtrate is normally centrifuged to obtain a crude nuclear pellet, and this pellet is then washed repeatedly and purified. The centrifugation should be just sufficient to sediment the nuclei, with a minimum of sedimentation of other particles. In the present work with 52.5% glycerol, it was found that almost all intact nuclei were sedimented after centrifugation at 1,500 Xg for 30 min in an MSE High Speed 18 centrifuge. When the

supernatant was examined by phase contrast microscopy it was found to contain no intact nuclei, but mainly cytoplasmic granules.

An experiment was designed to investigate the effect of glycerol concentration on the recovery of nuclei. This was expressed as the percentage of DNA in the initial filtrate which was recovered in the nuclear pellet after centrifugation. The experimental procedure was as follows. The ruptured suspension obtained from a 14-day old asynchronous culture was filtered and divided into 4 equal samples. Samples were then treated to give different final glycerol concentrations as follows:

(A) initial filtrate without dilution (final glycerol concentration is 70%)

(B) 1/3 volume of grinding medium was added (final glycerol concentration is 70%)

(C) 1/3 volume of dilution buffer was added (final glycerol concentration is 52.5%)

(D) 1 volume of dilution buffer was added (final glycerol concentration is 35%).

The samples were mixed well after dilution and then centrifuged at 4,250 Xg in an MSE High Speed 18 centrifuge for 20 min. The nuclear pellets obtained were then washed once with washing buffers, with their respective glycerol concentrations, prior to the second centrifugation. The following recoveries of nuclei were obtained: 64.3%, 24.2%, 81.7% and 66.2% for samples of (A), (B), (C) and (D) respectively. These data indicated that 70% glycerol gave poor recovery of the nuclei, especially when the initial nuclear concentration was low. The nuclei were not stable if the glycerol concentration was too low. They were stabilized better in 52.5% glycerol than in 35% glycerol. Consequently, nuclei were subsequently harvested and washed in 52.5% glycerol, together with

other reagents, as described in the Methods (p. 24). The use of 52.5% glycerol agrees with the findings of Antoni (1962) concerning the effect of glycerol concentration on the RNA, DNA and protein contents of ascites tumour cells. As the glycerol concentration was increased there was no loss of cellular DNA, but at about 40% glycerol there occurred a sharp loss of RNA and protein which did not further increase at higher glycerol concentration. This loss of RNA and protein was found to be mainly of cytoplasmic origin. This suggests that the presence of 52.5% glycerol in my washing medium may also favour removal of cytoplasmic contaminants.

After the nuclear fraction was washed three times with washing medium, the resulting nuclear pellet was resuspended in washing medium and purified by centrifugation through grinding medium, as described in the Methods (p. 25). This final step in the nuclear preparation is to purify further the nuclear fraction, and at the same time to terminate the exposure of nuclei to the Triton X-100. Since Triton X-100 may cause release of lysosomal enzymes (Hyodo and Ono, 1970), and the exposure of nuclei to detergents has been reported to result in a partial solubilization and loss of both basic and acidic nuclear proteins (Ginzburg-Teitz *et al.*, 1967; Johnson and Hnilica, 1970), it should be removed during the final purification. When the purified nuclei were resuspended in grinding medium and examined by phase contrast microscopy using EB as a fluorescent probe, they appeared to be morphologically intact (Fig. 7a). The nuclei were surrounded by many shiny particles which were considered to be starch grains (Fig. 7b).

The isolated nuclear fraction after being stored at -18 °C for 1 week still possessed RNA synthesizing activity. An aliquot (100 µl) of the nuclear suspension (in grinding medium) was incubated with 100 µl of a medium containing the 4 nucleoside triphosphates; after shaking

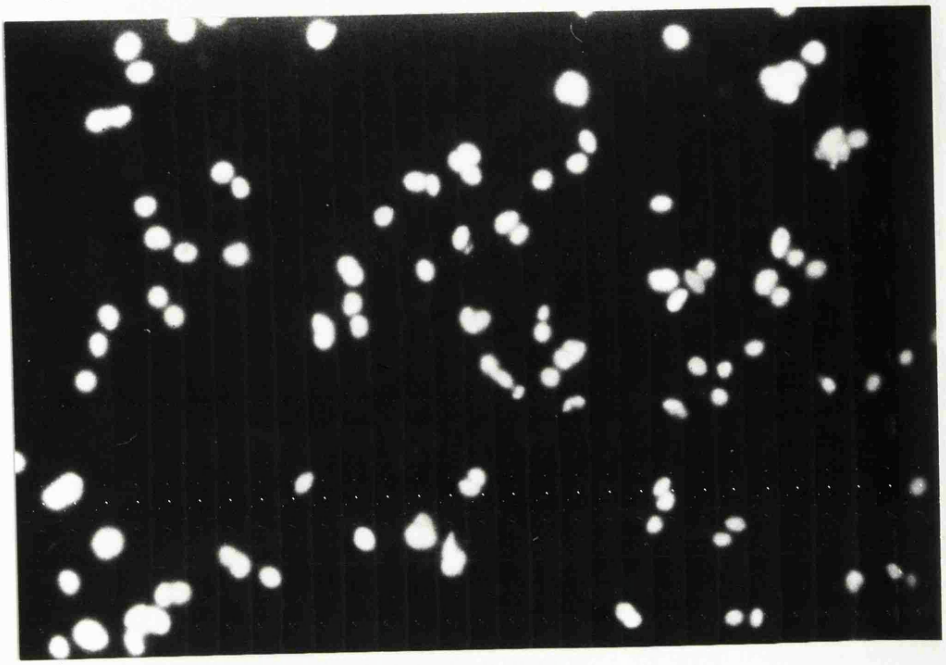
Figure 7

Phase contrast micrographs of isolated nuclei of *Acer pseudoplatanus* L.

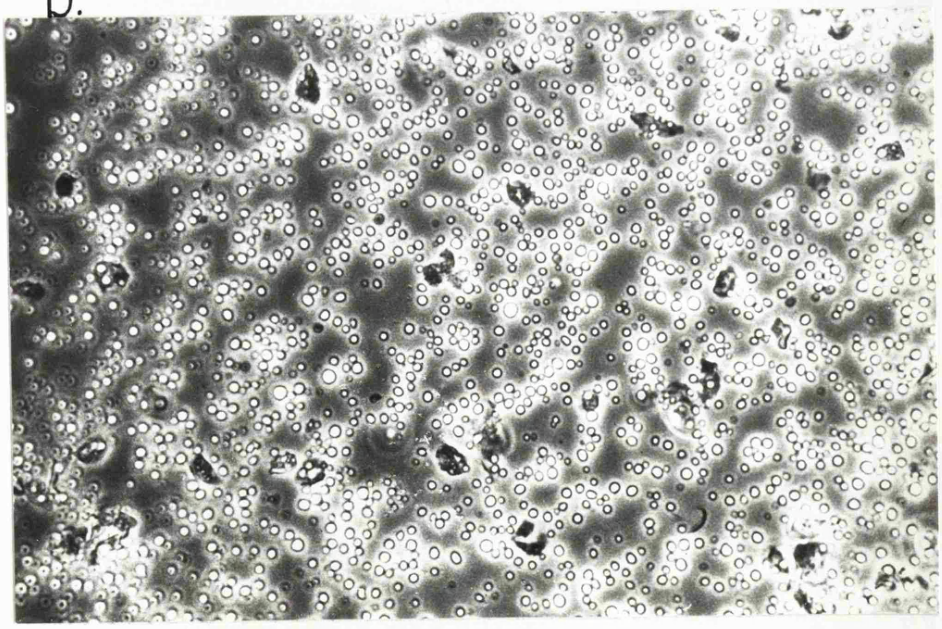
The isolated nuclei were suspended in grinding medium, and photographed (a) in UV light following addition of an equal volume of ethidium bromide solution (X320), (b) in tungsten light (X320).

Fig. 7

a.



b.



at 30 °C for 15 min the incorporated activity was 6 pmoles ^{3}H-UTP per 10^{6} nuclei, which is 2.5 times that obtained by Tautvydas (1971) for pea bud nuclei (S. Fry, personal communication).

6. Conclusion

In general, the main difficulty in isolating nuclei is the release of intact nuclei. For cultured cells of Acer pseudoplatanus L., the choice of grinding medium plays a very important role in the isolating process. Intact nuclei could not be obtained if the conventional aqueous grinding media were used (Bonner et al., 1968; Mohberg and Rusch, 1971; Flamm et al., 1963; Monahan and Hall, 1973). If, however, glycerol-containing medium is used, the resulting homogenate, whether produced with a Waring blender, a pestle and mortar or a Potter-Elvehjem homogenizer, does contain intact nuclei. Of the three methods of homogenization, use of the Potter-Elvehjem homogenizer causes the least damage to the nuclei. The efficiency of homogenization and release of intact nuclei is, however, generally low. Doubling the homogenization time increases the percentage of cell breakage, but at the same time it also breaks the exposed free nuclei (Table 3 and Avis, 1972). Therefore, it was decided to use only two strokes of the homogenizer during each rupture process. The grinding medium developed for this nuclear isolation technique contains 20 mM Tris-buffer at pH 7.8, 1 mM Mg acetate, 2 mM $CaCl_2$ and 70% (v/v) glycerol. The final nuclear yield in terms of DNA recovery is consistently about 20 percent. If a large number of nuclei is required for preparative purposes, the residue after filtration can be resuspended in grinding medium and re-homogenized. The second homogenate is then combined with the previous homogenate and the nuclear ~~pellet~~ fraction is prepared as described in the Methods (p. 25).

The isolated nuclei obtained by this method appear to retain their native properties in so far as they appear normal in shape with visible nucleoli, as seen by phase microscopy. After being kept at -18 °C for 1 week the nuclei still show RNA synthesizing activity. However, it would be necessary to examine the nuclear fraction with an electron microscope to confirm that the nuclei were really intact. Although the nuclear ~~pellets~~ fractions did not show significant contamination other than by starch grains, nuclei do occur both free and in clumps, and such clumping appears to result from contamination with very small amounts of cellular fragments. The clumping problem might be improved by varying the ionic strength of the isolating medium (Philpot and Stanier, 1956). Starch grains were rather difficult to separate cleanly from nuclei by centrifugation. Fortunately, the presence of starch grains does not interfere with the extraction of histones.

CHAPTER 4

Extraction and Characterization of Histones

1. Introduction
2. Efficiencies of different histone extraction methods
3. Characterization of *Acer* histones
   (A) polyacrylamide gel electrophoretic pattern
   (B) Identification of histone bands on polyacrylamide gel
   (C) Molecular weights of the histone fractions
   (D) Amino acid analysis
4. Conclusion

1. Introduction

Although various methods have been described for the preparation of histones (Phillips, 1962), they can be grouped into two approaches. Histones may be extracted with acid from cell nuclei or washed nucleoprotein, or alternatively, nuclei or nucleoprotein may be dissociated in solutions of high salt concentration, and the histone then separated from the other components (mainly DNA) of the viscous solution. The acid extraction, stemming from the early experiments of Kossel, is generally achieved with 0.1 to 0.3 N HCl or H_2SO_4. Since nucleic acid is insoluble in the acid, a clear solution of histone is readily obtained from which histone salts may be precipitated with ethanol or acetone (Bijvoet, 1957; Phillips, 1962; Fambrough and Bonner, 1966; Oliver and Sommer, 1972). Thus, this is considered as a very convenient method if a very pure nucleohistone or a pure nuclear fraction is available (Bonner et al., 1968; Phillips, 1962; Murray, 1965) and hence contamination with basic proteins of cytoplasmic origin is minimized. The acid extraction also eliminates possible proteolytic degradation of histones. A variety of salts have been used in the second method. Based on the observation that 1 M $CaCl_2$ dissociates the RNA and basic protein of brome grass masaic virus (Yamazake and Kaesberg, 1963), Mohberg and Rusch (1969) developed a method to extract histones from Physarum nuclei by treatment of the nuclei with 1 M $CaCl_2$, followed by precipitation of the histone from the $CaCl_2$ soluble fraction with TCA. These investigators obtained high yields of histones and also found that histones prepared in this way were less contaminated by other proteins.

Both electrophoretic and chemical studies on histones demonstrate that there are five main histone fractions in most eukaryotic tissues.

By polyacrylamide gel electrophoresis (Panyim and Chalkley, 1969), the five main fractions of calf thymus histones are the fastest moving band of H4, followed by the triplet of bands of H2A, H2B and H3, and the slowest moving band of H1.

Histones are of limited heterogeneity. Striking similarities have been observed between plant and calf thymus histones (Fambrough and Bonner, 1966, 1969; Spiker, 1971). The primary structures of the histones other than H1 are considered to have been highly conserved during evolution (DeLange and Smith, 1971; Panyim et al., 1971). Only 2% of the residues for H4 in pea histones differed from that of the calf thymus histones (DeLange et al., 1969a), and 3% of the residues for H3 in chicken erythrocytes differed from that of the calf thymus histones (DeLange et al., 1972, 1973; Brandt and Van Holt, 1972). Both H3 and H4 show consistent mobilities in polyacrylamide gel no matter from what sources they are isolated (Panyim et al., 1971). Among vertebrate histones analyzed, histones H2A and H2B also migrate with constant mobilities in polyacrylamide gel within a given class of animals and a definite trend is discernible as one progresses towards the mammals; H2B becomes progressively slower and H2A progressively faster moving. H1 histones varied considerably in their electrophoretic heterogeneity and mobility, indicating substantial changes in the primary structure of this fraction during evolution (Panyim et al., 1971). H1 histones were also found to be tissue and species specific (Kinkade and Cole, 1966a, 1966b; Bustin and Cole, 1968, 1969; Kinkade, 1969, DeLange and Smith, 1971).

2. Efficiencies of different histone extraction methods

Three extraction methods were compared in the present study. The purified nuclei obtained as described in the Methods (p. 25) were treated as follows:

(A) The nuclei were dissociated with 1 M $CaCl_2$, and then the histones were precipitated with TCA as described in the Methods (p. 26).

(B) The nuclei were extracted with 0.4 N H_2SO_4, and the histones were precipitated with ethanol as described in the Methods (p. 27).

(C) A method which combined (A) and (B). The nuclei were extracted with 0.4 N H_2SO_4, by magnetic stirring in a cold room overnight, and then the histones were precipitated with TCA as method (A).

Using 1 M $CaCl_2$ to dissociate the histones and DNA from Acer nuclei, the UV spectrum of the $CaCl_2$ soluble fraction showed the presence of DNA, while if H_2SO_4 was used instead, the H_2SO_4 soluble fraction did not show a peak at 260 nm (Fig. 8). However, all DNA-free histone samples showed UV spectra similar to that of calf thymus histone (Fig. 9).

Method (A) is that of Mohberg and Rusch (1969), employed for extracting histones from Physarum nuclei. From their experiment, they found that calcium chloride penetrates nuclear membranes more easily than does mineral acid, and that the precipitation of histone with TCA is superior to that with ethanol. Calcium chloride causes the Acer nuclei to swell as described by Mohberg (1969); however, their method did not extract maximum yields of histone from Acer nuclei, the yield varying from 60% to 95% of that of method (B). Method (B), which involves the precipitation of histones with ethanol, was the most efficient method of the three, and yielded 1.13 μg histone per μg of DNA. Method (C) yielded only about 30% of that of method (B).

Figure 8

Ultraviolet absorption spectra of $CaCl_2$ and H_2SO_4 soluble fractions prepared from nuclei of Acer pseudoplatanus L.

Acer nuclei were extracted with either 1 M $CaCl_2$ or 0.4 N H_2SO_4 as described in the text. The $CaCl_2$ soluble fraction was scanned against 1 M $CaCl_2$ (1), and the H_2SO_4 soluble fraction was scanned against 0.4 N H_2SO_4 (2), using a Unicam SP 800 Ultraviolet Spectrophotometer.

Fig. 8

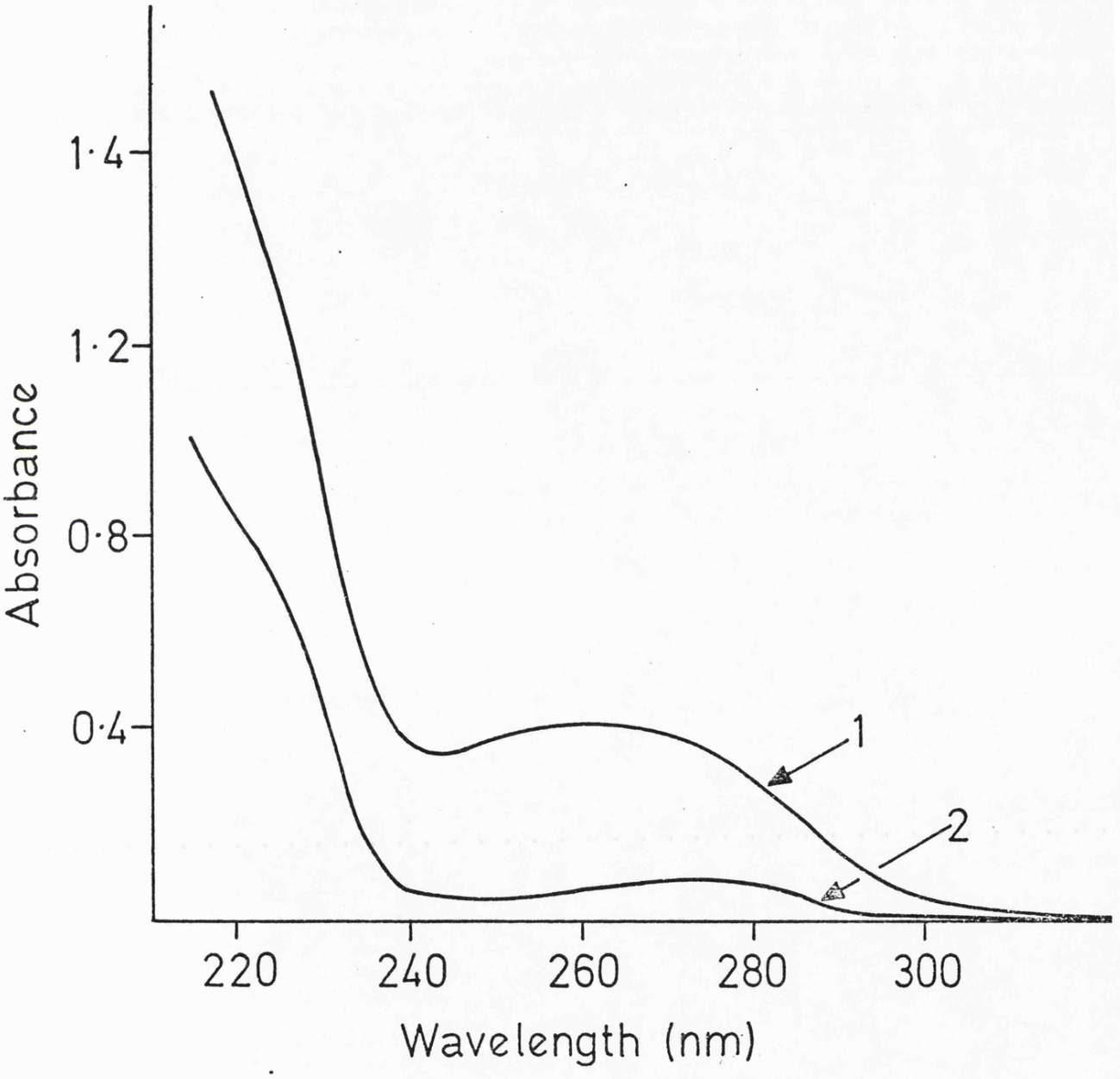
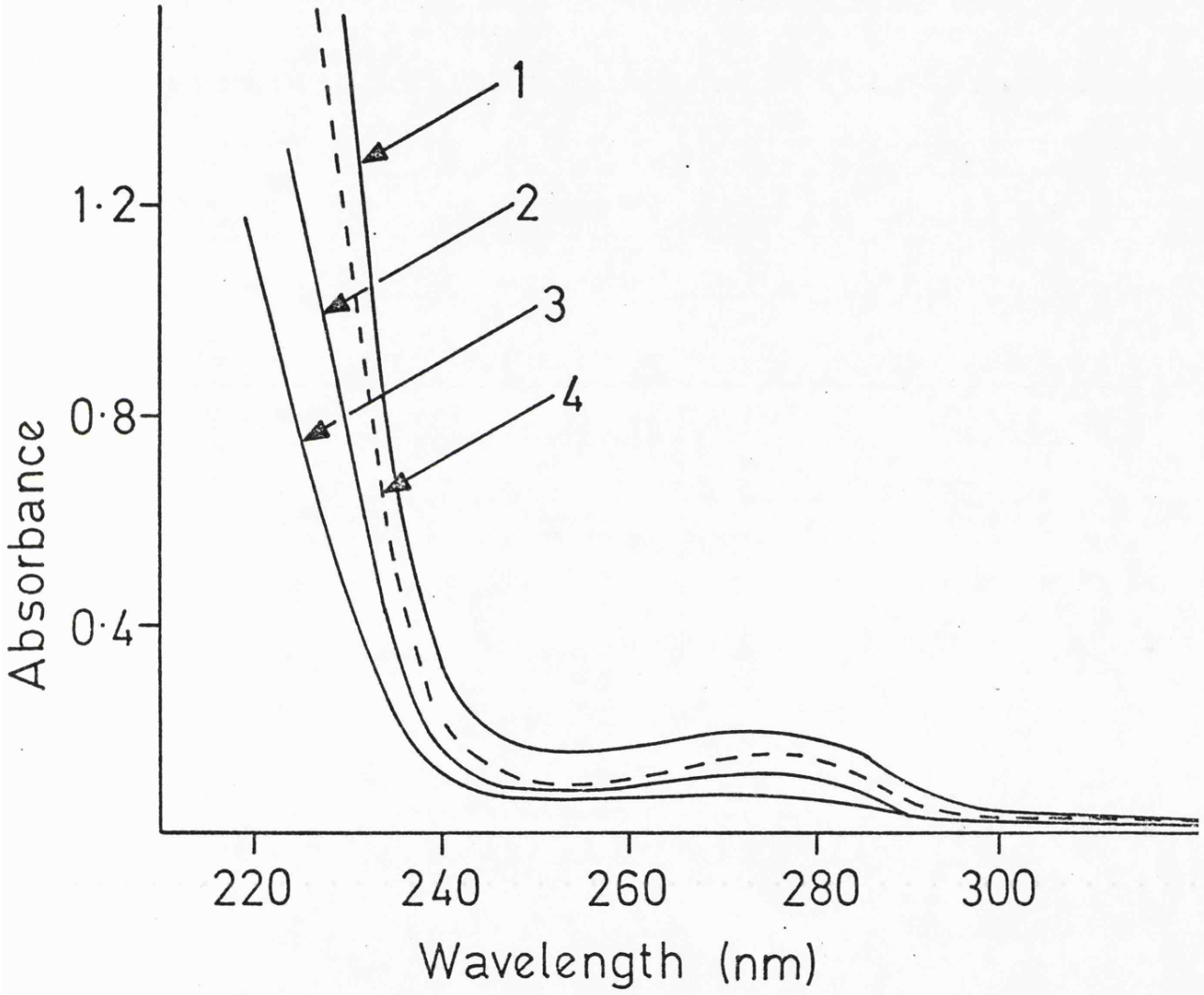


Figure 9

Ultraviolet absorption spectra of the extracted *Acer* histone samples and the calf thymus histones.

Acer histones were extracted using $CaCl_2$-TCA (1), H_2SO_4-EtOH (2) and H_2SO_4-TCA (3) as described in the text; calf thymus histones (4) were purchased from Sigma Chemical Company. All histone samples were dissolved in 0.02 N H_2SO_4 and scanned with a Unicam SP 800 Ultraviolet Spectrophotometer, using 0.02 N H_2SO_4 solution as blank.

Fig. 9



In general, the main disadvantage of the salt dissociation methods is that histones tend to aggregate above pH 4 (Davison et al., 1954) and this may seriously interfere with any subsequent fractionation procedure. However, the result of the present investigation suggests that the yield and the purity of the extracted histones are rather dependent upon the methods used for the precipitation and the recovery of histones. Since TCA has been reported to recover histones from aqueous solutions almost quantitatively (Hnilica, 1972), and serious losses of histones during dialysis in acid solution have been reported by many investigators (Butler et al., 1954; Bakay et al., 1957; Luck et al., 1958; Smillie et al., 1958; Neelin and Neelin, 1960; Crampton et al., 1957; Phillips, 1962), it can be suggested that it is the dialysis process which leads to loss of histone, especially when working with small scale samples. The variation of 60% to 95% by method (A) could be due to differences in the duration of dialysis, and the yield of 30% by method (C) was probably due to the small amount of material used.

The extraction methods also affect the purity of the resulting histones. In polyacrylamide gel electrophoresis all histone samples presented five major bands (Fig. 10 and Fig. 11). However, the appearance of histone bands on the gels differed depending upon the method used for their extraction. Histones prepared by the H_2SO_4-EtOH method exhibit very sharp bands whereas histone samples prepared by the other two methods occasionally present tailing problems. The acid extraction as described in the Methods (p. 27) is simple and efficient, and the histones separate in a more consistently satisfactory manner on polyacrylamide gels. However, in the first part of the work only the $CaCl_2$-TCA method was used, and since the histones so obtained also presented reasonably reproducible patterns on polyacrylamide gels, and

Figure 10

Photograph of the acid-urea polyacrylamide gel electrophoretic patterns of *Acer* histones obtained by different extraction methods

Histone samples each containing *c.* 20 µg, extracted from *Acer* nuclei by methods involving $CaCl_2$-TCA (right), H_2SO_4-EtOH (centre) and H_2SO_4-TCA (left), were electrophoresed on acid-urea polyacrylamide gels. These gels were stained with Amido Black and photographed after destaining. The bands were numbered from the anode to the cathode end.

Fig. 10

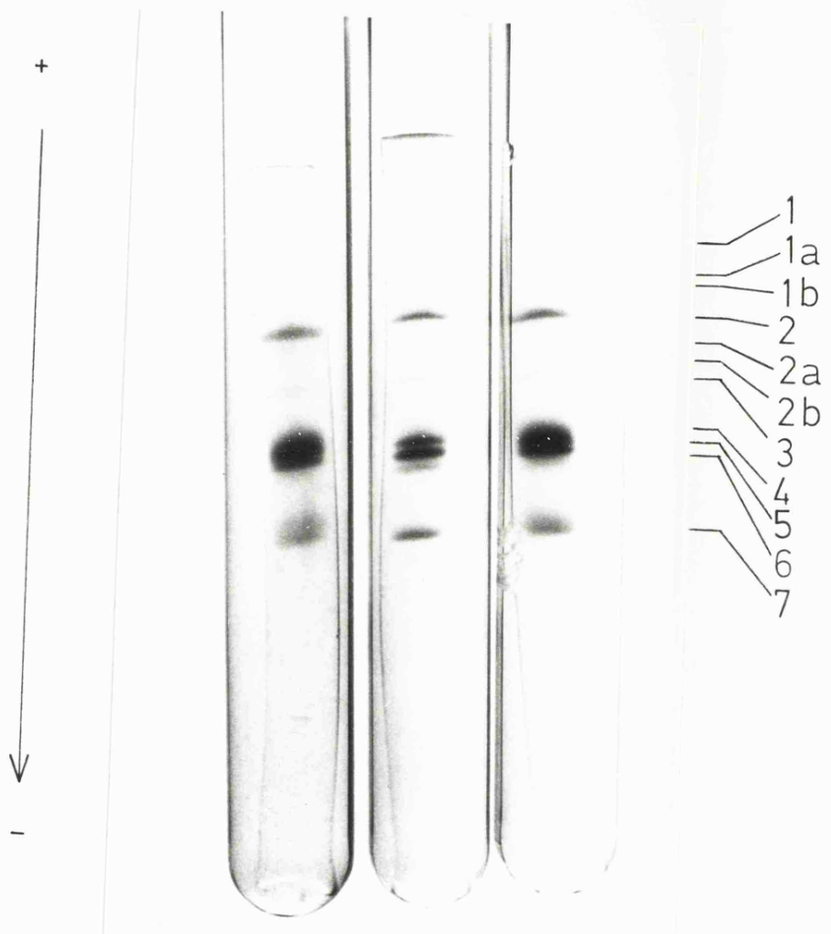
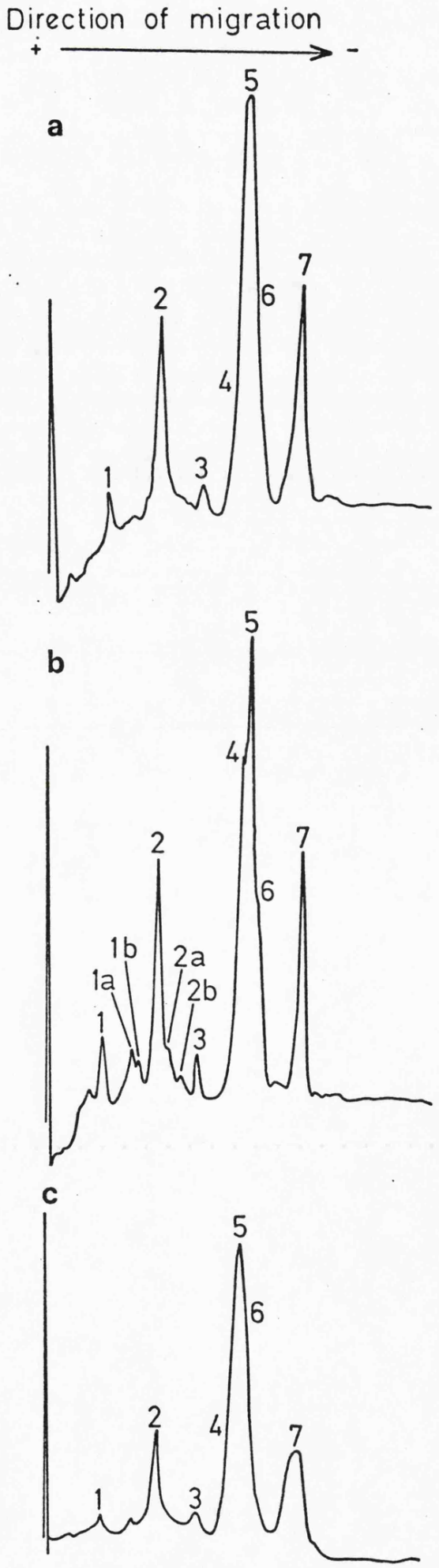


Figure 11

Microdensitometer tracings of the gels illustrated in Fig. 10.

Histone samples were those obtained using $CaCl_2$-TCA (a), H_2SO_4-EtOH (b) and H_2SO_4-TCA (c). Gels were scanned at 615 nm with a Beckman Spectrophotometer Model 24.

Fig. 11


Direction of migration
+
-
a
1
2
3
4
5
6
7
b
1
1a
1b
2
2a
2b
3
4
5
6
7
c
1
2
3
4
5
6
7

the yield was reasonably high, both the $CaCl_2$-TCA and the H_2SO_4-EtOH methods were employed for histone extraction in the following experiments.

3. Characterization of *Acer* histones

(A) Polyacrylamide gel electrophoretic pattern

Polyacrylamide gel electrophoresis offers the advantage of speed, simplicity, high resolution and high sensitivity for characterization of protein molecules. Since the transparent polyacrylamide gel displays the full path of stained protein zones, effective analyses can be obtained with samples containing one-tenth or less of the protein required for starch gel electrophoresis. Furthermore, polyacrylamide gel seems to retain defined banding of small polypeptides, degradation products, and histone aggregates as well as authentic components.

Polyacrylamide gel electrophoresis was introduced by Reisfeld *et al.* (1962) for cationic proteins analysis. The technique was first used by Cruft (1964) for histone analysis. This method, in various modifications, was then employed by a great number of investigators to study the heterogeneity, tissue specificity, metabolism and *in vivo* modifications of histones. However, the identification of true histones was interfered with by the presence of histone aggregates when electrophoresis was carried out at nearly neutral pH values; under these conditions histone aggregation can produce multiple protein bands, which can be erroneously interpreted as tissue or species specific components. To overcome this problem, the method was modified by decreasing the pH of the electrophoretic buffer (Johns, 1967a), and incorporating concentrated urea in the gel and sample buffers

(Fambrough et al., 1968; Panyim and Chalkley, 1969a; Panyim and Chalkley, 1969b). Panyim and Chalkley (1969b) also found that pre-electrophoresis (p.33) could further improve the resolution of histone bands. By using the electrophoretic technique described in the Methods (p.34), the electrophoretic pattern of Acer histones resembled that of calf thymus (Fig. 12 and Fig. 13). Seven bands of Acer histones were found; the major bands are the fastest moving band 7, followed by 'triplet bands' 6, 5, and 4, and the slowest moving band 2. Bands 6 and 7 co-migrate with H3 and H4 of calf thymus histones respectively, which is expected as they are highly evolutionarily conserved histones (Panyim and Chalkley, 1971). Minor bands 1 and 3 appeared uniquely in Acer histones. The remaining minor bands 1a, 1b, 2a and 2b sometimes showed on electrophoresis in histones obtained by acid extraction (Fig. 10).

The microdensitometer tracing of Acer histones on acid-urea polyacrylamide gels showed very sharp peaks (Fig. 13), suggesting that the Acer histones are of very limited heterogeneity; a similar limited heterogeneity has also been reported for pea-bud (Fambrough and Bonner, 1969) and vertebrate histone (Panyim et al., 1971).

(B) Identification of histone bands on polyacrylamide gel

To characterize a new histone sample on polyacrylamide gel one can apply a well characterized histone sample to compare the mobilities of the bands. However, the histones isolated from different sources have minor differences in their amino acid composition which can cause slight variations in their electrophoretic mobility (Panyim et al., 1971). Furthermore, some proteins though being dissimiliar in their amino acid composition, have the same mobilities. To help overcome these problems, one can use

Figure 12

Comparison of electrophoretic patterns of *Acer* and calf thymus histones

Acer histones were obtained from nuclei by the H_2SO_4-EtOH method, and calf thymus histones were purchased from Sigma Chemical Company. Histone samples were loaded on separate acid-urea polyacrylamide gels and electrophoresed. Protein fractions were stained with Amido Black.

Right-hand gel - calf thymus histones. Histone fractions are classified according to Panyim and Chalkley (1969a)

Left-hand gel - *Acer* histones. Histone fractions are numbered from anode to cathode.

Fig. 12

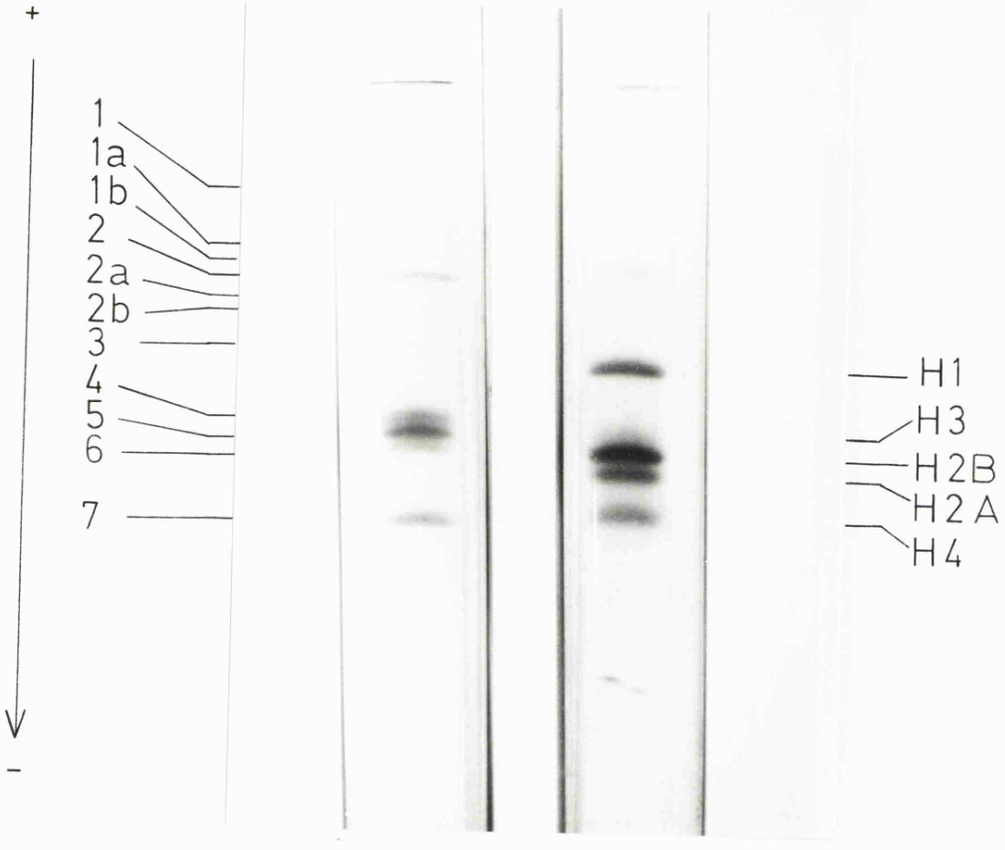
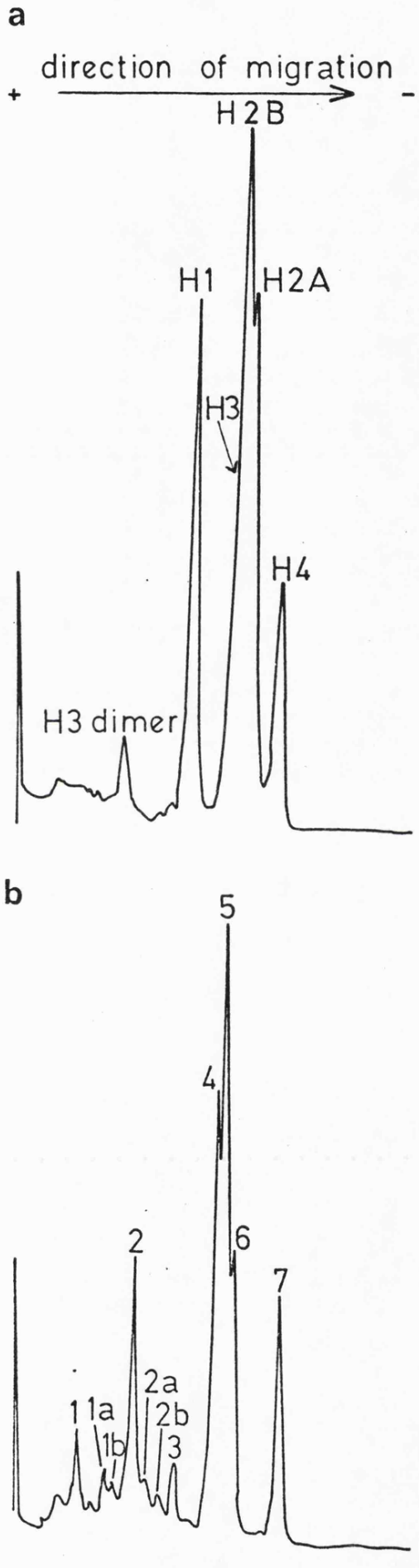


Figure 13.

Microdensitometer tracings of the gels illustrated in Fig. 12.

Gels were scanned at 615 nm with a Beckman Spectrophotometer Model 24. Samples are (a) calf thymus histones and (b) *Acer* histones. The banding classification is the same as in Fig. 12.

Fig. 13


a
direction of migration
+
-
H2B
H1
H2A
H3
H4
H3 dimer
b
5
4
2
6
7
1
1a
1b
2a
2b
3

various staining techniques. For example, Coomassie Brilliant Blue stains H2B of calf thymus histone violet-blue and the other histones faint blue. Amido Black stains every histone band blue but to different intensities, and if the gel is maintained in the destaining solution for a longer period, the arginine-rich histones become blue-green and the lysine-rich histones become grey as shown in Fig. 14 where the gel was kept in the destaining solution for 20 months before being photographed. In 1974, Barrett and Johns developed a differential staining technique to discriminate arginine-rich and lysine-rich histones using a mixture of 0.5% Alizarine Black and 0.0125% Ponceau S which stained lysine-rich histones H1 and H2B red and the other fractions blue. A modification of this technique was used in identification of Acer histones by replacing 0.5% Alizarine Black with 0.1% Amido Black. This stained the five major fractions of calf thymus histones in 5 different colours; H1, rosy pink; H3, light violet; H2B, purple; H2A, deep blue green; and H4, violet. A colour photograph is shown in Fig. 15, where three sets of gels are arranged with calf thymus histones alternating with Acer histones, and the pairs stained respectively with Amido Black, Coomassie Brilliant Blue and the mixture of Amido Black and Ponceau S, reading from right to left. Based on the specific staining characteristics together with the comparison of those of calf thymus samples, the five major fractions of Acer histones were classified as 2 (H1), 4 (H2A), 5 (H2B), 6 (H3) and 7 (H4). The unique minor bands 1 and 3 stain as arginine-rich and lysine-rich proteins respectively. The remaining minor bands are too vague to present clear colours. All the major bands of the Acer histones

Figure 14

Colour response of *Acer* histones on polyacrylamide gel which were stained with Amido Black and were maintained in the destaining solution for a period of time

Acer histones obtained from nuclei by the $CaCl_2$-TCA method were characterized by acid-urea polyacrylamide gel electrophoresis. The gel was stained with Amido Black, destained and stored in the destaining solution for 20 months before being photographed.

Fig. 14

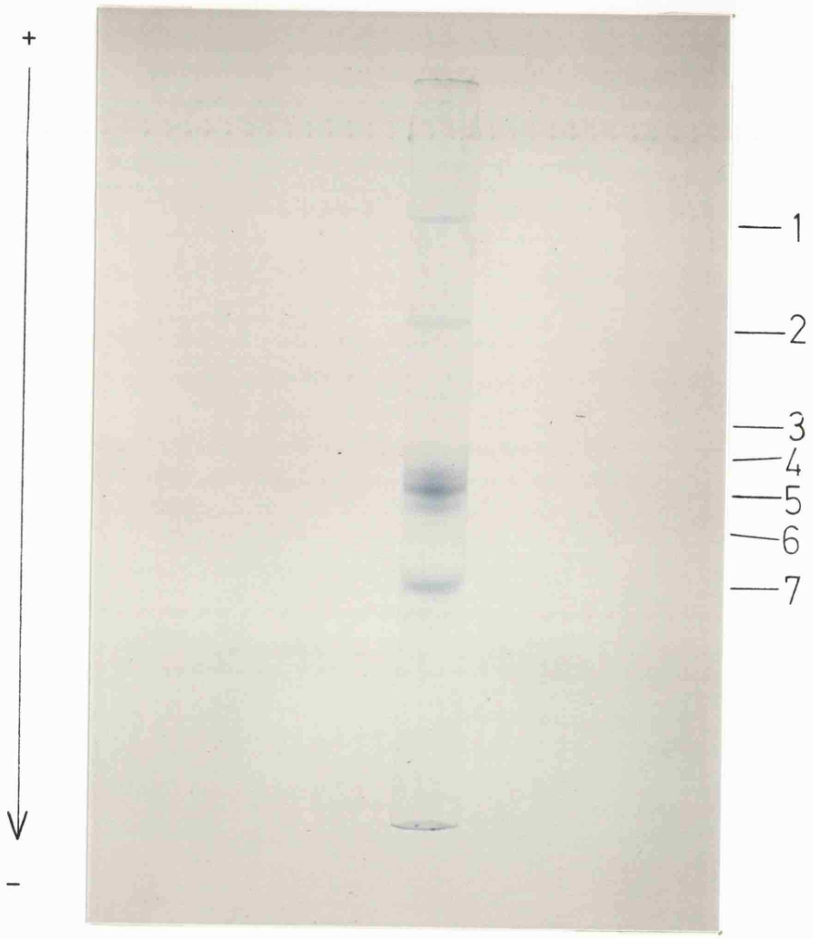
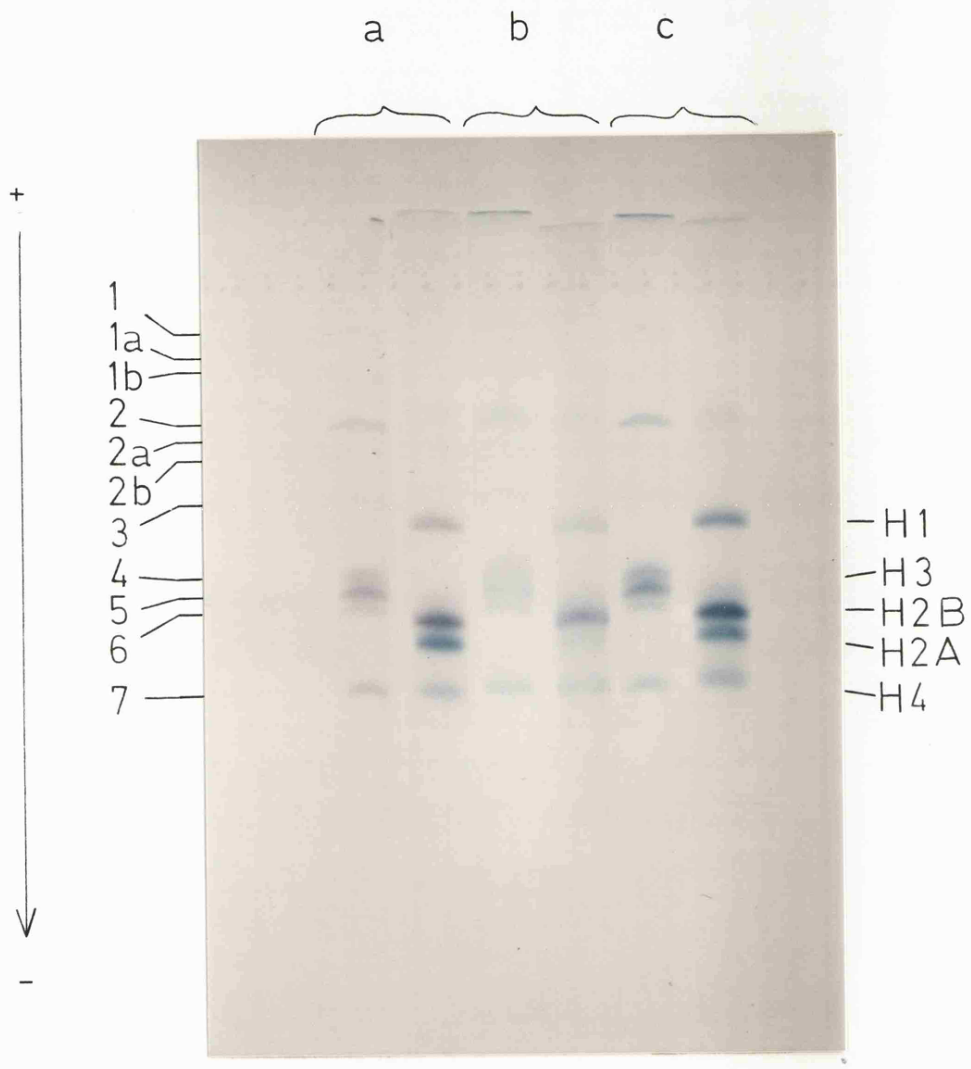


Figure 15.

Colour photograph of *Acer* and calf thymus histones on acid-urea polyacrylamide gels, showing the colour of histone fractions in response to three different kinds of staining technique.

Gels were stained with (a) mixture of Amido Black and Ponceau S, (b) Coomassie Brilliant Blue and (c) Amido Black. Left-hand gel of each pair : *Acer* histones; right-hand : calf thymus histones.

Fig. 15



correspond to the colours of those of calf thymus except H2A. It appeared pinkish rather than bluish or blue green colour, suggesting that H2A of Acer contains less arginine than that of calf thymus.

Another method developed by Barrett and Johns in 1973 is a differential destaining technique which washes out H1, H2A, H2B and leaves only the arginine-rich histones H3 and H4. When this method was applied to Acer histone samples, it again pointed to fractions 6 and 7 as being arginine-rich histones. However, owing to the staining and destaining process, the gel became swollen, and thus it is not possible to line it up with other gels for mobility comparison. All the staining and destaining techniques are described in detail in the Methods (p. 36).

Since the whole histones of Acer were extracted from nuclei directly, non-histone proteins with a relatively high content of basic amino acids would also be extracted, and those of low molecular weights could also migrate into the same gel used for histone analysis. Among the minor bands of Acer histones, band 1 could be one of the non-histone proteins which possesses a relatively high content of arginine because, firstly, the proportion of band 1 to total histone varied from experiment to experiment, and secondly, the mobility of band 1 is much slower even than H1 (band 2). Bands 1a and 1b could also be acid extractable non-histone proteins, while bands 2a, 2b and 3 could be aggregations of one or more of the major histones. It is also possible that all minor bands are nucleolar precursors of ribosomes for they are partially acid soluble proteins. Nevertheless, none of these minor bands interferes with the five major bands. In contrast

with that of calf thymus, H3 of Acer has only one band while calf thymus presents both reduced and oxidized forms (Fig. 12 and Fig. 13). This suggests that H3 of Acer is probably like that of other plants and contains only one cysteinyl residue (Panyim et al., 1971), whereas about 80% of the calf thymus H3 consists of molecules with two cysteinyl residues and thus has higher potentiality to form dimers or polymers (Marzluff et al., 1972).

(C) Molecular weights of the histone fractions

The detergent sodium dodecyl sulphate (SDS, sodium lauryl sulphate) binds to protein in a constant ratio and produces complexes with constant charge per unit mass. The resulting complex is rod-shaped, its length varying uniquely with the polypeptide molecular weight (Reynolds and Tanford, 1970a, 1970b; Pitt-Rivers et al., 1968). Therefore, proteins dissolved in high concentrations of SDS exhibit electrophoretic mobilities in polyacrylamide gels which are a direct function of their sub-unit molecular weights (Shapiro et al., 1967; Weber and Osborn, 1969).

The molecular weight of a protein under investigation can be calculated by comparing its electrophoretic mobility with that of a protein standard of known molecular weight. However, to utilize SDS-gel electrophoresis for determining the molecular weight of histones, Panyim and Chalkley (1971) declared that it is necessary to construct a molecular weight-mobility standard curve based on known molecular weights of histones because the over-all negative charge on the SDS-histone complex is reduced by the high positive charge on the histones. Therefore this

curve differs somewhat from the standard curve for non-basic proteins. This proposal on the effect of charge on SDS molecular weight determinations is in agreement with reports of Tung and Knight (1971) and Cohen and Gotchel (1971).

The SDS-gel electrophoretic patterns of Acer histones, differentially stained as described in the Methods (p. 36), together with standard protein markers and calf thymus histones, are shown in Fig. 16. Their microdensitometer tracings are shown in Fig. 17. Calibration lines were derived from molecular weight markers as well as calf thymus histones as shown in Fig. 18. These two standard curves are considerably different; they are both linear but they differ significantly in slope and intercepts. When molecular weights of calf thymus histones were calculated based on the calibration line of standard protein markers, their molecular weights came out as H1=29,500 and 32,300 (two sub-fractions), H3=H2B=14,450, H2A=14,000 and H4=11,900. These values, especially that of H1, are abnormally high. The calculation of the molecular weights of the Acer histones was therefore based on the calibration line derived from calf thymus histones, and gave the values shown in Table 4.

H1 of calf thymus is divided into 2 molecular weight sets, differing by about 600 daltons. While there is only one H1 band in Acer, its molecular weight is higher than that of calf thymus. The molecular weights of both H2A and H2B in Acer are also higher than those of calf thymus. These also coincide with their mobilities in acid-urea polyacrylamide gel electrophoresis. Both H3 and H4 are virtually identical in Acer and calf thymus histones.

Figure 16

Colour photograph of Acer histones, calf thymus histones and marker proteins on SDS polyacrylamide gels

Gel samples are calf thymus histones (right), marker proteins (centre) and Acer histones (left). They were stained with a mixture of Amido Black and Ponceau S.

Fig. 16

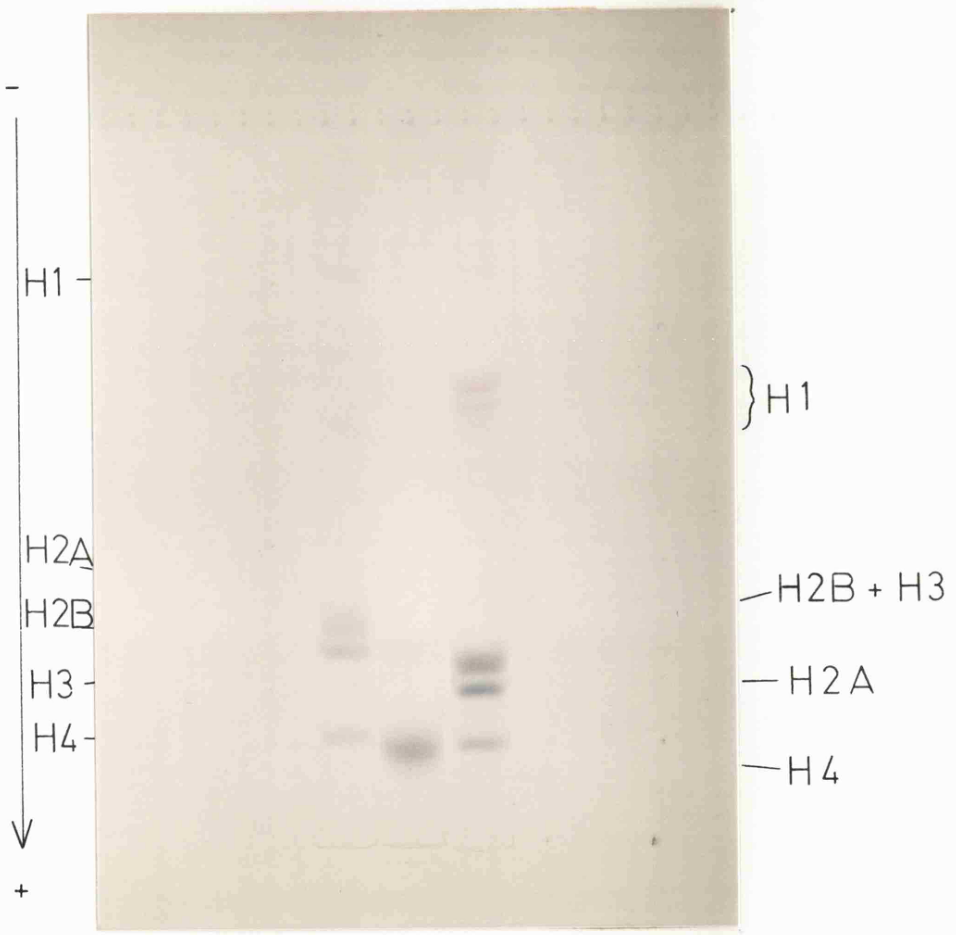

-
H1
H2A
H2B
H3
H4
+
}H1
—H2B + H3
—H2A
—H4
Acer histones
Calf thymus histones

Figure 17

Microdensitometer tracings of the gels illustrated in Fig. 16.

Gel samples are (a) standard protein markers, (b) calf thymus histones and (c) *Acer* histones. Gels were scanned at 570 nm with a Unicam SP 800 Ultraviolet Spectrophotometer Model 24.

Fig. 17

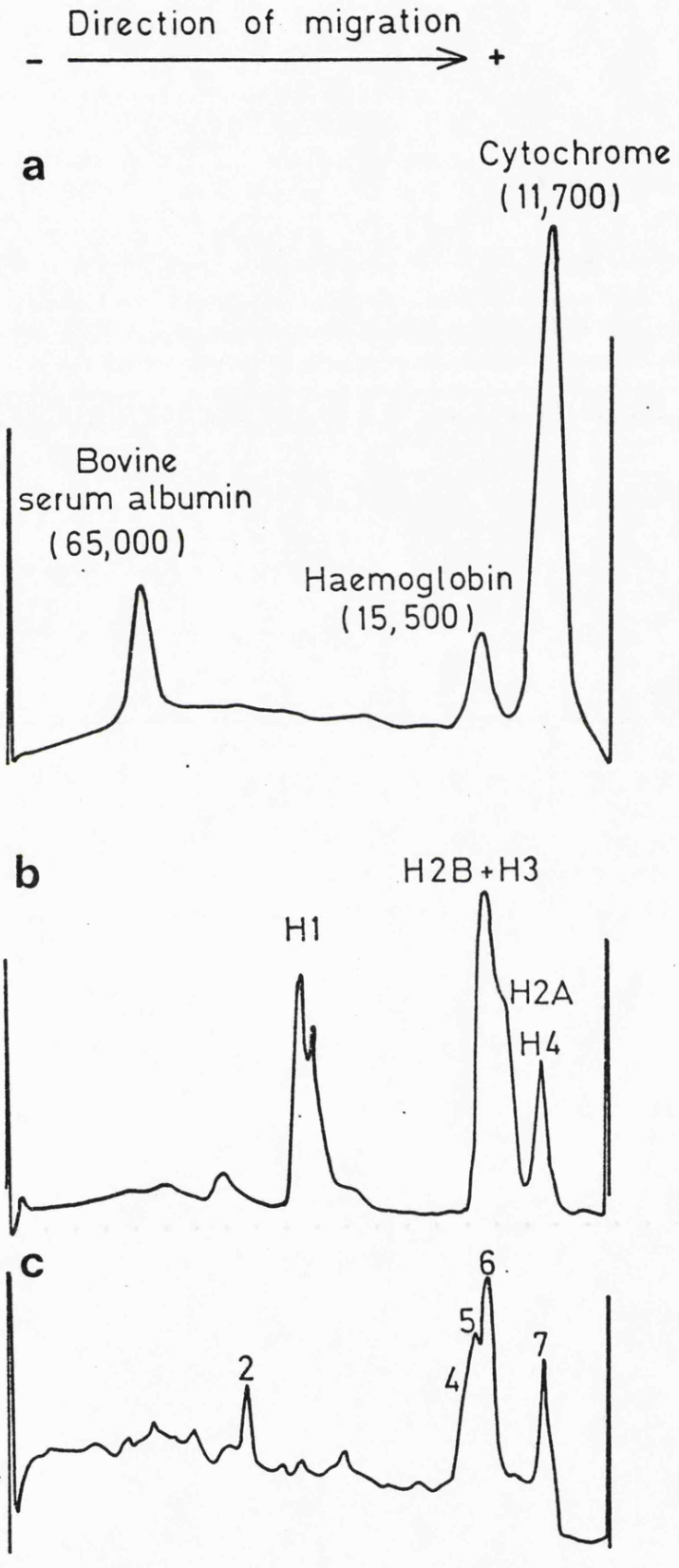

Direction of migration
-
+
a
Cytochrome
(11,700)
Bovine
serum albumin
(65,000)
Haemoglobin
(15,500)
b
H2B+H3
H1
H2A
H4
c
6
5
7
2
4

Figure 18

Calibration lines for molecular weight marker of proteins and calf thymus histones.

Mixtures of protein markers containing (i) cytochrome-c, haemoglobin and bovine serum albumin, and (ii) calf thymus histones were electrophoresed on SDS polyacrylamide gels. The relative mobility of each protein fraction was measured and plotted against the logarithmic value of its molecular weight.

Fig. 18

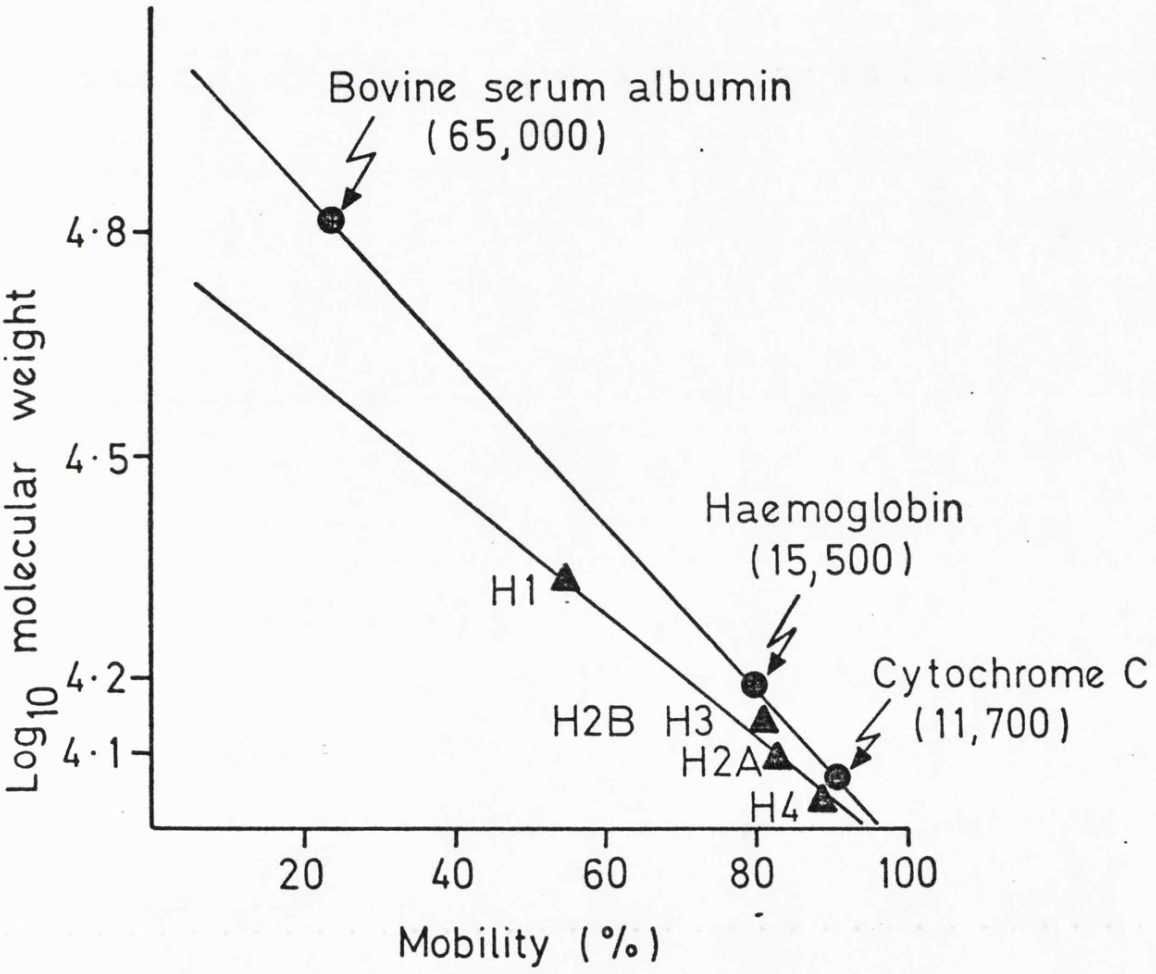

Bovine serum albumin
(65,000)
Haemoglobin
(15,500)
Cytochrome C
(11,700)
H1
H2B
H3
H2A
H4
4·8
4·5
4·2
4·1
Log10 molecular weight
20
40
60
80
100
Mobility (%)

Table 4. Comparison of molecular weights of calf thymus histones (Panyim *et al.*, 1971) and *Acer* histones

	H1	H3	H2B	H2A	H4
calf thymus	20,400 21,000	12,800*	12,800*	12,500	11,000
Acer	24,500	12,800	13,300	13,500	11,000

* this molecular weight value was obtained from the calibration line based on calf thymus H1, H2A and H4. The molecular weight of H2B and H3 quoted by Panyim *et al.* (1971) was 14,000.

(D) Amino acid analysis

The amino acid composition of the isolated histone will give a general indication of the purity of the histone. *Acer* and calf thymus histones were compared by analysis with a Technicon Automatic Analyzer as described in the Methods (p. 31). The *Acer* histones were extracted from nuclei by the $CaCl_2$-TCA method, while the calf thymus histones were purchased from Sigma Chemical Company. Table 5 presents the amino acid composition of the total histones of *Acer pseudoplatanus*, calf thymus, *Vicia* root, *Lilium* leaf, and wheat germ. Column 1 is the data of Crampton *et al.* (1957), columns 2 and 3 are the present experimental data, and columns 4, 5, and 6 are data of Dick (1968), Sheridan and Stern (1967) and Johns and Butler (1962) respectively. Although all the proteins are in most respects similar, the histones of plant origin are closely alike in that they all have a lower arginine content than the calf thymus histone. From the colour response of each fraction of the *Acer* histones on poly-

Table 5. Amino acid analysis of whole histones from various sources (as moles per 100 moles of total amino acids).

	calf thymus (Crampton et al., 1957)	calf thymus (experimental data)	Acer (experimental data)	Vicia roots (Dick, 1968)	Lily leaf (Sheridan and Stern, 1967)	wheat germ (Johns and Butler, 1962)
Aspartic acid	5.3	4.7	6.6	6.7	4.7	4.4
Glutamic acid	8.5	6.9	6.4	8.2	7.9	8.6
Threonine	5.6	5.4	5.3	5.5	4.7	5.4
Serine	5.3	5.8	6.3	6.0	4.5	4.9
Proline	5.0	5.9	7.4	5.4	7.2	5.1
Glycine	8.6	8.2	7.8	8.4	7.7	8.1
Alanine	13.2	15.3	13.5	12.7	12.6	15.5
Valine	6.2	6.6	7.4	6.2	7.2	5.5
Methionine	1.1	0.7	0.12	0.6	0.8	-
Isoleucine	4.3	4.2	5.5	4.6	5.4	-
Leucine	7.9	7.5	7.0	7.1	8.4	12.3
Tyrosine	2.6	2.2	1.9	1.6	3.1	1.7
Phenylalanine	1.9	1.5	1.1	2.3	3.2	2.2
Histidine	2.0	1.7	1.7	1.9	1.1	1.5
Lysine	13.5	15.6	15.9	15.9	15.0	17.6
Arginine	8.9	7.7	6.0	6.6	6.6	7.5
Basic/acidic	1.8	2.0	1.7	1.6	1.7	2.0
Lysine/arginine	1.5	2.0	2.6	2.4	2.4	2.4

No corrections were made for hydrolytic losses.

acrylamide gel (discussed earlier), the low content of arginine in the total Acer histones seems to be due to the low content of arginine in H2A rather than any other fractions. To support this idea, analysis of each fraction is necessary, but would require a larger quantity of histones than was available.

4. Conclusion

The yield and the purity of Acer histones extracted from the nuclear fraction is dependent on the methods employed. The H_2SO_4-EtOH method yields 1.13 µg of histone per µg of DNA, whereas methods involving precipitation of histones with TCA are less efficient. The ratio of DNA to histone protein to non-histone protein in Acer nuclei is 1.0 : 1.13 : 0.78. All the histone samples present typical histone patterns on polyacrylamide gels. The separation of the 'triplet bands' is better observed by eye than by scanning. The Acer histones extracted by the H_2SO_4-EtOH method show very sharp bands on the gels, indicating that the resulting histones are limited in aggregation, and that the individual fractions are homogeneous (Fig. 10 and Fig. 11).

The Acer histone fractions were characterized by comparing their mobilities with those of fractions of calf thymus (purchased from Sigma Chemical Company), and also by their response to different staining techniques. The differential stain developed by Barrett and Johns (1974) was modified to contain 0.1% Amido Black and 0.0125% Ponceau S which dyes the five major histone fractions of calf thymus in 5 different colours; and the colour response seemed to correlate with the content of basic amino acids. The major fractions of Acer histones were identified as H1, H2A, H2B, H3 and H4 from anode end to cathode end on an acid-urea polyacrylamide gel.

The molecular weights of Acer histones are H1=24,500, H2A= 13,500, H2B=13,300, H3=12,800 and H4=11,000. Both H3 and H4 have the

same molecular weights as those from calf thymus, while the molecular weights of Acer histones H2A, H2B and H1 are higher than those of calf thymus. The order of the Acer triplet bands on acid-urea gels agrees with their molecular weights measured by SDS-gel electrophoresis.

The results of amino acid analysis indicated that Acer histones contain 22% of basic amino acids. As with histone of other plant sources, the arginine content of Acer histones is lower than that of calf thymus histones (Table 5). Since the H2A of Acer gives $\overset{\alpha}{\underset{\wedge}{\sim}}$ pinkish rather than the blue green colour as that of calf thymus with the differential stain, the low arginine content of total Acer histone is probably largely due to a very low arginine content in its H2A.

CHAPTER 5

Biosynthesis of Histones

1. Introduction
2. Kinetic studies on the incorporation of ^{14}C-lysine and ^{14}C-arginine into proteins
3. Incorporation of ^{3}H-lysine and ^{14}C-arginine into histone proteins
4. Cellular localization of histone synthesis
5. Effect of hydroxyurea on histone biosynthesis
6. Biosynthesis of histones during the growth cycle
7. Biosynthesis of histones during the cell cycle
8. Conclusion

1. Introduction

Protein synthesis is generally accepted to occur on cytoplasmic polyribosomes, but there is considerable uncertainty as to whether protein synthesis also occurs in the nucleus. Owing to the localization of histones in the chromatin, the cell nucleus might appear to be a logical location for histone synthesis. Allfrey et al. (1955, 1957) first demonstrated the incorporation of labelled amino acids into histones by isolated calf thymus nuclei. Further studies on isolated nuclei of pea stems (Birnstiel et al., 1962) and cultured tobacco cells (Flamm and Birnstiel, 1964) demonstrated that histones were synthesized in the nucleolus (-i) and then migrated into the nucleoplasm. This conclusion was drawn from kinetic studies of incorporation of ^{14}C-amino acids into the various protein fractions of different nuclear components. Laval and Bouteille (1973) incubated Triton X-100 washed rat liver nuclei with radioactive amino acids, and showed by autoradiography that there was about three times as much incorporation into the nucleoli as into the rest of the nucleus. There is another report of amino acid incorporation into isolated nuclei from rat liver (Rendi, 1960), and also HeLa cells (Bach and Johnson, 1967). However, the purity of isolated nuclei is always questionable. In order to remove any cytoplasmic ribosomes contaminating isolated nuclei, McCarthy et al. (1966) treated their isolated rat liver nuclei with EDTA and ribonuclease, and found that the ribosomes which could subsequently be released from such nuclei were as effective as cytoplasmic ribosomes in protein synthesis. Such released nuclear ribosomes were insensitive to DNase, but sensitive to RNase and puromycin. They were considered to be 'precursors' of the cytoplasmic ribosomes (Flamm and Birnstiel, 1964; McCarthy et al., 1966), and to be only slightly involved in amino acid incorporation while within the nuclei (McCarthy

et al., 1966). If this interpretation is justified, it may be reasonable to consider that of all the systems that have been studied, only the thymus nucleus may be a valid example of an isolated nucleus in which significant protein synthesis can occur. The calf thymus nucleus is an unusally large one for a somatic cell, occupying about two-thirds of the volume of the cell (nuclei of most cells only occupy 2 to 20 percent of the cell volume), and therefore it is just possible that in such a cell, many nomally cytoplasmic activities occur within the nucleus.

Bloch and his associates (1960, 1963, 1964), working with grasshopper sperm, obtained the first evidence suggesting cytoplasmic synthesis of basic proteins which became associated with the DNA, following incorporation of ^{3}H-arginine, by an in situ autoradiographic technique. The initial incorporation of ^{3}H-arginine was shown to occur in the RNA-containing layer of cytoplasm surrounding the nucleus; only later was this radioactivity found accummulating on the DNA in the nucleus, indicating a transfer of arginine-rich proteins, synthesized in the cytoplasm, into the nucleus. The most convincing demonstration of the locus of histone synthesis comes from the work of Robbins and Borun (1967) and Borun et al. (1967) using synchronized HeLa cell cultures. By carefully controlled pulse-chase experiments with ^{14}C-tryptophan and ^{3}H-lysine, they found that the incorporation of radioactivity into histones only occurred during the S period, and involved a class of small cytoplasmic polysomes (s-polysomes). These polysomes contained 7-9S RNA as well as histone-like polypeptides. During the ensuing chase the radioactivity appeared in the HCl-soluble (histone) fraction of the nuclei. Nemer and Lindsay (1969) obtained evidence that the s-polysomes of early sea urchin embryos are probably the main site of

chromosomal histone synthesis. Based on direct observation of the incorporation of labelled precursor into DNA-associated proteins by autoradiography, on the solubility of labelled proteins in acid, and on radioactivity analysis of polypeptides synthesized *in vitro* in the presence of ^{14}C-lysine and ^{3}H-tryptophan, Kedes and Gross (1969) concluded that, in early sea urchin embryos, histones are synthesized in the cytoplasm and transferred rapidly into the nucleus.

Impressive evidence for cytoplasmic synthesis of histones has been obtained in work with isolated polysomes. Gallwitz and Mueller (1969a, 1969b, 1969c, 1970) isolated a class of polysomes from mitotically active HeLa cells which could assemble histone-like polypeptides *in vitro*. The newly synthesized proteins co-electrophoresed with histones in polyacrylamide gel and their appearance was found strictly coupled with active DNA replication. The cytoplasmic synthesis of histones was supported by the evidence of protein migration between cytoplasm and nucleus, as exemplified by reports of Goldstein and Prescott (1967), Zetterberg (1966a, 1966b) and Kroeger *et al.*(1963).

A relationship between the migration rate and the molecular weight of proteins was demonstrated by Paine and Feldherr (1972). They injected labelled proteins of different molecular weights into the cytoplasm of cockroach oocytes and traced the migration of the injected molecules into the nucleus; they observed that proteins of low molecular weight (20,000) moved rapidly into the nucleus, whilst those of moderate or high molecular weight (40,000) moved into the nucleus slowly and even after 5 hours the nuclear concentrations of those proteins were still far below the cytoplasmic levels.

The site of histone synthesis is still ambiguous although clearly, in some cases, the evidence favours the view that at least some of it is made outside the nucleus. In contrast there is also rather

convincing evidence that, in thymocytes, the nucleus is a site of protein synthesis. There is also a mass of data suggesting that the nuclei of other cell types also synthesize protein.

Although all histone fractions are synthesized at their maximum rates during the peak of DNA synthesis (S phase), the relationship between these two events is still controversial. Concurrent synthesis of histone and DNA has been reported in tissues of liver and mouse fibroblasts (Bloch and Godman, 1955), in Euplotes eurytomas (Prescott, 1966), and in HeLa cells and Chinese hamster cells in tissue culture (McClure and Hnilica, 1970; Robbins and Borun, 1967). Inhibition of DNA synthesis by hydroxyurea in HeLa cells in vivo rendered subsequently isolated microsomes incapable of histone synthesis in vitro (Gallwitz and Mueller, 1969c). The effect of puromycin and 5-fluoro-deoxy-uridine on onion root tip meristems suggested that continuation of DNA synthesis is dependent on a concurrent protein synthesis, but histone synthesis can proceed at a normal rate under conditions in which DNA synthesis is inhibited (Bloch et al., 1967; Flamm and Birnstiel, 1964). In HeLa cells synthesis of only part of the histone fractions appears to be dependent on concurrent DNA synthesis (Sadgopal and Bonner, 1969); different histone fractions differ in their degrees of dependence on the synthesis of DNA (Chalkley and Maurer, 1965). Histones thus may be divided into two classes, those which are replaced (turnover) in cells, and are synthesized independently of DNA replication; and those which do not turn over at all, or turn over very slowly, and are synthesized only during DNA replication.

2. Kinetic studies on the incorporation of ^{14}C-lysine and ^{14}C-arginine into proteins

The Acer suspension cultures which are routinely subcultured

and grown under the conditions described in the Methods (p.19) have a growth curve as shown in Fig. 19. The linear phase cells are those cells which have grown for 5 to 7 days, and were usually used for studying histone biosynthesis. In order to provide such rapidly dividing cells in a high concentration, the cell density of 7-day old culture was increased by sedimentation and decantation as described in the Methods (p. 38). For study of ^{14}C-lysine incorporation, 60 ml of such a concentrated culture suspension (1.94 X 10^6 cells ml^{-1}) was transferred to a clean conical flask (250 ml), and to this was added 200 µl of ^{14}C-lysine (287 mCi $mmole^{-1}$, 50 µCi ml^{-1}) to give a cell suspension containing ^{14}C-lysine at 5.8 X 10^{-7} M, and at 0.17 µCi ml^{-1}. Similarly 200 µl of ^{14}C-arginine (324 mCi $mmole^{-1}$, 50 µCi ml^{-1}) was added to 56 ml of concentrated culture suspension (1.68 X 10^6 cells ml^{-1}) to give a final concentration of ^{14}C-arginine of 5.3 X 10^{-7} M, 0.18 µCi ml^{-1}. These two experiments were performed at different times but with the same procedure as follows. The culture flask was incubated by shaking in a water bath at 25 °C. Four replicate samples (1 ml each) were taken from the incubating suspension at intervals during a period of 120 min. Samples were then treated as described in the Methods (p.38-39) for determining the radioisotopes remaining in the culture medium after incubation, the uptake by the cells and the incorporation into total proteins. All samples were then ignited in an Oxymat and counted with a Beckman LS-100 Scintillation Counter (p.45). Since the plant cell suspension is not as fine as a bacterial suspension, samples taken with the same automatic syringe pipette or Fin-pipette (disposible tips were cut at the end to produce a wider aperture for sampling purposes) do not always give the same quantity of sample material; it was therefore necessary to convert the resulting radioactivity of each sample to a value per unit packed cell volume. Fig. 20 presents the average value

Figure 19

Growth curve of asynchronous suspension culture of Acer pseudoplatanus L. subcultured routinely into Heller's medium.

Subculture was made by transferring 14-day old stock culture into fresh Heller's medium and the cultures were shaken on a rotary shaker in a temperature controlled room as described in the text. A culture flask was sacrificed at intervals for cell sampling. Cell densities were determined as described in the text, and their standard errors were also calculated.

Fig. 19

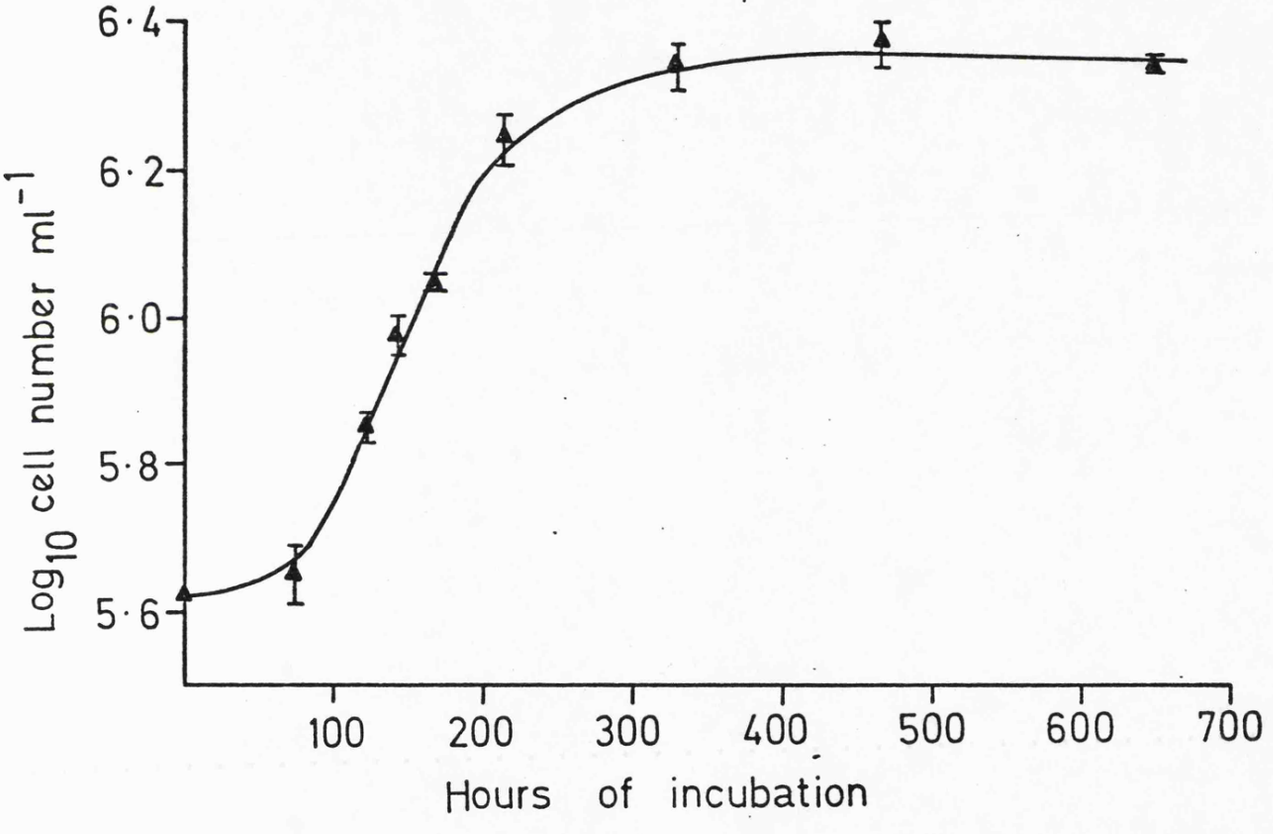

6·4
6·2
6·0
5·8
5·6
Log10 cell number ml-1
100
200
300
400
500
600
700
Hours of incubation

Figure 20

Total uptake of ^{14}C-arginine and ^{14}C-lysine, and their incorporation into proteins by an actively dividing suspension of Acer pseudoplatanus L.

To 56 ml of concentrated 7-day old suspension culture (1.68×10^6 cells ml^{-1}) was added 200 µl of ^{14}C-arginine (324 mCi $mmole^{-1}$, 50 µCi ml^{-1}), and to 60 ml of concentrated 7-day old suspension culture (1.94×10^6 cells ml^{-1}) was added 200 µl of ^{14}C-lysine (287 mCi $mmole^{-1}$, 50 µCi ml^{-1}). Incubation was carried out in 250 ml conical flask by shaking at 25 °C. Samples were taken at intervals during a period of 120 min. The uptake of the labelled amino acid and the incorporation of the labelled amino acid into total protein was determined. The remaining isotope in the incubation medium was also measured.

(a) cultured cells incubated with ^{14}C-arginine

(b) cultured cells incubated with ^{14}C-lysine

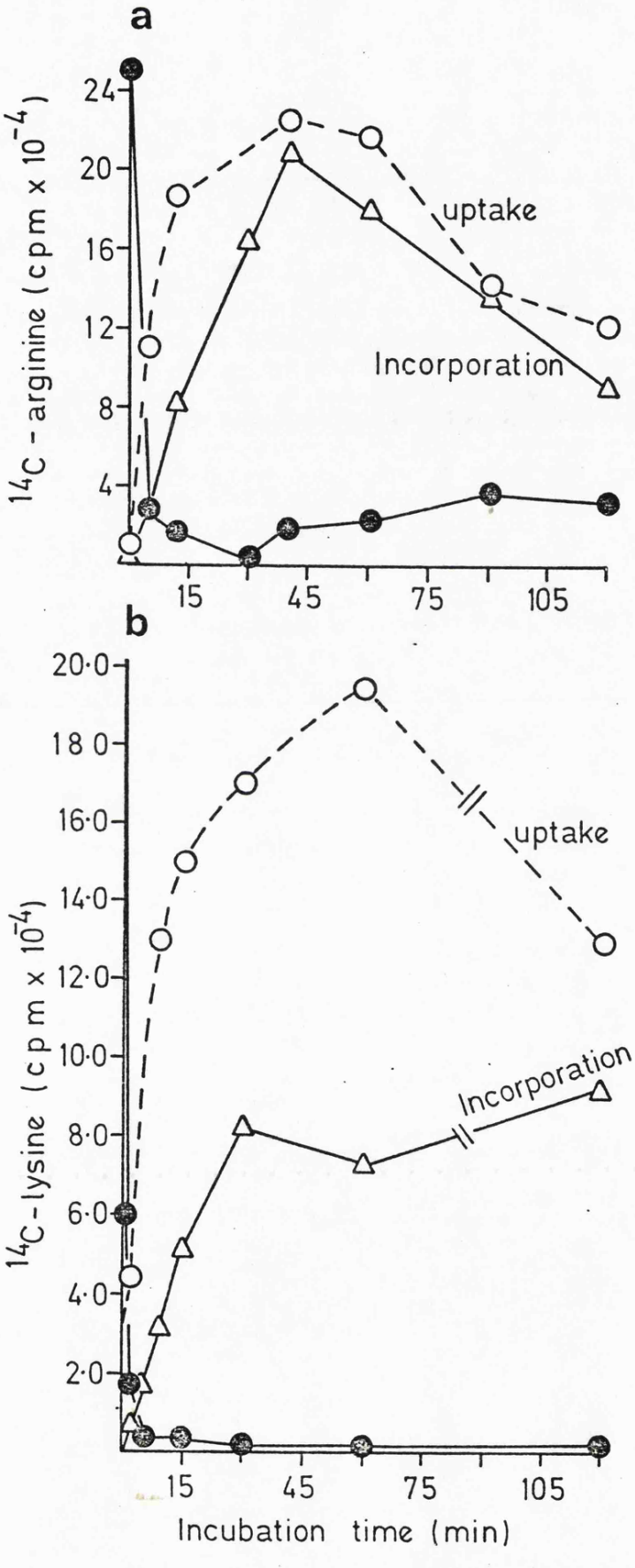
(●——●) the remaining isotope in the culture medium

(o– – –o) uptake by the cells

(△——△) incorporation into proteins.

vertical axis - cpm per 2 ml sample.

Fig. 20


a
^{14}C - arginine (cpm x 10^{-4})
24
20
16
12
8
4
uptake
Incorporation
15
45
75
105
b
^{14}C - lysine (cpm x 10^{-4})
20·0
18·0
16·0
14·0
12·0
10·0
8·0
6·0
4·0
2·0
uptake
Incorporation
15
45
75
105
Incubation time (min)

of the replicate samples with their radioactivities so corrected. A similar pattern was observed with lysine and with arginine. Both Fig. 20a and Fig. 20b illustrate very rapid loss of labelled amino acids from the culture medium. This presumably is due to a physical adsorption of the labelled amino acids onto the cell walls. The labelled amino acids were soon taken up by the cells and reached their maximum uptake in 60 min with 74% efficiency for ^{14}C-lysine and in 40 min with 89.7% efficiency for ^{14}C-arginine. Following uptake both labelled amino acids were detected in the hot-TCA insoluble material (proteins). The maximum incorporation was at 32.8% efficiency for ^{14}C-lysine in 30 min and at 82.9% efficiency in 40 min for ^{14}C-arginine. Both uptake and incorporation decreased with further incubation. This may be due to the turnover of the radioactive proteins during long term incubation. Both ^{14}C-lysine and ^{14}C-arginine will be incorporated into all sorts of protein in the cells and although histones are rich in these basic amino acids, they have the slowest turnover rates of all the protein fractions (Piha et al., 1966) so that incorporation into other proteins such as non-histone proteins and basic ribosomal proteins will be relatively high owing to their high turnover rates.

3. Incorporation of ^{3}H-lysine and ^{14}C-arginine into histone proteins

Since lysine and arginine are commonly found in all proteins, it is interesting to know how efficiently these labelled amino acids are incorporated into histone proteins. Flamm and Birnstiel (1964), in agreement with others, indicated that the incorporation of ^{14}C-lysine into the histone proteins in cultured tobacco cells is much slower than its incorporation into the residual (non-histone proteins) fraction. In studying the incorporation of these labelled amino acids into the nuclear proteins of isolated calf thymus nuclei it was found that non-

histone nuclear proteins became nearly 10 times as radioactive as histones (Reid and Cole, 1964; Reid et al., 1968). An experiment was therefore performed to compare the incorporation efficiencies of labelled lysine and arginine into histone proteins in cultured Acer cells. A labelled amino acid mixture containing 20 µl of ^{14}C-arginine and 10 µl of ^{3}H-lysine was added to 100 ml of 7-day old stock culture (9.12 X 10^5 cells ml^{-1}), and incubated by shaking in a water bath at 25 °C. The concentrations of ^{14}C-arginine and ^{3}H-lysine in the culture suspension were respectively 3.1 X 10^{-8}M, 0.01 µCi ml^{-1} and 5.56 X 10^{-9} M, 0.1 µCi ml^{-1}. Duplicate samples (2 ml each) were taken after 20 min for measurement of incorporation into total protein, while the remaining culture was left shaking for a total 2 hours. Cells were harvested after incubation and from these the histone proteins were extracted. Sample cells taken after 20 min of incubation were found to have incorporated ^{14}C-arginine with 78.6% efficiency and ^{3}H-lysine with 54.4% efficiency. Their radioactivities were 2.5 X 10^4 cpm and 9.1 X 10^4 cpm respectively for ^{14}C and ^{3}H and the ratio of ^{14}C to ^{3}H was 1 to 3.64. Counts of 627 cpm and 3430 cpm were present respectively for ^{14}C and ^{3}H in a 100 µl sample of the extracted histone giving 1 to 5.47 for the ratio of ^{14}C to ^{3}H. This suggests that the incorporation of ^{3}H-lysine into histone proteins is more efficient than that of ^{14}C-arginine, although the latter shows higher efficiency of incorporation into total protein.

Further investigation of the incorporation of labelled lysine and arginine into the histone fractions was carried out by separating the double labelled histones on acid-urea polyacrylamide gel. The positions of the major bands on the gel were recorded after staining and destaining. This gel was then cut into 1.0 mm slices with the

MICKLE gel-slicer (p. 37), and the slices were ignited and counted as described in the Methods (p. 45). Fig. 21 illustrates the distribution of the radioactivity between the gel slices both for ^{14}C-arginine and ^{3}H-lysine. The data for ^{14}C-arginine show extensive incorporation into H2B, moderate incorporation into H2A, H3 and H4 and no detectable incorporation into H1. The data presented for ^{3}H-lysine suggest that the biosynthesis of Acer histone is similar to that of Walker carcinosarcoma (Hnilica and Busch, 1963; Ohly et al., 1967). The ^{3}H-lysine was incorporated into H3 most actively, H2B was next to H3 in level of incorporation, while incorporation into H2A was at an intermediate rate and the incorporation into H1 and H4 was least active. Similar differential biosynthesis of histone fractions has also been observed in liver and hepatoma by Irvin and associates (Holbrook et al., 1960, 1962; Evans et al., 1962; Chalkley and Maurer, 1965). Although the individual histone fractions other than H1 appear to be present in equal amounts in the nucleus, it seems that their biosynthetic rates are different. The present study indicated that the very arginine-rich histone H3 incorporated labelled lysine most actively and incorporated arginine at only a moderate rate (possibly suggesting the presence of a large arginine pool), whereas the very lysine-rich histone H1 incorporated labelled lysine very slowly and showed no arginine incorporation (suggesting that very little of this histone was synthesized during the test period of 2 hours). The result also indicated that lysine is more actively incorporated than arginine into histone proteins.

4. Cellular localization of histone synthesis

When cultured Acer cells after pulsing for 5 min with labelled lysine or arginine were fixed, autoradiography showed radioisotope distributed fairly evenly within the cells, and not clearly localized

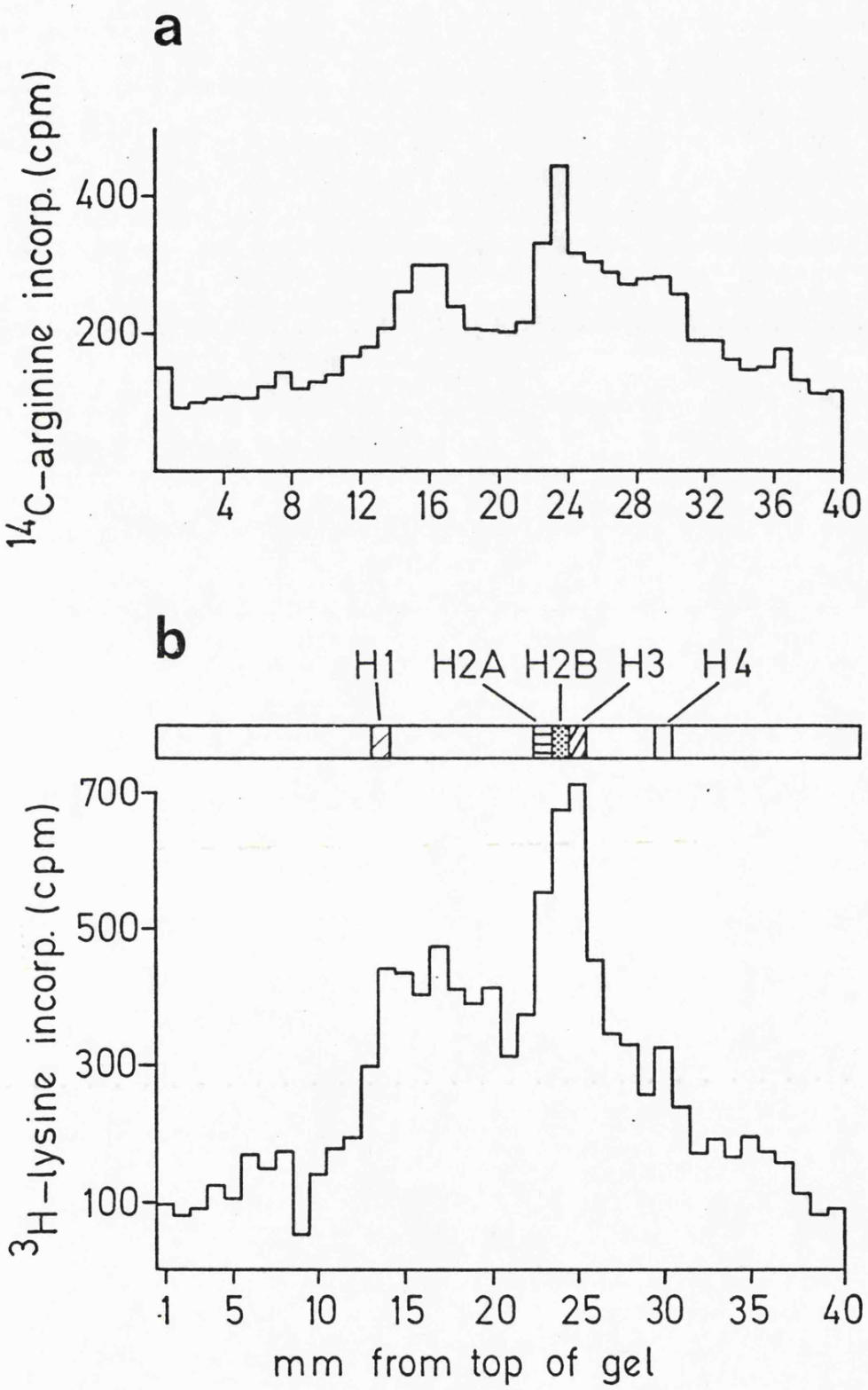
Figure 21

Distribution of labelled acid-soluble proteins on acid-urea polyacrylamide gel.

Seven-day old stock culture (100 ml, 9.12 X 10^5 cells ml^{-1}) was incubated with a mixture of ^{14}C-arginine and ^{3}H-lysine for a period of 2 hours by shaking at 25°C. The final concentrations of ^{14}C-arginine and ^{3}H-lysine were respectively 3.1 X 10^{-8}M, 0.01 µCi ml^{-1} and 5.56 X 10^{-9} M, 0.1 µCi ml^{-1}. Histones extracted from these labelled cells were characterized on acid-urea polyacrylamide gels. The histone pattern is presented between profiles (a) and (b). The gel slices (1 mm thick) were combusted in an Oxymat, and their radioactivities were recorded respectively for ^{14}C-arginine (a) and ^{3}H-lysine (b).

vertical axis - cpm per 1 mm gel slice.

Fig. 21


a
^{14}C-arginine incorp. (cpm)
400
200
4
8
12
16
20
24
28
32
36
40
b
H1
H2A
H2B
H3
H4
^{3}H-lysine incorp. (cpm)
700
500
300
100
1
5
10
15
20
25
30
35
40
mm from top of gel

(Gould, 1975 and my own observation). This approach is therefore not capable of establishing the site of histone synthesis. However, since histones contain no tryptophan but are rich in basic amino acids, the synthesis of histones can be distinguished from that of other proteins by feeding the cells with labelled lysine plus a different kind of labelling of tryptophan. To investigate the location of histone synthesis in cultured cells of Acer pseudoplatanus L. a mixture of ^{14}C-lysine and ^{3}H-tryptophan was applied to the linear phase cells, and both 'pulse' and 'pulse-chase' experiments were carried out. In order to avoid the complexity of the turnover problem during incubation, the experiment was confined to a short incubation period. The 'pulse' treated cells were incubated with the labelled mixture for 5 min while the 'pulse-chase'treated cells were chased with 'cold'medium (Heller's medium plus lysine and tryptophan) for 10 min after this 5 min pulse. These treated cells were then fractionated into nuclear and cytoplasmic fractions, and their proteins were precipitated and counted. The detailed procedure was described in the Methods (p.39-41). Table 6 illustrates the incorporation of ^{14}C-lysine and ^{3}H-tryptophan into proteins in different fractions of 'pulsed' and 'pulse-chased' cells. Since it is the ratio of ^{14}C-lysine to ^{3}H-tryptophan incorporated into the proteins which should be analyzed for the present experimental purpose, the radioactivities presented for the different cell fractions have not been converted into values per unit biomass. The result shows that the incorporation of ^{14}C-lysine and ^{3}H-tryptophan into total protein, cytoplasmic protein and nuclear protein of the 'pulsed' cells is within a ^{14}C to ^{3}H ratio range of 3.0 to 3.6. However, the cytoplasmic protein possesses a higher ratio than does the nuclear protein. By contrast, in the 'pulse-chased' cells the ratio of ^{14}C to ^{3}H in nuclear protein is

Table 6. Pulse-chase kinetics of ^{14}C-lysine and ^{3}H-tryptophan labelling of proteins in cultured cells of Acer pseudoplatanus L.

Cell fractions	'pulsed' cells			'pulse-chased' cells		
	^{14}C-lysine (cpm)	^{3}H-tryptophan (cpm)	$^{14}C/^{3}H$	^{14}C-lysine (cpm)	^{3}H-tryptophan (cpm)	$^{14}C/^{3}H$
Whole cell	1.7×10^4	5.29×10^3	3.21	1.2×10^5	1.8×10^4	6.66
Cytoplasm	2.1×10^4	5.90×10^3	3.56	1.0×10^5	1.7×10^4	5.72
Nucleus	1.9×10^3	6.20×10^2	3.06	2.8×10^4	2.3×10^3	12.17
Uptake*	3.76×10^5	6.0×10^4	6.26	3.62×10^5	5.0×10^4	7.24

*The uptake of labelled amino acids by cells.

more than twice that of the cytoplasmic proteins; presumably this is due to the transport of histones (proteins with a very high ratio of ^{14}C-lysine to ^{3}H-tryptophan) from the cytoplasm into the nucleus during the chase period. Both 'pulsed' and 'pulse-chased' cells had ratios of ^{14}C to ^{3}H in the total cell protein between those of cytoplasmic protein and nuclear protein as predicted. This result is in agreement with the work of Robbins and Borun (1967) on HeLa cells. As many investigators have reported on the migration of low molecular weight proteins from the cytoplasm to the nucleus (see introduction of this Chapter), the histones of cultured Acer cells are predicted to be synthesized in the cytoplasm and then rapidly transported into nucleus.

5. Effect of hydroxyurea on histone biosynthesis

One way of studying the coupling between the synthesis of different macromolecules is to use specific inhibitors. Hydroxyurea (HU) is a specific inhibitor of DNA synthesis in vivo (Yarbro, 1967). It has been reported to have no influence on RNA and protein biosynthesis except histone biosynthesis (Yarbro et al., 1965; Rosenkranz et al., 1965, 1967). Gallwitz and Mueller (1969c), in studies on HeLa cell microsomes, have found that HU at 5×10^{-3} M blocked DNA synthesis and that this resulted in termination of histone synthesis within 30 min. They proposed that the histone mRNA is synthesised during DNA synthesis, and that the utilization of the RNA for translation in the cytoplasm is probably activated by a DNA-dependent mechanism. To undertake a coupling investigation of this kind with cultured Acer suspension cells, the effect of the concentration of HU on protein biosynthesis in vivo was first studied. The experiment was performed by incubating 5 ml of concentrated 7-day old Acer cells (1.36×10^{6} cells ml^{-1}) with 25 µl of

^{14}C-leucine (324 mCi mmole^{-1}, 50 μCi ml^{-1}) in the presence of different concentrations of HU. The final concentration of ^{14}C-leucine in the cell suspension was 6.76 X 10^{-7} M, 0.25 μCi ml^{-1}, and the different concentrations of HU were 1.0 X 10^{-6} M, 1.0 X 10^{-5} M, 1.0 X 10^{-4} M, 1.0 X 10^{-3}M and 1.0 X 10^{-2} M. A control flask containing distilled water instead of HU was also included in the experiment. Both HU and ^{14}C-leucine were first pipetted into 25 ml conical flasks, and the incubation was initiated by adding culture suspension into the flask and shaking in a water bath at 25 °C. The incubation was stopped by adding 10 ml of ice-cold 'cold' Heller's medium after 20 min shaking. Immediately triplicate samples (each 4 ml) were pipetted into ice-cold 'cold' 10% TCA to give 3 sets of samples for each different concentration of HU. Two sets of these samples were used for determination of the radioactivity of their total protein, while the 3rd set was extracted with 0.25 N HCl, and then the remaining acid insoluble fractions were collected on membrane filters and their radioactivities were measured (detail in p. 41-43).

Both Fig. 22 and Table 7 illustrate the effect of HU on the synthesis of total protein, basic protein (HCl-soluble protein) and non-basic protein (HCl-insoluble protein) in cultured Acer cells. There occurred a substantial decline in the synthesis of basic protein at low concentrations of HU. The incorporation of ^{14}C-leucine at 1.0 X 10^{-6} M and 1.0 X 10^{-5} M HU was respectively 56.6% and 11.6% of the control value; this decline, however, did not continue with the increase of HU concentration. This result suggests that there are at least two classes of basic proteins in Acer cells in terms of their sensitivities to HU: those whose synthesis is very sensitive to HU and those whose synthesis is resistant to HU. Hydroxyurea in terms of its effect on

Figure 22

Inhibition of the synthesis of total protein and non-basic protein in an actively dividing suspension of Acer pseudoplatanus L. by different concentration of hydroxyurea.

Concentrated 7-day old Acer suspension (5 ml, 1.36 X 10^6 cells ml^{-1}) was incubated with 25 ul of ^{14}C-leucine (324 mCi $mmole^{-1}$, 50 μCi ml^{-1}) and 250 μl of hydroxyurea at different concentrations. Incubation was carried out in 25 ml conical flasks with shaking at 25°C for 20 min, and the incorporation of ^{14}C-leucine into total protein and non-basic protein was measured.

(●——●) incorporation into total protein

(▲– – –▲) incorporation into non-basic protein

vertical axis - cpm per 4 ml sample (after dilution as described in the text).

Fig. 22

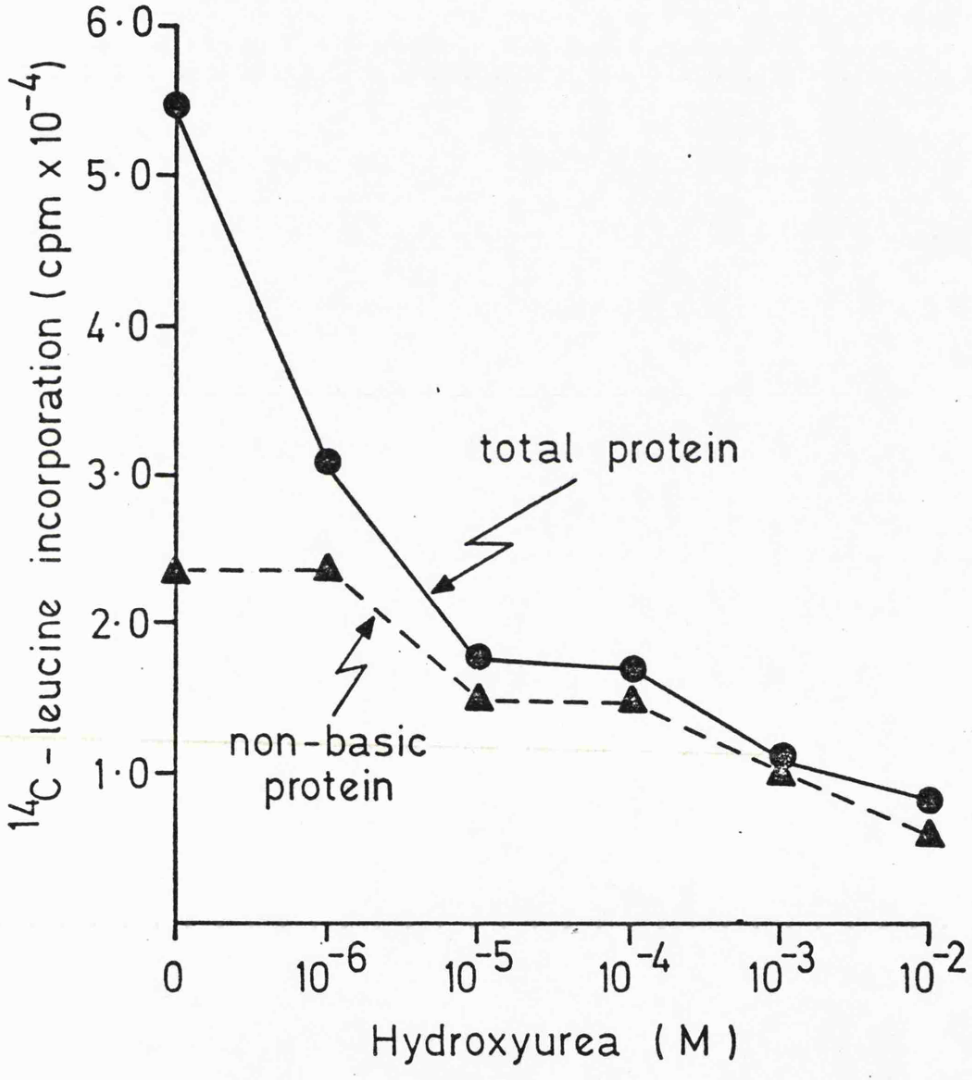

6·0
5·0
4·0
3·0
2·0
1·0
^{14}C - leucine incorporation (cpm x 10^{-4})
total protein
non-basic protein
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Hydroxyurea (M)

Table 7. Inhibition of the synthesis of total protein, basic protein and non-basic protein in cultured cells of Acer pseudoplatanus L. by different concentrations of hydroxyurea

	percentages of protein synthesis					
conc. of hydroxyurea	0	1.0×10^{-6}M	1.0×10^{-5}M	1.0×10^{-4}M	1.0×10^{-3}M	1.0×10^{-2}M
total protein	100	56.6	32.2	31.1	19.8	15.0
non-basic protein	100	100.0	64.0	63.7	44.0	26.0
basic protein	100	56.6	11.6	11.3	11.1	11.1

the synthesis of non-basic protein can be classified into 3 concentration groups : a low concentration such as 1.0×10^{-6} M has no effect at all, a moderate concentration such as 1.0×10^{-5} M or 1.0×10^{-4} M inhibits synthesis to 64% of the control value, and higher concentrations such as 1.0×10^{-3} M or 1.0×10^{-2} M intensly and progressively inhibits the synthesis. This result is not in agreement with those of other systems : protein synthesis in Acer cells appears to be hypersensitive to HU. Rosenkranz and Levy (1965) indicated that hydroxyurea has a bacteriostatic rather than a bactericidal effect; it requires 3×10^{-3} M for inhibition of DNA synthesis and more than 0.2 M is required for a similar effect on protein synthesis. However, the present result concerning the effect on protein synthesis is convincing in so far as all replicate samples gave reproducible data and the inhibition patterns for total protein and for non-basic protein coincided.

Although HU has been used to induce cell division synchrony in a suspension culture of Haplopappus gracilis (Eriksson, 1966), and to study the effect on nucleic acid synthesis in Vicia, neither of these studies was concerned with the effect on protein synthesis. It has been reported by Sinclair (1965) that cultured mammalian cells which are actively synthesizing DNA (S phase) are lethally damaged when exposed to HU; if the majority of the linear phase Acer cells studied were synthesizing DNA, this could account for their particular sensitivity to HU.

A similar experiment was also performed to study the effect of HU on DNA synthesis. Unfortunately, the result showed that ^{3}H-thymidine was not even incorporated in detectable amounts into DNA in the control sample. If a new stock of ^{3}H-thymidine had been used, inhibition by HU of DNA synthesis might have been obtained, and the basic protein of Acer cells could then have been considered to be of two

classes as described by Chalkley and Maurer (1965) : those whose synthesis is sensitive to HU and coupled with DNA replication, and those whose synthesis is resistant to HU and independent of DNA replication.

If sufficient time had been available it would have been interesting to repeat these experiments, and choose a particular concentration of HU to investigate its effect on the synthesis of individual histone proteins and non-basic proteins so that the relationship between their synthesis and that of DNA could be considered.

6. Biosynthesis of histones during the growth cycle

During the early stage of this project, the ratio of DNA to histone in the nucleus as well as the histone patterns on polyacrylamide gels was examined in cells at different stages during their growth in batch culture. This included cells in exponential phase, linear phase, early stationary phase and late stationary phase. Cultures were grown in a 1 litre culture bottle or a 10 litre culture bottle to provide a large quantity of cells at the same growth stage. These cultures were initiated from 14-day old stock cultures as described in the Methods (p. 20). Both the exponential phase cells and the linear phase cells were collected from 5-litre suspension cultures which were grown in 10-litre bottles for 5 days and 8 days respectively. The early and late stationary phase cells were harvested from 500 ml suspension cultures which were grown in 1-litre bottles for 14 days and 28 days respectively. Cells after harvest by filtration were used to isolate nuclei. A portion of the isolated nuclear fraction was withdrawn for DNA determination, while the remaining nuclear fraction was extracted for histones by the $CaCl_2$-TCA method, and the histones were determined

by the Lowry method. The result showed that the ratios of histone to DNA are 0.76, 0.86, 0.95 and 0.93 for cells of exponential phase, linear phase, early stationary phase and late stationary phase respectively. The DNA content of the residues after histone extraction (a combined mixture of $CaCl_2$-insoluble fraction and 0.02 N H_2SO_4-insoluble fraction) were also determined. However, values only 40% to 55% of those calculated from the untreated nuclear fractions were obtained.

Histones extracted from these cells were characterized on acid-urea polyacrylamide gels and their electrophoretic patterns are shown in Fig. 23. Histone samples are similar in terms of their banding patterns on polyacrylamide gels except for the sample of 8-day cells where H4 was missing. Since the plan at that time was to isolate nuclei not only for histone extraction but also for chromatin template availability studies, all starting materials were on a fairly large scale, particularly the sample of 8-day cells, which required 5 hours to complete the rupture process for the 180 g fresh weight of cells. The missing H4 in this sample may be due to proteolysis occurring while the nuclei were still in the rupture suspension. It must be pointed out that these samples of cells, because of their large quantity, could only be extracted one sample a day, and the duration of dialysis was not strictly controlled so that the variation of histone to DNA ratios presented may not be the true values. The electrophoretic patterns seemed to be unchanging.

Since the investigation was then directed to work on synchronous cultures, this experiment was not repeated.

Figure 23

Electrophoretic patterns of histones extracted from cultured cells of Acer pseudoplatanus L. at different growth stages.

Acer histones were obtained from nuclei by the $CaCl_2$-TCA method, and calf thymus histones were purchased from Sigma Chemical Company. Histones were characterized on acid-urea polyacrylamide gels and stained with Amido Black. The sample gels (from left) are calf thymus histones (20 µg), and Acer histones of exponential phase cells (21 µg), linear phase cells (32 µg), early stationary phase cells (16 µg) and late stationary phase cells (32 µg).

Fig. 23

7. Biosynthesis of histones during the cell cycle

As mentioned earlier in this Chapter, when asynchronous suspension cultures of Acer pseudoplatanus L. were pulsed with labelled lysine or arginine for short periods, the autoradiographic results showed that silver grains generally appeared in all cells. This is in agreement with the observation by Gould (1975), and suggests that basic proteins are synthesized throughout the cell cycle. Since it is impossible to correlate the cytochemical and autoradiographic measurements with the biosynthesis and turnover of the individual histone fractions, an attempt was made to pursue this investigation biochemically.

Suspension cultures of Acer pseudoplatanus L. have been successfully synchronized by nitrate starvation and re-growth in a 4-litre batch culture (King, et al., 1973, 1974; Gould and Street, 1975). It would be an ideal to use synchronous cultures for studying the temporal relationship between synthesis of DNA and histones. An experiment had been performed by Kovacs and Gould (Gould, 1975) involving extraction of histones and DNA during interphase in synchronously dividing cultures. They concluded that there is no evidences for the co-ordinated synthesis of these two macromolecular fractions. However, the extractable histone fraction they obtained could not be characterized on the polyacrylamide gel which permits the separation of calf thymus histones into their fractions. Thus, it was necessary to re-investigate this problem using appropriate methods.

On account of the properties of the culture cells and the techniques available at this moment, it is necessary that each representative sample contains at least 1.5×10^8 cells (thus providing not less than 100 µg of histone for chemical analysis). A sample of this size is necessary because of the low DNA content of Acer cells

(2c value=5-6 X 10^{-12} g), the low efficiency of the isolation of nuclei (20% of the total cell population) and the variable efficiency of histone extraction (60-100% of the isolated nuclear fraction). The synchronous culture system was therefore modified by using a 10.0-litre culture vessel instead of the standard 5.0-litre vessel. Cell division synchrony was initiated by transferring 3 flasks (60 ml culture per flask) of stationary phase Acer pseudoplatanus cells (28-day old stock culture) into 9.0 litres of Heller's medium. Eight cultures of this kind were established as described in the Methods (p. 20-21) during the present experimental work. The fates of these cultures were :

Culture I. became asynchronous after 2 cell cycles.

Culture II. was asynchronous from the beginning.

Culture III. contaminated after 160 hours' incubation.

Culture IV. proceeded through 3 cell cycles synchronously.

Culture V. did not grow.

*Culture VI. appeared to proceed through 5 cell cycles synchronously.

Culture VII. proceeded through 5 cell cycles synchronously and then became asynchronous.

Culture VIII. proceeded through 5 cell cycles synchronously.

*The automatic sampling system was not operating properly during 190-280 hrs, so that the incomplete data did not satisfactorily demonstrate the apparent 5 cell cycles.

The growth patterns (in terms of cell number) of the synchronous cultures IV, VII, and VIII are illustrated in Fig. 24 by plotting $\log_{10}$ of cell number data against incubation time. The fractions of the total cell populations which divided at each burst of cytokinesis and the interphase intervals between those bursts are shown in Table 8. Although cell numbers showed distinct step-ups, the duration of cycle times was

Figure 24

Cell number curves of synchronous cultures IV, VII and VIII.

The cultures were initiated by inoculating 3 flasks of 28-day old stock cultures of Acer pseudoplatanus L. into 9.0 litres of Heller's medium (contained in a 10.0 litre culture vessel). Cell numbers (Δ) were counted during the progress of growth. The symbol (O) indicates the times at which samples were taken for chemical analysis.

Fig. 24

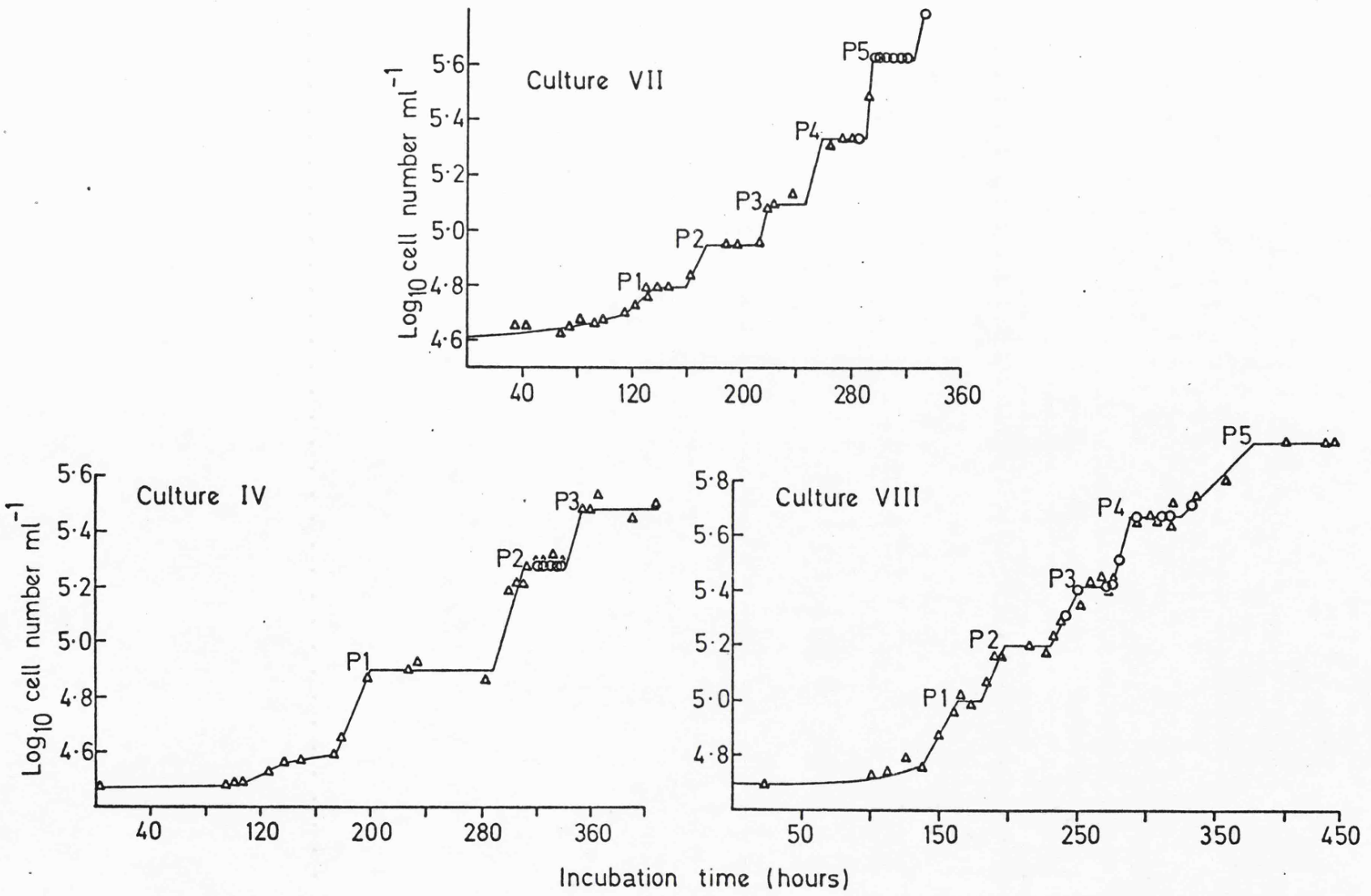

Culture VII
Culture IV
Culture VIII
Log10 cell number ml-1
P1
P2
P3
P4
P5
Incubation time (hours)

Table 8. Fraction of the cells dividing (%) at each burst of cytokinesis (A), and the time intervals (B) between steps for the growth curves illustrated in Fig. 24.

(A) Fraction of cell division (%)

	plateaux				
culture	P1	P2	P3	P4	P5
IV	101	120	79.5	-	-
VII	57.7	71.4	70.7	86.9	95.2
VIII	86.9	79.0	83.2	89.9	91.9

(B) Duration of intervals (hours)

		plateaux				
culture	lag	P1	P2	P3	P4	P5
IV	200	110	42	-	-	-
VII	136	38	46	38	37	37
VIII	164	34	54	36	90	-

random. This made it very difficult to obtain samples corresponding to the sequential phases of the cell cycle. In order to obtain an adequate number of cells for histone extraction and at the same time to avoid removing too large a volume of culture suspension at each sampling, samples were not taken until the cell density had reached about 2.0 X 10^5 cells ml^{-1}. Therefore, samples were withdrawn from $*P_2$, P_4-P_5 and P_3-P_4, as shown in Fig. 24, for cultures IV, VII and VIII respectively.

Two sets of samples were collected from culture VIII. One set of samples was incubated with ^{3}H-thymidine for 1 hour. These labelled cells were then used for autoradiographic analysis as well as for studying the activity of DNA synthesis (in terms of the specific activity of DNA being synthesised). Unfortunately, these two sets of data did not coincide with each other (Data of the specific activities of DNA were provided by Dr. B. Stratton). None of the samples was proved to be unambiguously in a synchronized S phase. The other set of sample cells was incubated with ^{3}H-lysine for 1 hour, in an attempt to compare the specific activities of each histone fraction at intervals during interphase. It was very disappointing to find that the incorporation efficiencies were too low to allow fractionation and to measure the radioactivity of each fraction. However, the data for specific activities of the total histones indicate that histones are synthesized throughout the cell cycle. Table 9 illustrates these data. The histone samples (extracted by the H_2SO_4-EtOH method) were electrophoresed on acid-urea polyacrylamide gels, and the gels were scanned after staining and de-staining. Fig. 25 illustrates the microdensitometer tracings of those gels. All major fractions of histones presented sharp peaks on the gels. Their banding patterns were similar but the ratio of H1 to H4

*P stands for plateau.

Table 9. Specific activities of DNA, histones, and the percentages of labelled nuclei analysed by autoradiography for samples from synchronous culture VIII.

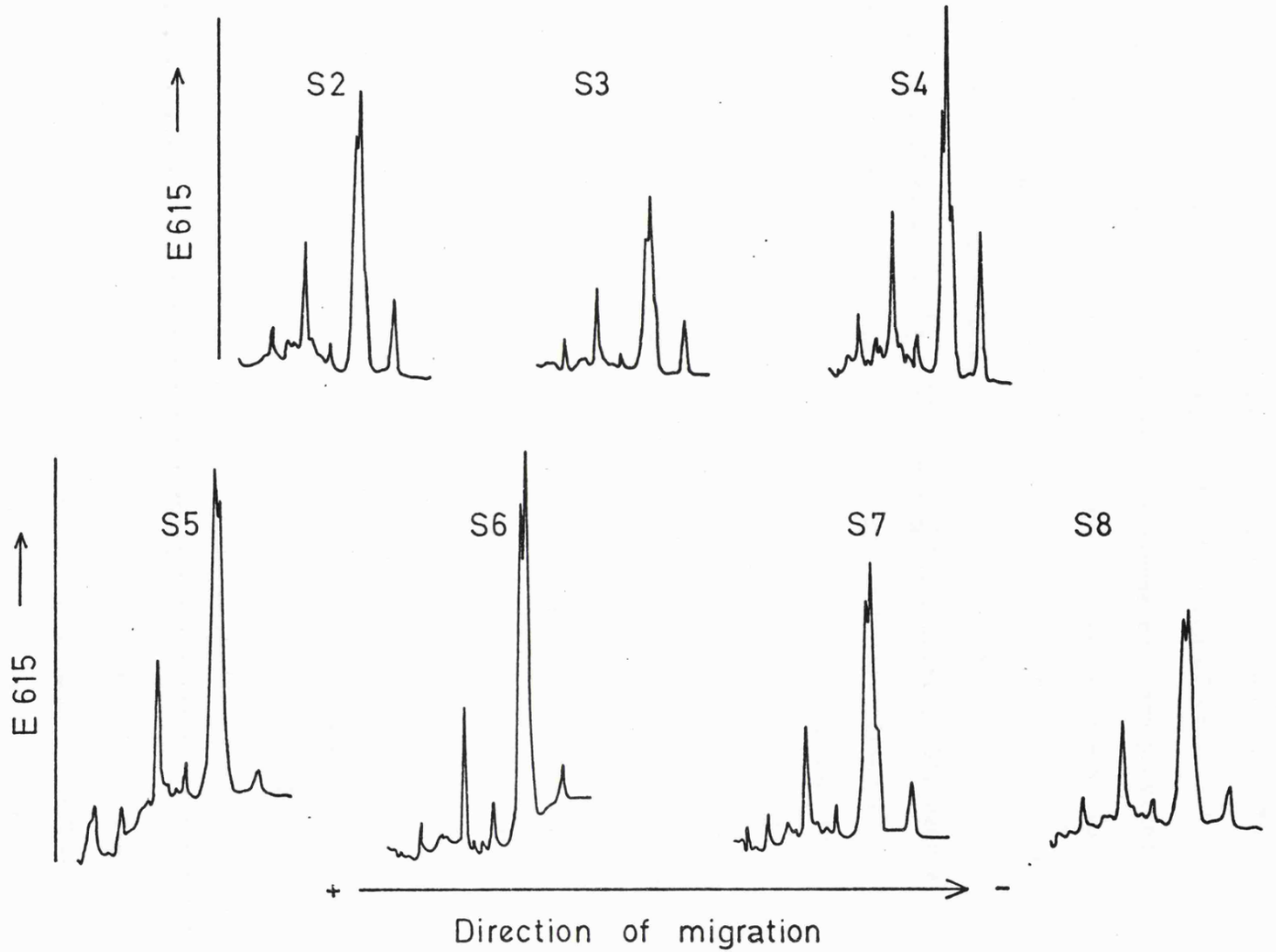
		sample no.						
		2	3	4	5	6	7	8
DNA	specific activity (cpm x 10^{-3} per μg)	1.45	1.79	1.66	1.88	1.77	1.67	1.35
	labelled nuclei (%)	12.8	14.2	12.8	13.6	14.0	14.8	12.5
Histone	specific activity (cpm per μg)	54.5	52.8	43.7	68.0	51.5	51.5	51.5

Figure 25

Microdensitometer tracings of histones extracted from samples of synchronous culture VIII.

Histones obtained by the H_2SO_4-EtOH method from samples 2, 3, 4, 5, 6, 7 and 8 of synchronous culture VIII were characterized on acid-urea polyacrylamide gels and scanned at 615 nm with a Beckman Spectrophotometer Model 24.

Fig. 25



varied (Table 10).

Samples of culture VII were analyzed by cell-counts (Fig. 24), autoradiography, mitotic index and the fraction of cells with G2-DNA content. The data for the last two parameters were provided by Dr. L. Withers who used the same culture for freeze-preservation studies. Those data except cell density are illustrated in Fig. 26. From the analysis of these data, the samples collected from Culture VII were classified as sample 1='M', sample 2='G1', sample 3='S', sample 4='S', sample 5='S-G2', sample 6='G2' and sample 8='M'. Although sample 4 may not be precisely at the peak of S phase, it seemed to be around the peak, for it was actively synthesizing DNA. The biosynthetic activities of macromolecules for those samples were also studied. The sampled cells were incubated respectively with ^{3}H-thymidine, ^{3}H-uridine, ^{3}H-lysine and ^{3}H-tryptophan as described in the Methods (p. 43), in an attempt to investigate the biosynthesis of DNA, RNA, basic protein and non-basic protein respectively. Fig. 27 demonstrates the incorporation patterns obtained for the individual macromolecules.

The duration of S phase according to the incorporation pattern of ^{3}H-thymidine was about 7 hours : it started before sample 3 and ended by sample 5, and the incorporation was highest in sample 4. Both ^{3}H-uridine and ^{3}H-lysine were incorporated before S phase, and the maximum incorporations corresponded to that of ^{3}H-thymidine. In contrast, ^{3}H-tryptophan was found to be actively incorporated in all phases except S phase. The appearance of the minor peak of ^{3}H-thymidine incorporation after S phase may suggest that the culture was not perfectly synchronized.

The same cell samples of culture VII were also extracted by the $CaCl_2$-TCA method and the histones were characterized on acid-urea polyacrylamide gels. Fig. 28 illustrates the microdensitometer tracings

Figure 26

Parameters for assessing cell-cycle stage of samples from synchronous culture VII.

Two sets of samples were collected from the culture during P_4-P_5. One set of samples was prepared for microdensitometric analysis : 300 nuclei were scored from each sample for their DNA contents, from which the percentage of nuclei with the G2 DNA content was calculated (Δ——Δ). The same sample-slides were also scored for mitotic index (●——●). The other set of samples was incubated with ^{3}H-thymidine and processed for autoradiographic analysis of the percentage of labelled nuclei (o- - -o). Data for mitotic index and fraction of labelled nuclei were based on the examination of 1000 nuclei per sample.

Samples collected for chemical analysis are numbered along the mitotic index curve.

c : cytokinesis.

Fig. 26

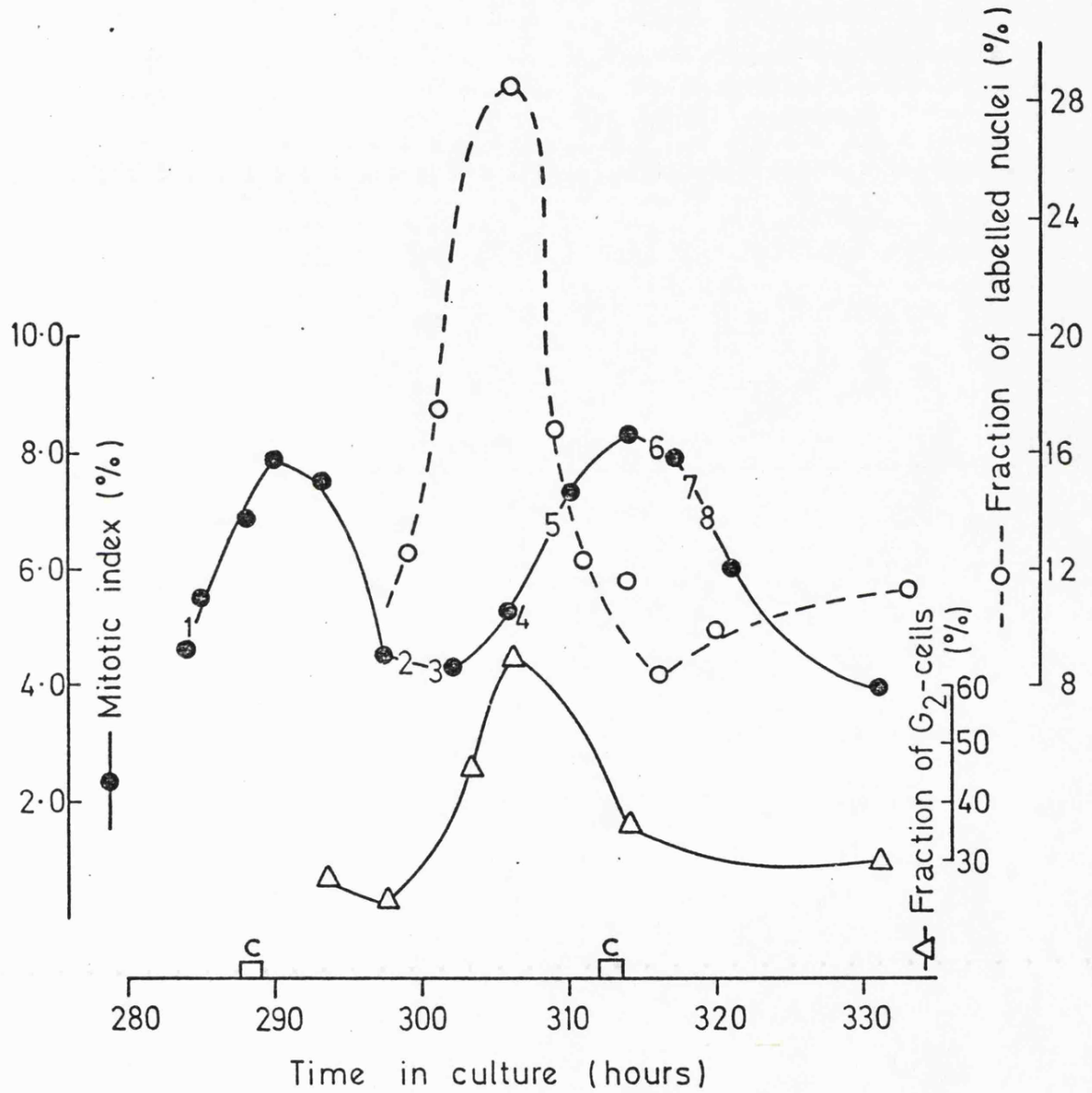

10·0
8·0
6·0
4·0
2·0
Mitotic index (%)
28
24
20
16
12
8
Fraction of labelled nuclei (%)
60
50
40
30
Fraction of G2-cells (%)
1
2-3
4
5
6
7
8
C
C
280
290
300
310
320
330
Time in culture (hours)

Figure 27

Biosynthesis of macromolecules by cells sampled during interphase of synchronous culture VII.

The biosynthesis of DNA, RNA, basic protein and non-basic protein were detected respectively by incubation of 5 ml culture suspension with 5 µl of ^{3}H-thymidine, ^{3}H-uridine, ^{3}H-lysine and ^{3}H-tryptophan at 25 °C. Samples were taken at zero time and after 15 min incubation. The incorporation of each labelled compound into macromolecules was determined, and the true incorporation was obtained by subtracting the radioactivity measured for the corresponding zero time sample.

In order to present these incorporation patterns in a clear form, the incorporation curves for ^{3}H-uridine and ^{3}H-lysine are plotted with 1000 cpm as background.

(▲——▲) ^{3}H-uridine incorporation

(●- - - -●) ^{3}H-lysine incorporation

(△——△) ^{3}H-thymidine incorporation

(o- - - -o) ^{3}H-tryptophan incorporation

vertical axis - cpm per 1ml Sample.

Fig. 27

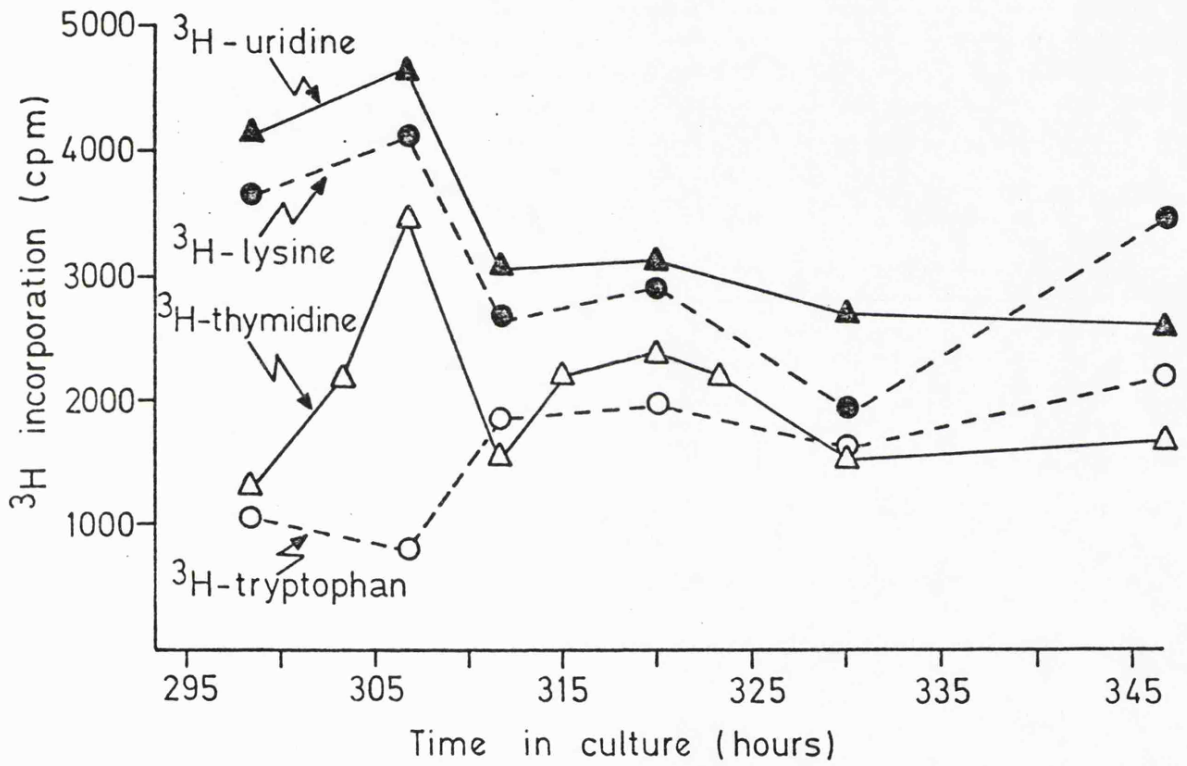
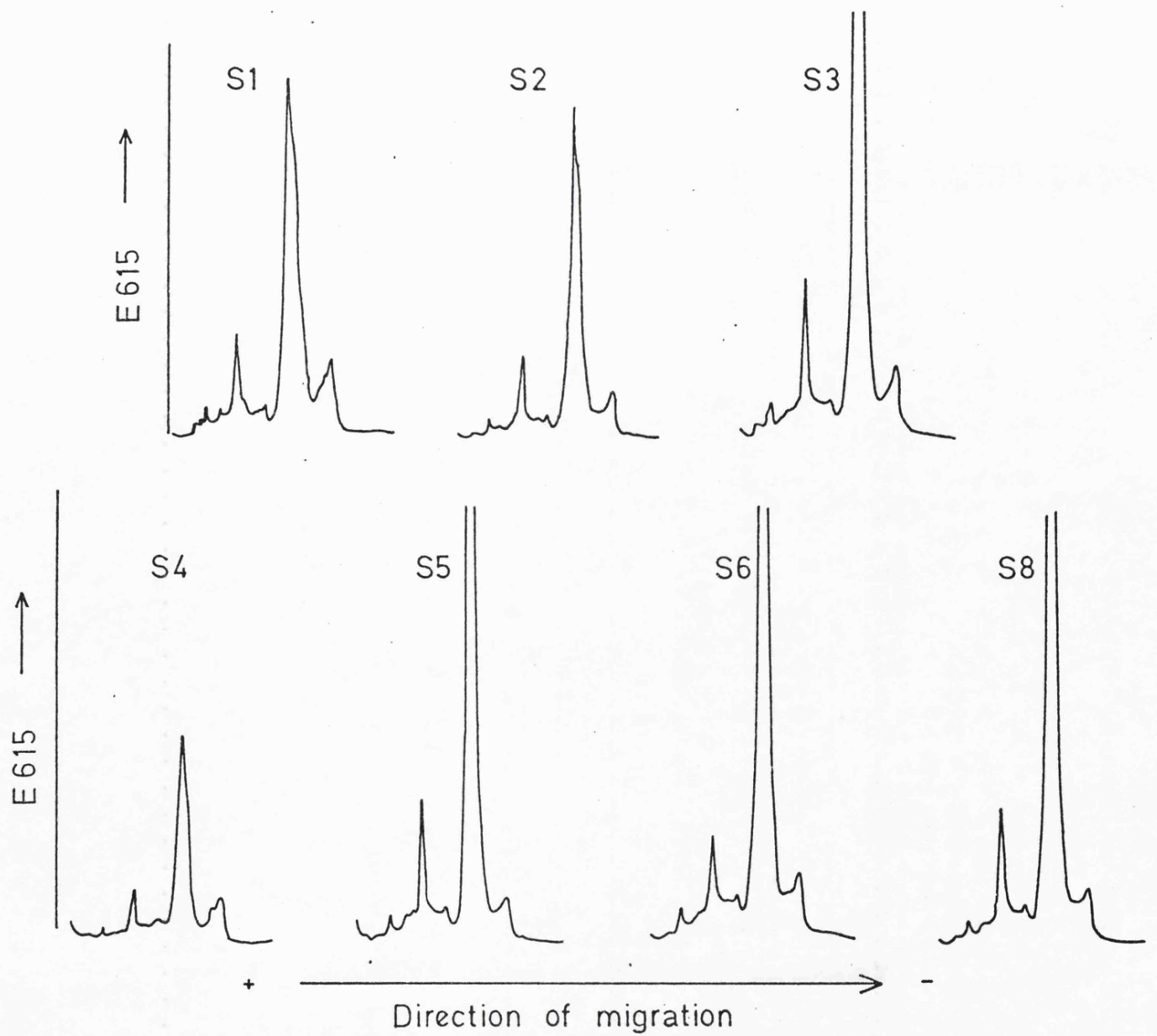

5000
4000
3000
2000
1000
^{3}H incorporation (cpm)
^{3}H - uridine
^{3}H - lysine
^{3}H-thymidine
^{3}H-tryptophan
295
305
315
325
335
345
Time in culture (hours)

Figure 28

Microdensitometer tracings of histones extracted from cells sampled during interphase of synchronous culture VII.

Histones extracted by the $CaCl_2$-TCA method from samples 1, 2, 3, 4, 5, 6 and 8 of synchronous culture VII were characterized on acid-urea polyacrylamide gels and scanned at 615 nm with a Beckman Spectrophotometer Model 24.

Fig. 28



of the resulting gels. The differences observed between these scanning patterns lie in the heterogeneity of H4 and in the ratio of H1 to H4. Although the heterogeneity of H4 could be due to the extraction method employed as discussed in the previous Chapter, it could also be a reflection of the samples per se, because all these batch samples were extracted at the same time with the same procedure. Modification of histone such as acetylation and phosphorylation could result in the heterogeneity of histone bands; if so, the modification of H4 seems mainly to occur in S phase of the cell cycle. It would be interesting to know what sort of modification actually happens and its function in relation to progress through the cycle.

For those samples collected from culture IV the histones were extracted by the $CaCl_2$-TCA method and characterized on acid-urea polyacrylamide gels. This also showed variation in the ratio of H1 to H4 amongst the samples.

The histone fractionation was quantified by measuring the occupied areas on expanded microdensitometer tracings and expressing each fraction as a percentage of the total histone. Table 10 shows the percentages of the histone fractions for samples of cultures IV, VII and VIII. The variations in H1 and H4 are considerable. The ratio of H1 to H2A+H2B+H3 to H4 is 12.7±6.75% to 77.37±3.09% to 11.1±9.27%, and the ratio of H1 to H4 varies from 0.59 to 4.0. Since the bands of H2A, H2B and H3 are close together on polyacrylamide gels their individual areas could not be calculated, and thus three fractions are expressed as one.

Histone samples of cultures VII and VIII were also examined for possible modification of H1. The total histones were electrophoresed on acid-urea polyacrylamide gel for 8 hours to allow better separation

Table 10. Ratio changes of histone fractions amongst histone samples extracted from cells during interphases of synchronous cultures.

culture no.	phase	sample no.	histone fraction H1	H2A+H2B +H3	H4	H1/H4
IV		1	11.2	85.4	5.9	1.8
		3	15.7	72.5	11.4	1.38
		4	12.5	78.2	9.3	1.35
		5	11.7	75.2	13.1	0.89
VIII		2	23.7	63.5	12.8	1.85
		3	14.1	70.4	15.6	0.9
		4	10.1	75.7	14.2	0.71
		5	13.6	83.0	3.4	4.0
		6	16.6	71.4	12.0	1.38
		7	12.4	79.3	8.3	1.49
		8	10.0	80.1	9.9	1.01
VII	M	1	8.07	83.9	8.07	1.00
	G1	2	12.2	71.1	16.7	0.73
	S	3	12.9	74.2	12.8	1.01
	S	4	10.5	71.6	17.9	0.59
	S-G2	5	14.8	77.9	8.04	1.84
	G2	6	11.3	74.4	14.3	0.79
	M	8	7.08	86.1	6.79	1.04
	average		12.7 + 6.75%	76.4 + 3.09%	11.0 + 9.27%	

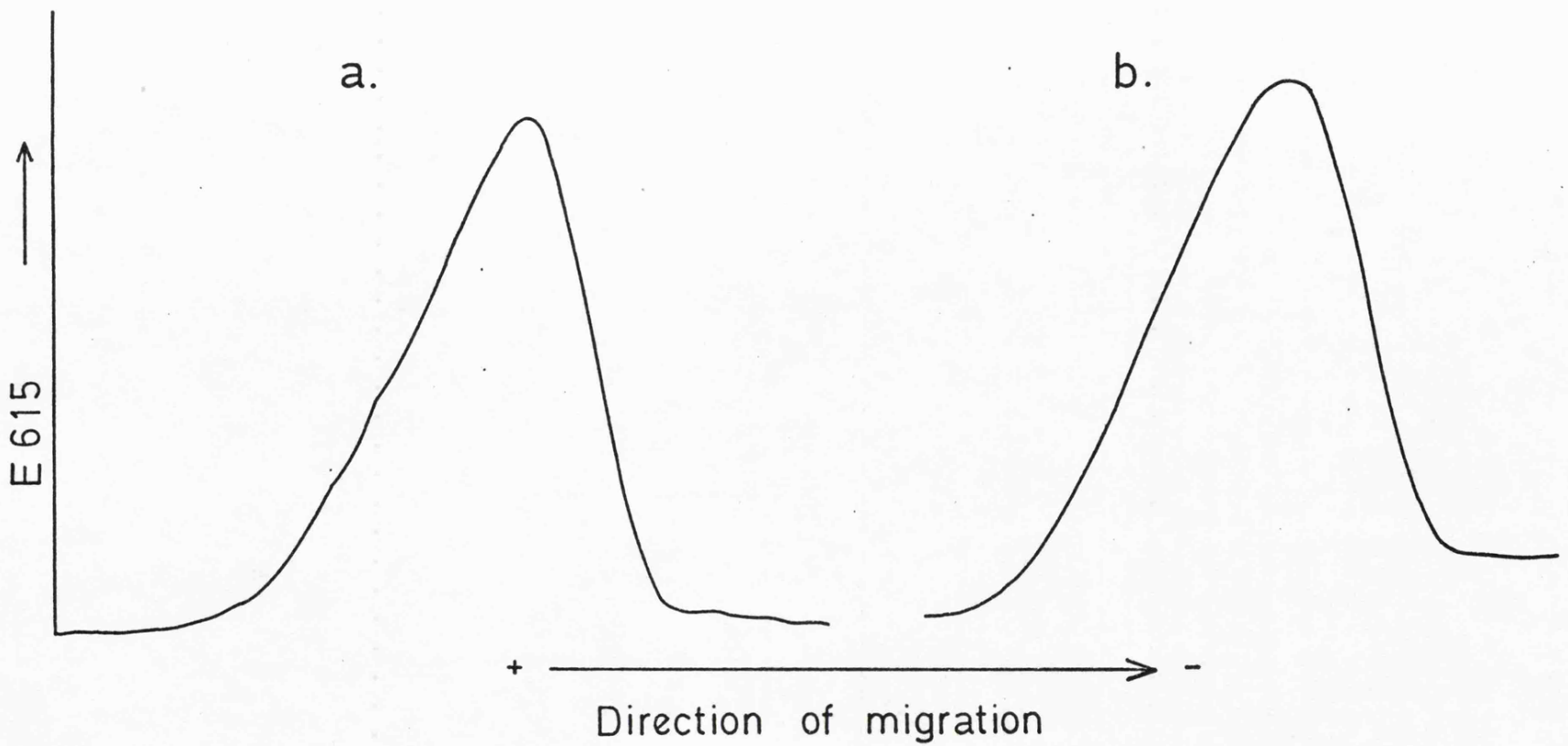
of H1 and its modifications. The resulting electrophoretic patterns demonstrated no apparent difference between samples. The scanning pattern of H1 are shown in Fig. 29 with samples 6 and 7 of culture VIII as examples. These gels were scanned at 12.7 X horizontal scale expansion, and asymmetric microdensitometer tracings were obtained. Since the trailing appeared to be on the anode end, it can be suggested that H1 has subfractions which migrate slower than the main fraction. The subfraction may comprise methylated, acetylated or phosphorylated derivatives of the main H1. If so, they only account for a very small percentage of the main fraction (it does not even appear as a shoulder of the main H1 peak on an expanded microdensitometer tracing); alternatively there could be no modification and the asymmetry be due to 'tailing' on the gel of a single H1 species. The smoothness of the tracing perhaps favours the latter interpretation. This result is different from that of animal systems and Physarum as described in Chapter 1; it is also different from that of cultured explants from Jerusalem artichoke (Stratton, 1976), where at least 2 distinct bands migrated slower than the main H1 in auxin-treated cultured explants, and even the control explants showed obvious heterogeneity of H1. It is therefore assumed that Acer histones are rather homogeneous, and that any modifications of H1 account for a very small percentage of the total H1.

Figure 29

Microdensitometer tracings of histone H1, with sample 6 and 7 of synchronous culture VIII as examples.

Histone samples were electrophoresed on acid-urea polyacrylamide gels for 8 hours. Gels were stained with Amido Black and scanned after being destained. Scanning was carried out in a Beckman Spectrophotometer Model 24 with a horizontal scale expansion of 12.7X.

Fig. 29



8. Conclusion

Both labelled lysine and arginine were actively incorporated into proteins of Acer suspension cells. During the incubation, the labelled amino acids were rapidly adsorbed onto the cells, and soon taken up and incorporated into proteins. The maximum incorporation was at 32.8% efficiency for ^{14}C-lysine in 30 min and at 82.9% efficiency in 40 min for ^{14}C-arginine. Both uptake and incorporation decreased with further incubation; this is possibly due to the turnover of labelled non-histone proteins and basic proteins of the ribosomes.

Labelled lysine was found to be more suitable than arginine for studying histone biosynthesis owing to its high efficiency of incorporation into histone proteins. The incorporation of ^{3}H-lysine into Acer histone was similar to that of Walker carcinosarcoma (Hnilica and Busch, 1963; Ohly et al., 1967). ^{3}H-Lysine was incorporated most actively into H3, followed by H2B and H2A, and least actively into H1 and H4, indicating that, in Acer cells, the biosynthetic rate of each histone fraction is different.

A mixture of ^{14}C-lysine and ^{3}H-tryptophan was applied to an asynchronous 7-day old culture in 'pulse' and 'pulse-chase' experiments, after which the cells were fractionated into nuclear and cytoplasmic fractions, and the radioactivity present in the protein of these fractions was measured. It was found that in 'pulse-chased' cells the ^{14}C to ^{3}H ratio in nuclear protein was more than twice that of the cytoplasmic protein, while in 'pulsed' cells the ^{14}C to ^{3}H ratio is slightly higher in cytoplasmic protein than in nuclear protein. The evidence therefore favours the idea that Acer histones are synthesized in the cytoplasm and are then rapidly transported into the nucleus.

To investigate the relationship between the synthesis of

histone and that of DNA in Acer cells, studies were performed on both asynchronous and synchronous cultures. When the DNA synthesis inhibitor, hydroxyurea, was added to the 7-day old asynchronous culture, it was found that synthesis of the majority of the basic proteins was very sensitive to inhibition by hydroxyurea, while about 11% of the total basic protein was resistant. After incorporation of ^{3}H-lysine into interphase cells of synchronous culture VIII, the specific radioactivity of histones was about the same in all the samples examined. The incorporation pattern obtained from samples of synchronous culture VII showed that incorporation of ^{3}H-lysine occurred before DNA synthesis and reached the maximum rate during DNA synthesis. All these data suggest that, in Acer cells, the histones can be divided into two classes : those whose synthesis is dependent on DNA synthesis and those whose synthesis is independent of DNA synthesis.

Amongst the eight cultures set up in hope of synchrony, half of them showed cell number step-ups. Histone samples were extracted from interphase cells of cultures IV, VII and VIII, and characterized on acid-urea polyacrylamide gels; none of them showed deviant banding patterns. Nevertheless, the ratio of H1 to H4 varied from 0.59 to 4.0 (Table 10). The ratio of H1 to H2A+H2B+H3 to H4 was 12.7±6.75% to 77.37±3.09% to 11.1±9.27% (Table 10). Histone H4 also presented heterogeneity in samples taken from S phase of culture VII. However, it is still not clear whether this is caused by the method used for extraction or a true reflection of the samples per se.

A general characteristic of Acer histones is the homogeneity of each fraction as shown on polyacrylamide gels. Acer histones, especially when they are extracted by the H_2SO_4-EtOH method, always present very sharp bands on the gels. Histone H1 after 8 hours'

electrophoresis does not show any distinct subfractions. However, all H1 samples examined have been shown to possess similar asymmetric patterns on expanded microdensitometer tracings. This could suggest that H1 has slower-migrating subfractions which occur in very small amounts; alternatively there could be no modification and the asymmetry be due to 'tailing' on the gel of a single H1 species.

CHAPTER 6

General Discussion

To investigate the biosynthesis of histones in cultured cells of Acer pseudoplatanus L., the isolation and characterization of histones was first developed and established. Since histones extracted directly from cells were found to be heavily contaminated, it was decided to isolate nuclei prior to histone extraction. The grinding medium developed as described in Chapter 3 for the nuclear isolation contains 20 mM Tris-buffer at pH 7.8, 1 mM Mg acetate, 2 mM $CaCl_2$ and 70% (v/v) glycerol. Cultured cells suspended in this grinding medium, at -18 °C for 5 months, were found to retain their characteristic histone pattern as revealed on polyacrylamide gels. When the cells were cooled to -20 °C in grinding medium and ruptured with a glass Potter-Elvehjem homogenizer, the cell walls were comparatively easily and preferentially ruptured; most of the nuclear membranes remained intact. It was also found that the cell walls were ruptured but not broken into small pieces and that the nuclei were released into the medium, intact and free from cytoplasmic slurry; this made the purification of nuclei less complicated than in other systems such as that using a non-aqueous medium for the isolation of rat liver nuclei (Maggio, 1963).

The established nuclear isolation technique gave (in terms of DNA recovery) a yield of about 20% of the nuclei. With asynchronously cultured cells the yield was similar no matter what the growth stage of the culture, suggesting that the isolation technique does not just release nuclei of a particular cell-cycle stage, although this has to be confirmed by microdensitometric analysis. The nuclear fractions so prepared contained no apparent contamination other than starch grains, thereby permitting the isolation of nuclear histones free from cytoplasmic contamination. The nuclear fraction stored in the grinding medium at

-18 °C for 1 week has also been shown to retain RNA synthesising activity, which implies that the biosynthetic activities of Acer nuclei could be studied in vitro.

Despite the difficulty arising during homogenization (the grinding medium became very viscous at -20 °C and was rather difficult to handle while homogenizing), the isolation procedure is simple, and gives a reasonably pure nuclear fraction. Further improvement on the technique should aim to increase the nuclear yield, and to overcome the problem of nuclear clumping. This could probably be achieved simply by changing the concentration of the components in the grinding medium. Such isolated nuclei would be used for analysis of the non-histone proteins, and in vitro studies on DNA, RNA and possibly protein synthesis.

Since glycerol-containing media have the advantages of (i) preserving cells at subzero temperatures without solidifying them, (ii) removing the cytoplasmic contaminants from the nuclei, (iii) retaining the structure and the enzyme activity of the nuclei and (iv) not interfering with enzymatic assays, the use of glycerol-containing media for nuclear isolation appears worthy of more attention by cell biologists. To my knowledge, no similar technique has previously been developed for nuclear isolation from plant material. The technique developed for Acer suspension cells has also proved satisfactory in spinach suspension cells. It would be of interest to test this technique on other plant sources. Since individual cell types do sometimes present particular problems, certain modifications of the basic procedure may be necessary for its more extensive use.

Of the three methods tested for extraction of Acer histones from the nuclear fraction, the H_2SO_4-EtOH method gave 100% extraction efficiency, and the extracted histones were less aggregated than those

obtained by other methods (as shown on polyacrylamide gels), while methods involving precipitation of histones with TCA were less efficient. Nevertheless, all Acer histone samples present typical histone electrophoretic patterns on acid-urea polyacrylamide gels. Of the seven major bands of Acer histones, bands 6 and 7 co-migrate on both acid-urea and SDS gels with calf thymus H3 and H4 respectively; this proves again that H3 and H4 are evolutionarily conserved.

A differential staining method was developed for identification of the histone fractions on polyacrylamide gels. This is a modification of that of Barrett and John (1974) who found that the basis for the differential staining is the ratio of lysine to arginine. With their staining method, they found that the lysine-rich histones (H1 and H2B) stain red, while the arginine-rich histones (H3 and H4) as well as the intermediate fraction (H2A) stain blue. The staining solution has been modified for the present purpose by replacing 0.5% Alizarine Black with 0.1% Amido Black, and has been shown to stain individual histone fractions different colours. The lysine-rich histones became reddish, the arginine-rich histones violet, and the H2A bluish. The calf thymus histones are stained as follows : H1, rosy pink; H3, light violet; H2B, purple; H2A, deep blue green; and H4, violet. The differences in colours obtained on the gel are better seen by eye than they are recorded by colour photography.

By comparing the mobilities and responses to different staining conditions with those of calf thymus histones, the five major Acer histones were identified as band 2= H1, band 4= H2A, band 5 = H2B, band 6 = H3 and band 7 = H4. Bands 1 and 3 are identified as relatively arginine-rich and lysine-rich proteins respectively, and appear to be unique Acer histones.

The results of amino acid analysis show that total Acer histones are similar to those from other plant sources, containing a lower arginine content than those of calf thymus. The Acer histones contain 22% of basic amino acids, and the ratio of lysine to arginine is 2.6 compared to 2.0 for calf thymus. Since the colour response of all major Acer histone fractions other than H2A correspond to those of calf thymus, and the H2A showed a pinkish rather than purplish or bluish colour, it can be suggested that the low content of arginine in the total Acer histones is probably due to a low content of arginine in the H2A rather than in the other fractions. This tentative conclusion should be tested by amino acid analysis of the individual fractions. This experiment could be carried out by preparing and hydrolyzing the individual histone fractions according to the method of Houston (1971), but would require a large quantity of Acer histone (c.8.0 mg). Each hydrolyzed protein would then be analyzed for the amino acid composition and compared with that of the corresponding calf thymus protein.

The modified differential staining technique without further improvement is already very useful for identification of a new histone source on polyacrylamide gels. However, it would be worthwhile to investigate the basis of this modified method. By running a number of proteins on the gel, the relationship between colour formation and the major amino acid composition of the proteins could possibly be established. If so, this method could become very useful and very important for all kinds of protein identification. Polyacrylamide gel electrophoresis is so far the most simple and sensitive method of protein fractionation (a 10-20 μg sample of protein is sufficient), and, coupled with the differential staining technique, the gels would provide information for each protein such as : (i) the relative charge or molecular weight, (ii) its approximate concentration and (iii) its relative amino acid composition.

Identification of proteins such as histones is based on the ratio of lysine to arginine, and hence the colour response after differential staining indeed enables each fraction on the gel to be characterized.

A further application of polyacrylamide gel electrophoresis in histone studies would be the running of histone samples on longer gels (such as 25 cm long), so that the subfractions of the major histones could possibly be more effectively separated. This might be further improved by a two-dimensional gel electrophoresis as developed by O'Farrell (1975).

By using mixtures of ^{14}C-lysine and ^{3}H-tryptophan in 'pulse' and 'pulse-chase' experiments, Acer histones can be shown to be synthesized in the cytoplasm and then rapidly transported into the nucleus. It would be of interest to investigate the exact location of histone synthesis in cytoplasm. Direct observation could be achieved by pulsing slow-growing cultured cells with labelled lysine and examining the cells in the electron microscope as well as by autoradiography. It could also be carried out by isolation of cytoplasmic organelles after the cells have been incubated with the labelled mixture. The experiment should then aim to find the organelle which contains proteins with a high ratio of ^{14}C-lysine to ^{3}H-tryptophan, and to characterize the proteins so labelled. Since it is histone synthesis which is interesting at this moment, the labelled protein should be extracted and proved to be histone; the established characterization method for Acer histones would be applicable for this purpose.

The biosynthesis of individual histones has been shown to proceed at different rates in cultured Acer cells. When ^{3}H-lysine was fed to the asynchronous cultured cells, the most active incorporation was into H3, followed by H2B and H2A; least active incorporation was into

H1 and H4. It would be interesting to undertake similar studies with synchronously dividing cells, to investigate the relationship between the synthesis of individual histone fractions and the cell-cycle stages. This experiment was attempted on culture VIII, but it was not successful owing to the low incorporation efficiency of ^{3}H-lysine into histones and because the cell-cycle stages of the samples were not clearly identified. Therefore, whenever a synchronous culture becomes available, this experiment should be repeated.

During the present investigations, the most difficult experiments were those involving cultures designed to show division achieved by release from prior nitrate starvation (through which cells are arrested exclusively in G1 phase). During re-growth the starved cells may proceed to divide in synchrony. However, the synchrony-controlling mechanism in this system is still far from being properly understood, and the puzzling features observed by King (1973) and Gould (1975), such as unpredictable lag phase, varying interphase duration and sharp synchrony-decay have been encountered in a more exaggerated form than in earlier work. This may be due to the long term maintenance of the suspension resulting in loss of uniformity in the stock culture. Since suspension cultured Acer cells have a tendency to develop aneuploidy and polyploidy (Gould, 1975), it is possible that the genetic composition of the present stock culture differs from that originally initiated (this was however not examined), and was responsible for the lack of well defined and persistent synchrony.

Amongst the 8 cultures set up for synchrony initiation purposes, half of them showed division synchrony. However, not only did the growth rate vary between separate cultures, but within the same culture the duration of the plateaux of cell number also varied. The interphase

samples for chemical and cell-cycle stage analysis were thus withdrawn from the cultures at times which could not be precisely related to the cell cycle, and the samples did not therefore necessarily include all the cell-cycle stages.

The work on synchronous cultures aimed to investigate the relationship between histone synthesis (as well as histone modifications) and cell-cycle stages. The results show that histones are synthesized throughout the cell cycle but that the maximum rate occurs at S phase. In the 18 histone samples extracted from interphases of 3 synchronous cultures, none of them showed deviant electrophoretic patterns on polyacrylamide gels, nor did these samples after being electrophoresed for 8 hours show subfractions of H1. However, all H1 samples exhibit a similar asymmetric pattern on the expanded microdensitometer tracings, suggesting that the asymmetry could be due to 'tailing' on the gel of a single H1 species; alternatively , the H1 could have derivatives which migrate slower than the main H1 on polyacrylamide gels. Since these derivatives seemed to represent a very small amount of the total H1, the complete separation of H1 and its derivatives is probably difficult to achieve. However it should be possible to detect the derivatives if they have taken up isotopes such as ^{32}P-phosphate for phosphorylated derivatives, and sodium-^{14}C-acetate for acetylated derivatives. Similar incorporation experiments could also be applied to other types of modification on other histone fractions. For this purpose, the histone samples would have to be run on long gel tubes so that the derivatives could be separated from the main histones.

To investigate the relationships between the occurrence of histone modification and the cell-cycle stage, experiments have to be performed on synchronous cultures and would require the problem of

reproducible initiation of synchronous cultures to be solved. A synchrony system might alternatively be induced by metabolic inhibitors. Hydroxyurea seems to be very effective on the synthesis of macromolecules in Acer cells, and it has also been reported to induce cell division synchrony in suspension cultures of Haplopappus gracilis (Eriksson, 1966). A new suspension culture line initiated from Acer callus tissue could possibly provide a uniform cell population, which would show the excellent synchrony earlier demonstrated in the AM line used by Gould and Street (1975).

Besides the work on synchronous cultures, there are still several interesting experiments which could be done on asynchronous cultures. These include:

(1) RNA synthesis by isolated cell nuclei — The incorporation of RNA precursors into RNA molecules in response to plant hormones or metabolic inhibitors.

(2) The effects of added histones on nuclear RNA synthesis in vitro — Individual histones could be isolated from cells and added to the isolated nuclei to examine their effects on RNA synthesis.

(3) Stimulation of RNA synthesis by selective histone removal — Histones could be selectively removed from chromatin by either salts or enzymes; the effect of this removal on RNA synthesis would then be investigated.

(4) The effect of histones on RNA polymerase activity — The DNA-dependent RNA polymerase could be prepared from nuclei, and the effect of histones on this enzyme activity would be examined.

(5) The effect of modified histones on RNA synthesizing activity — Histone modifications such as acetylation could be carried out chemically on the isolated histone fractions (Reid, 1951), and

the modified histones added to the RNA polymerase system to examine their effect on RNA synthesis.

The present investigation must therefore be seen as the beginning of a more comprehensive project. Since the temptation in such an investigation is to interpret observations in the light of concepts developed from work with animal sources, further experiments similar to those designed here for Acer suspension cells should also be applied to other plant materials to establish the general nature of the results for higher plant cells.

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CHARACTERIZATION AND BIOSYNTHESIS OF HISTONES IN CULTURED CELLS OF ACER PSEUDOPLATANUS L.

by

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ABSTRACT

A new technique has been developed for the isolation of nuclei from suspension culture cells of Acer pseudoplatanus L. This technique involves the use of a glycerol-containing grinding medium at -20 °C. The whole isolation process is simple and consistently produces about 20% nuclear yield with reasonable purity.

Histone extraction from this nuclear fraction by the H_2SO_4-EtOH method is superior to other methods examined. The extracted Acer histones exhibit a typical histone pattern on polyacrylamide gels, and the major fractions are identified as H1, H2A, H2B, H3 and H4 (in sequence from the anode to the cathode end on the gel); their molecular weights are respectively 24,500, 13,500, 13,300, 12,800 and 11,000. Both the H3 and H4 histones of Acer cells are identical with those of calf thymus in terms of their mobilities on acid-urea and SDS gels. Identification of the Acer histone fractions has been assisted by a newly developed differential staining method which stains the 5 major histone fractions of calf thymus in 5 different colours.

The total extracted Acer histone fraction contains 22% of basic amino acids, and the ratio of lysine to arginine is 2.6 which is higher than that for calf thymus and is probably due to the low content of arginine in Acer H2A.

The Acer histones have been shown to be synthesized in the cytoplasm and then transported into the nucleus. The synthesis occurs throughout the cell cycle but reachs its maximum rate while DNA is being synthesized. Histone samples obtained from both asynchronous and synchronous cultures at intervals during the progress of their growth show uniform electrophoretic patterns on the gels, suggesting that the Acer histones are generally homogeneous and that any modification of the histones probably affects only a very small proportion of the total histones. The possible existence of such modified histone derivatives and their functions remains to be investigated. Directions along which the present studies could be developed are discussed.