THE BIOCHEMISTRY OF CELLULAR CONTROL MECHANISMS: THE CHARACTERISATION OF CHROMATIN FROM AVIAN ERYTHROID CELLS

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SUMMARY

One highly specialised system (the avian erythroid cell series) was studied with the view of characterising those parameters of the nuclear chromosomal material which alter during terminal differentiation of the cells of the series. The early cells of the series (erythroblasts) originate in the bone marrow and are active in DNA, RNA and protein synthesis; these cells mature to become circulating, non-dividing polychromatic erythrocytes, active in RNA and protein synthesis. Polychromatic erythrocytes finally form mature erythrocytes, which are inactive in macromolecular synthesis. Since cells retain the same complement of nuclear DNA, our *aim* was to correlate differences in the DNA-protein complex with the particular biochemical activity of the cell from which it was isolated, to investigate possible *transcriptional controls* operating.

2. No qualitative changes were found within the histone component of chromatin, from the erythroblast through to the mature erythrocyte. It was clear that the appearance of any one histone component could not be correlated with the marked changes in transcription from DNA occurring within the cell series.

3. Some minor quantitative changes in the distribution of histones were established. No change in the whole histone:DNA ratio could be found from erythroblasts through to mature erythrocytes. There was, however, a reduced amount of f2c histone in erythroblasts, relative to the other histones,

compared to the amount in non-dividing cells. In addition, within the non-dividing cells, mature erythrocytes contained a relatively lower amount of histone f1-peak 4 than polychromatic erythrocytes.

4. Of the histones present in chromatin, polychromatic erythrocytes synthesised only the f2c histone; erythroblasts synthesised all the identifiable histone species. Furthermore, the metabolism of f2c histone (in polychromatic erythrocytes) was shown to involve the removal of this histone from chromatin as well as the addition of f2c to the chromatin (at approximately equal rates). The f2c histone in chromatin thus appeared to be in a state of metabolic flux.

5. Points 3 and 4 above are considered in some detail in relating f2c and/or f1 histones to a broad level of transcriptional control. We postulate the important feature relating these histones to the overall potential of chromatin for transcription is not the static level of histones, but rather the dynamic state of their synthesis and release from DNA.

6. Although histones were the only chromosomal components considered in detail, quantitative data relating to the so-called non-histone component is presented which reveals an important difference between polychromatic erythrocytes and mature erythrocytes. For washed nuclei the protein:DNA ratio of polychromatic erythrocytes was

greater than that of mature erythrocytes. For purified chromatin, however, this was not the case. More protein was removed with the nuclear membrane fraction (separated from chromatin) from polychromatic erythrocyte nuclei than from mature erythrocyte nuclei. This result is discussed in relation to published correlations between protein:DNA ratios and cellular gene activity.

7. Besides the characterisation of chromosomal components, whole chromatin was also partially characterised. These studies on isolated chromatin were carried out to examine the usefulness of this material, and the avian erythroid cell series in general, for studies on transcriptional controls. Chromatin preparations from polychromatic and mature erythrocytes were used. The parameters examined included, sucrose density gradient analyses, ultraviolet absorption spectra, melting profiles, dye binding properties, precipitability by Mg^{++} and template activities with added *E. coli* RNA polymerase. The latter parameter, in particular, indicated that the isolated material retained at least to some extent the *in vivo* properties. In general the avian erythroid cell series did appear to provide a useful model system for studying the mechanisms related to major structural changes in the chromatin complex.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge, this thesis contains no material previously published or written by any other person except where due reference is made in the text.

R. APPELS.

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NOMENCLATURE AND ABBREVIATIONS

(1) Avian erythroid cells.

The nomenclature used by Lucas & Jamroz (1961) was followed in this thesis.

Cell type	Reference in this thesis	Alternative nomenclature (e.g., Sadgopal & Kabat, 1969)
dividing	erythroblast	
non-dividing, active in RNA synthesis	polychromatic erythrocyte	reticulocyte
inactive in macromolecular synthesis	mature erythrocyte	erythrocyte

(2) Avian erythroid histones.

The following table, summarising the nomenclature used for histones was taken from Johns (1971).

Nomenclature	Histone fraction					
1	f1	f2c	f2b; f2a2		f2a1	f3
2	Ia, Ib	V	IIa, IIb, IIc		IV	III
3	1,1',1''		3,4		5,5'	2,2',2''
4			AL		GAR	

NOMENCLATURE AND ABBREVIATIONS

Nomenclature number 1 (f1, f2c etc.) was used in this thesis.

(3) Other abbreviations.

OD - optical density (subscript donating wavelength at which optical density was monitored).

S.E. - standard error of mean

BSA - bovine serum albumin

INTRODUCTION

The principle interest underlying the work presented in this thesis is how higher cells are able to differentiate. One highly specialised system (the avian erythroid cell series) was studied with the view of characterising those parameters of the nuclear chromosomal material which alter during terminal differentiation of the cells of the series. The early cells of the series (erythroblasts) originate in the bone marrow and are active in DNA, RNA and protein synthesis; these cells mature to become circulating, non-dividing polychromatic erythrocytes active in RNA and protein synthesis. Polychromatic erythrocytes finally mature to erythrocytes, which are inactive in macromolecular synthesis. Since cells retain the same complement of nuclear DNA, our *aim* was to correlate differences in the DNA-protein complex with the particular biochemical activity of the cell from which it was isolated, to investigate possible *transcriptional controls* operating. More specifically the histone component of chromatin was examined with regard to:

(a) Qualitative changes within this group of proteins,

(b) Quantitative changes within this group of proteins,

(c) The function of histone f2c (tissue specific to avian erythroid cells).

In addition, changes in chromatin isolated from polychromatic and mature erythrocytes were examined, particularly with regards ability to provide a template for added RNA polymerase.

Preceding the experimental work in this thesis, a brief literature survey of the following areas is given: (a) differentiation in eukaryotes, (b) control of DNA transcription, (c) structure of chromatin, (d) changes in chromatin in relation to cellular activity, (e) characterisation of the avian erythroid cell series.

LITERATURE SURVEY (To October 1971)

(a) Differentiation in eukaryotes.

In general, during differentiation, cellular controls are co-ordinated to effect the time of appearance, the shape, the function of cells and their organisation into tissues and specialised organs. This complex phenomenon must involve cellular controls at many levels. To date four specific points of control, affecting the phenotypic expression of genes within cells, have been described;

(i) Control at the level of DNA transcription (Gurdon, 1968; McCarthy & Duerksen, 1970)

(ii) Control at the level of newly synthesised RNA leaving the nucleus (Scherrer & Marcaud, 1968; Warocquier & Scherrer, 1969).

(iii) Control at the level of translation of RNA (Harris, 1970; Ilan & Ilan, 1971; Maxwell, Kamper & Rabinowitz, 1971).

(iv) Control at a post-transcriptional level - effecting, for example, the formation of an

active protein unit from precursor subunits (discussed by Scherrer & Marcaud, 1968; Soll & Sonnenborn, 1971).

The co-ordination of these controls to give the phenotypic response which is called differentiation is not well understood; the possibility of chemical messengers (chalones), diffusing between cells, being involved in this process has been reviewed by Bullough (1967).

(b) Control of DNA transcription

Transcriptional controls are readily divided into two categories. One of these includes changes in RNA polymerase to alter its specificity for different sequences of DNA, the other including changes in the DNA template itself. Precedents for both of these types of control have been observed in prokaryote systems.

Regarding changes in RNA polymerase, the σ-element of bacterial RNA polymerase has been characterised (Burgess & Travers, 1970). The σ-factor portion of the polymerase molecule has been found to be particularly important in determining the specific interaction between the polymerase and a DNA template. Some evidence that new σ-factors appear in T4 'phage infection of *E. coli* (Travers, 1970) and sporulation of *B. subtilis* (Losick & Sonensheim (1970), has been found. In both these instances, however, other portions of polymerase (e.g., β-subunit) also appear to be altered, and in fact entirely new RNA polymerase molecules with their own specificity have been

found in conditions such as T7 'phage infection of *E. coli* (Chamberlin *et al.*, 1970). The class of cytoplasmic proteins (ψ-factors) which specifically stimulate the synthesis of ribosomal RNA in *E. coli* may also belong in this category of factors affecting the specificity of RNA polymerase molecules (for review see Travers, 1971).

The second category relates to changes in the DNA template. Here in prokaryote systems two repressors, the *lac* and λ 'phage repressors, have been well characterised (Chadwick, Pirrota, Steinberg, Hopkins & Ptashne, 1970). Both specifically bind to DNA to prevent the RNA polymerase (attached to a promoter site) from transcribing DNA distal to its point of attachment (operator region). An alternative template modification involving a positive control element acting at the promoter site of the *lac* operon in *E. coli* is seen with cyclic-AMP and its binding protein. In this instance the attachment of the cyclic-AMP binding protein (with cyclic-AMP) to DNA is required for optimal transcription of the genes of the *lac* operon (Chen, de Crombrugghe, Anderson, Gotterman, Pastan & Perlman, 1971).

In eukaryote systems, different RNA polymerases have been characterised within the same cell types (Roeder & Rutter, 1970_1; Roeder & Rutter, 1970_2) some of which may be interconvertible (Chesterton & Butterworth, 1971), so that the concept of transcriptional control involving changes in the RNA polymerase (either modification of an existing molecule or production of an entirely new one) is tenable here. This is clearly not as well characterised in eukaryotic systems as in prokaryotic systems. Modifications

in template DNA (in the form of chromatin) have been correlated in higher cells with changes in the transcription of DNA. This latter aspect of control is discussed in more detail below.

(c) Structure of chromatin

(i) Definition of chromatin. *In vivo*, chromatin is the genetic material of the nucleus. It contains DNA with which is associated proteins and RNA. In this respect there is little difference between prokaryote 'chromatin' and eukaryote chromatin in that the former also has proteins (repressors, RNA polymerase, etc.) and RNA (newly synthesised transcription products) associated with the DNA. The difference between the two lies in the much greater complexity of eukaryotic chromatin, particularly in the presence of a special class of proteins called histones.

In vitro, chromatin is defined as the material isolated from washed nuclei by chemically gentle means. It is clear from such a definition that the isolated material may bear only a limited relation to the *in vivo* material due to polymerisation, loss of components or addition of cytoplasmic components during the isolation of nuclei. Where possible this problem is considered in this thesis.

(ii) Chemical studies on chromatin. The approximate composition of chromatin, isolated from various sources (Bonner *et al.*, 1968) is, by weight, 40% DNA, 58% chromosomal proteins (36% histone) and 2% RNA.

The DNA component. Methylation of cytosine residues in the DNA of higher cells has been reported (Burdon, 1971); to date no other chemical modification of DNA residues has been found. In the T-even 'phages, glucosylation as well as methylation of DNA has been described (Arber & Linn, 1969). A characteristic feature of eukaryotic DNA is the high content (relative to prokaryotic DNA) of repeated sequences found to be dispersed amongst the unique sequences of DNA (Britten & Davidson, 1969). The significance of these repeated sequences of DNA is not understood; although the transcription products of these portions of DNA have been suggested to be control elements (Britten & Davidson, 1970; Crick, 1971), there is no evidence for this to date.

The chromosomal proteins. This component of the chromatin is readily categorised into histone and non-histone protein.

Histones. The chemical composition of this class of chromosomal protein has been extensively studied in many tissues and several reviews are available on this subject (Stellwagen & Cole, 1969; Hnilica, 1967; Hearst & Botcham, 1970). The main characteristics of these proteins are as follows: (1) A high basic amino acid content (relative to average cellular proteins). (2) Only a relatively small number of individual species have been identified over a wide range of organisms studied (histone:DNA ratios (w/w) also change little). Present estimates stand at approximately 10 species - although not all have been sequenced, those moieties which have been are homogeneous (see also Greenaway & Murray, 1971). One histone which has been

sequenced and examined from widely divergent species (histone f2a1 or 1V) shows very little variation in its primary structure, indicating strong selective pressures against changes in this feature of the molecule. (3) There is an asymmetric distribution of basic amino acids (in those histones which have been sequenced). (4) Histones are relatively small proteins - the maximum molecular weight so far determined being 20,000 daltons. (5) Chemically modified amino acids have been found in histones - for example, o-phosphoserine (Sung & Dixon, 1970); ε-NH_2-methyl lysine (Tidwell *et al.*, 1968); 3-methyl histidine (Gershey *et al.*, 1969) and ε-NH_2-acetyl lysine (Vidali *et al.*, 1968). For the histone f2a1, ε-NH_2-acetyl lysine has been found only amongst the lysine residues in the portion of the molecule rich in basic amino acids (the N-terminal portion, Candido & Dixon, 1971). (6) Histones appear to interact with DNA mainly *via* ionic forces.

Non-histone proteins. This class of protein has not been studied extensively because of difficulties in handling them experimentally, namely, their tendency to aggregate. Recently work has been published (Shaw & Huang, 1970; Marushige *et al.*, 1968; Shirey & Huang, 1969, Teng *et al.*, 1971) related to developing methods for handling these proteins. Evidence indicates they are a heterogeneous group of proteins. Amino acid analyses have shown these proteins to be relatively rich in glutamic acid, aspartic acid and glycine (Shaw & Huang, 1970; Shirey & Huang, 1969; Elgin & Bonner, 1970). Phosphorylated forms of these proteins have been isolated (Gershey & Kleinsmith, 1969) some of which have been shown to interact

specifically with DNA (Teng, Teng & Allfrey, 1971) - this latter point is discussed further when mechanisms for changing the transcriptional potential of chromatin are considered (p.12).

Chromosomal RNA. Chromosomal RNA is a moiety of RNA which has been found in association with chromatin (Dahmus & Bonner, 1970) and in fact is claimed to be covalently bound to protein (Huang, 1967). This RNA does not appear to be m-RNA still attached to chromatin since it contains (characteristically), two unusual bases (dihydro-uridine and/or dihydro-ribothymidine), which are considered to provide covalent links to serine residues in chromosomal proteins (Huang & Bonner, 1965; Jacobson & Bonner, 1968). The species of RNA hybridises to repetitive sequences of DNA (Sivotan & Bonner, 1971) and appears to be wide-spread, having been found in both plant and animal tissues (Huang & Bonner, 1965; Huang & Huang, 1969). Recently, doubt has been cast upon the origin of chromosomal RNA. Heyden & Zachau (1971) have suggested chromosomal RNA to be a degradation product of transfer RNA. Although their work was not well defined, Artman & Roth (1971) have shown, more convincingly, that chromosomal RNA is not covalently bound to protein and suggested it to be a degradation product of ribosomal RNA. The significance of chromosomal RNA is thus not clear at present; the work published does emphasise, in particular, the need to consider artefacts introduced into isolated chromatin by the method of preparation. It is interesting to note that a species of RNA similar to chromosomal RNA has been found in membranes known to associate with chromatin (Tolstoshev, personal communication).

(iii) Physical studies on chromatin.

On isolated chromatin. X-ray studies have shown the primary nucleoprotein fibre to be 38 Å in diameter, super-coiled into a secondary fibre 100 Å diameter (Pardon *et al.*, 1967). This interpretation of structure is supported by electron microscope (Davies, 1967) and flow diochroism studies (quoted by Hearst & Botcham, 1970). In addition to containing 100 Å fibres, isolated chromatin appears to be further cross-linked inter-molecularly, as shown by the irreversible effects of procedures such as shearing and increasing ionic strengths on the sedimentation coefficient of chromatin preparations (Chalkley & Jensen, 1968).

Studies utilising the DNA binding properties of ethidium bromide (Olins, 1969) and actinomycin-D (Kleiman & Huang, 1971) and determinations of the thermal stability of DNA have shown that phosphate groups of DNA are, in effect, neutralised in chromatin (Olins, 1969). Experiments with reporter groups such as $2,4(NO_2)_2C_6H_5NH(CH_2)_2{}^{+}N(CH_3)_2(CH_2)_3{}^{+}N(CH_3)_3$. 2Br have indicated that chromosomal proteins are localised in the major groove of DNA in the primary nucleoprotein fibre (Simpson, 1971). Removal of chromosomal proteins destabilises the DNA against temperature increases (Shirey & Huang, 1969), releases dye binding sites (Kleiman & Huang, 1971) and sites for transcription by bacterial RNA polymerase (Seligy & Neelin, 1970; Smart & Bonner, 1971).

Two additional parameters of isolated chromatin, namely, susceptibility of chromatin DNA to added DNA'ase and binding polylysine have been examined (Clarke & Felsenfield,

1971). These parameters indicate that 50% of the DNA in chromatin is not covered (thus protected) by chromosomal protein. In these studies some chromatin preparations were fixed with formaldehyde prior to DNA'ase digestion and polylysine binding experiments, with the same results being obtained.

On isolated histone components (free from DNA). Nuclear magnetic resonance studies on the two histones (f2a1 and f2b - from calf thymus) which have been sequenced, have indicated how these proteins may function in folding the primary nucleoprotein fibre into a supercoiled structure (or any other structure which the material may take up *in vivo*). The f2a1 (Boublik *et al.*, 1970_1) and f2b histones (Boublik *et al.*, 1970_2; Bradbury *et al.*, 1971) were found to interact inter- and intra-molecularly *via* specific portions of their structure under conditions favouring helical formation within proteins and aggregation between molecules. These regions of the respective molecules were rich in hydrophobic residues (valine, leucine, isoleucine and aromatic amino acids) and contained few basic amino acids (particularly lysine). Those portions of the structure relatively rich in basic amino acids, which did not participate in the above interactions, were shown to interact with DNA when this was present (Boublik *et al.*, 1971). It seems likely then, that for these two histones, one portion of the molecule interacts with DNA while another portion of the molecule can interact with other histones or cellular informational molecules. Although the interaction between histone and DNA is ionic and thus possibly non-specific, each histone does have a

characteristic mode of binding to DNA as determined by its ability to stabilise DNA against thermal denaturation (Li & Bonner, 1971; Ansevia & Brown, 1971).

On chromatin in vivo. *In vivo*, chromatin is readily categorised into two states (for reviews see Brown, 1966; Lyon, 1968), namely, euchromatin and heterochromatin. The distinction is based essentially on electron microscope studies. The transition from heterochromatin (highly condensed) to euchromatin is well illustrated in the polytene chromosomes of Chironomus and Drosophila (Clever, 1968; Ashburner, 1970). In the highly condensed bands of these chromosomes little detail can be seen with the electron microscope. However, when specific bands form 'puffs' in response to, say, ecdysone, chromosomal material in these regions appears to unwind (euchromatin) and is seen to consist of numerous fibres, loosely coiled (DuPraw, 1970). The puffing of certain sections of polytene chromosomes is generally correlated with intense RNA synthesis occurring at these sites of the chromosomes (Ashburner, 1970). It is in fact often observed that genes present in heterochromatin are not expressed whereas those in euchromatin are expressed (Lyon, 1968). Exceptions to this observation have been found (Lyon, 1968) and it is possible that the difference between heterochromatin and euchromatin is one of degree of gene expression and that heterochromatin may allow some transcription of DNA. Whether a change from heterochromatin to euchromatin is the cause or effect of a change in transcription of DNA has not been determined.

(d) Changes in chromatin in relation to cellular activity

The fact that chromatin in different cells is transcribed to give different RNA products as a result of altered properties of chromatin itself, is best shown by *in vitro* transcription studies. In these studies, chromatin isolated from various tissues has been used to provide a template for purified bacterial RNA polymerase (Smith, Church & McCarthy, 1969). Competition hybridisation studies on the RNA synthesised in such systems showed the products from, say, liver and brain chromatins to be quite different. Although limitations in the hybridisation techniques used in these studies mean that only RNA transcribed from repeated sequences of DNA would be detected (Britten & Davidson, 1969), they do show that the transcriptional properties of chromatin depend on the tissue from which it was isolated. The question is -*what aspects of the chromatin are altered, in a cell, to alter its template properties?* Since transcription involves binding of RNA polymerase to DNA, initiation, propagation and termination of the polymerase reaction, any one (or combination) of these may be affected in changing the template properties of chromatin.

In most studies related to chromatin, the DNA component of the chromatin is considered to be stable in the sense that this is not a point of control for changing the template properties of chromatin. This appears to be a valid assumption since nuclei from, for example, hen erythrocytes have been activated to produce hen specific gene products simply by introducing such nuclei into cells actively transcribing their

DNA (Harris, 1970). More impressively, nuclei from differentiated cells of larval *Xenopus laevis* have been shown to have the potential for coding for the production of an entire organism (Gurdon & Woodland, 1970). In view of the occurrence of methylated cytosine in DNA, however, it is possible that chemical modification may occur to change the template properties of chromatin (Turkington, 1971). In the following discussion the protein portion of the chromatin is considered in most detail.

Structural changes in chromatin appear to accompany cell differentiation in that, as cells become more specialised, heterochromatin replaces euchromatin (Hearst & Botcham, 1970). Experimentally it is observed that dye binding sites on DNA are reduced (Kernell, Bolund & Ringertz, 1971) and the thermal stability of DNA increases (Kernell, Bolund & Ringertz, 1971). Such structural changes would, most likely, affect the binding of RNA polymerase to DNA and/or RNA chain elongation. Furthermore, these changes would probably constitute a coarse level of control, affecting relatively large blocks of genes in chromatin. Since histones appear to be the main structural units of chromatin they are implicated in this coarse level of control. Correlations found in biological systems consistent with such a concept include the limited, *yet observable* quantitative and/or qualitative tissue specificity of histones (Hnilica, 1967; Stellwagen & Cole, 1969). In addition, the chemical modification and metabolic stability of histones, both of which could influence the overall interaction between histones and DNA, have been found to depend on the metabolic state of the cell (Stellwagen &

Cole, 1969). It is interesting that native DNA, isolated chromatin and reconstituted histone-DNA complexes appear to have the same number of binding sites for bacterial RNA polymerase (Bonner *et al.*, 1968; Shih & Bonner, 1970), thus the coarse control mentioned above may be limited to affecting only the propagation of transcription. Consistent with the latter statement is the evidence that the f1 histone in calf thymus chromatin in particular appears to affect the length of the RNA chains formed *in vitro* (Koslov & Georgiev, 1971 - interpretation of these experiments is somewhat ambiguous because the salt concentrations used to remove specific histones may also cause a general redistribution of other chromosomal components, Clarke & Felsenfield, 1971).

In looking for more specific elements within chromatin structure, much research is being turned toward the non-histone protein and chromosomal RNA components. These elements would presumably function in controlling any one (or combination) of the aspects of the RNA polymerase reaction mentioned before. Precedents for such modes of control have been found in prokaryote systems and were discussed in section (b) of this literature survey (p.3). Evidence that non-histone proteins (Gilmour & Paul, 1970) and chromosomal RNA (Huang & Huang, 1969) may provide specific elements of control has been obtained from experiments with reconstituted chromatin preparations. The parameter of chromatin on which all these experiments were based was its ability to provide a template for added bacterial RNA polymerase (and examination of RNA formed by hybridisation studies). This

parameter has limitations which were discussed on p.12. More direct evidence for a concept of non-histone behaving as specific elements within chromatin has been obtained by Teng *et al.* (1971), in demonstrating that a particular class of non-histone proteins (phosphoproteins) interact specifically with DNA from the organism from which they were isolated. The phosphorylation of this particular class of protein (i.e., phosphoproteins) has also been correlated with the RNA synthetic activity of nuclei (Gershey & Kleinsmith, 1969).

(e) Characteristics of the avian erythroid cell series

The process of avian erythropoiesis is much the same as in human, rabbit and rat bone marrow, in that rapid onset of hemoglobin synthesis occurs at the expense of the synthesis of other proteins during the last stages of erythropoiesis (Borsook, 1966; Williams, 1971). In these last stages of erythropoiesis the synthesis of DNA and RNA declines rapidly. The work of Williams (1971) in characterising cells of the avian erythroid series has made possible the handling of relatively pure populations of cells, facilitating a critical study of the system. The principle advantages of the system for a correlative study of the type outlined in the Introduction (p.1) are as follows: (1) A wide range of macromolecular synthesis within one cell line is available. (2) The system is simple in that it involves terminal differentiation to the erythrocyte which is completely inactive in macromolecular synthesis. (3) Terminal differentiation from, at least, the polychromatic erythrocyte stage can be

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

MATERIALS

General chemicals used were of A.R. grade (commercially available). Bovine serum albumin, BSA, (fraction V) and phenylhydrazine (twice recrystallised) were from Sigma. Isotopes were purchased from the following sources - 'carrier free' P^{32} orthophosphate, Australian Atomic Energy Commission; C^{14}-lysine, specific activity 210 mC/m-mole and C^{14}-leucine, specific activity 311 mC/m-mole from International Chemical and Nuclear Corporation (U.S.A.); C^{14}-arginine, specific activity 195 mC/m-mole from Radiochemical Centre, Amersham.

METHODS

Unless otherwise stated all operations were carried out at 0 - 4°.

(1) Processing of avian erythroid cells

(a) Separation on isotonic BSA gradients. The procedures followed were essentially those described by Williams (1971). Mature erythrocytes were obtained from the blood of healthy White-leghorn Australorp pullets (six months old) and purified on discontinuous gradients of isotonic BSA (pH 7.4) to ensure removal of polychromatic erythrocytes and white cells. Purification was carried out by centrifuging (3,800 *g*, 90 minutes) cells through BSA of density 1.090 g/cm^3 (only erythrocytes pass through). Polychromatic erythrocytes from anaemic blood (course of induction of anaemia is described in

Appendix 1) [illegible] were obtained at the interphase of BSA of densities 1.083 and 1.071 g/cm^3. Erythroblasts prepared in discontinuous gradients were obtained at the interphase of BSA of densities 1.071 and 1.059 g/cm^3 after centrifuging bone marrow cells from the tibia-fibular and femur bones of highly anaemic chickens.

(b) Incubation of cells in vitro for the incorporation of C^{14}-amino acids. The medium used to incubate cells (purified on discontinuous gradients of BSA) was that described by Schulman (1968) for the *in vitro* maturation of mammalian red blood cells, with the modification that chicken transferrin (or chicken serum) was used instead of rabbit transferrin. Polychromatic erythrocytes (collected 28 hours after the last injection of phenylhydrazine) incubated in this medium for five hours showed only 3 - 5% lysis of cells as judged by the appearance of haemoglobin in the medium. In addition, these cells retained approximately 65% of their ability to incorporate C^{14}-leucine after a five hour incubation; the loss of activity is accounted for in part by cell lysis and possibly *in vitro* maturation, as reported for mammalian red blood cells (Schulman, 1968). For incubation, 0.1 - 0.12 ml of packed cells/ml of total incubation mix were generally equilibrated for 10 minutes at the incubation temperature (40°, normal chicken blood temperature, Freedman, Honig & Rabinowitz, 1966), after which the appropriate radioactive isotope was added. Incorporations were terminated by removal of samples of cells into a known volume of cold 0.5% saponin in isotonic sucrose (0.294 M sucrose,

0.001M $MgCl_2$, 0.001 M potassium phosphate, pH 6.8). Nuclei thus obtained were collected by centrifugation (1,800 *g*, 10 minutes) for the isolation of histones (see p.20); the supernatant was retained for the determination of radioactivity into cytoplasmic protein (5% trichloroacetic acid insoluble material). For the analysis of cytoplasmic protein on polyacrylamide gels the above nuclei free supernatant was dialysed against 0.9% acetic acid, freeze-dried and dissolved in loading buffer for gels used to analyse histones (see p.21).

(2) Preparation and analysis of histones

(a) Large scale isolation of avian erythroid histones. The preparation of histone is based on the procedures described by Dingman & Sporn (1964). Nuclei were first isolated by lysis of cells (in the form of a pellet after the last wash in isotonic buffer), in an equal volume of 0.5% saponin in isotonic sucrose for 10 minutes. The nuclei, collected by centrifugation (1,800 *g*, 10 minutes) were washed with 10 ml isotonic sucrose per 0.5 ml packed nuclei. Nuclear pellets were similarly washed six times with 0.08M NaCl - 0.02M EDTA, pH 6.4 (pH adjusted with 4N NaOH) and twice with 0.147 M NaCl. Where indicated solutions also contained 0.05M sodium metabisulphite (pH readjusted with 4N NaOH). Nuclei, now free of cytoplasmic material were resuspended in a known volume of 0.147 M NaCl for the isolation of histones. An equal volume of 4N NaCl was added to the nuclear suspension, allowed to stand for 30 minutes (with occasional agitation), and 10 N HCl added to a final concentration of 0.25N. After standing for a further 30 minutes, acid insoluble was removed by

centrifugation (38,000 *g*, 10 minutes). Protein soluble in 0.25 N HCl, 2N NaCl was dialysed against water for a cumulative time of 6 - 9 hours (2 changes). After lyophilisation the histones were dissolved in the loading buffer for polyacrylamide gel electrophoresis, at a concentration of 2 mg/ml. For histone: DNA ratios, the 2N NaCl, 0.25N HCl extract (neutralised with NaOH) was used for protein estimations (presence of salt did not affect the estimation).

(b) Small scale isolation of avian erythroid histones. In experiments where radioactive histones were isolated, the above procedure was carried out on a smaller scale. Generally 0.05 ml of packed nuclei per sample were handled for histone isolation. After centrifugation to remove cell lysate (as described before), the nuclear pellet was washed once with 10 ml of each of the following solutions - isotonic sucrose, 0.08M NaCl - 0.02 M EDTA, pH 6.4 and 0.147 M NaCl. The washed nuclear pellet was allowed to drain thoroughly, after which 1 ml of 2M NaCl was added and the pellet resuspended. This suspension was allowed to stand for 30 minutes (with occasional agitation). 10N HCl was added to a final concentration of 0.25N and allowed to stand for a further 30 minutes. The acid insoluble material was removed by centrifugation (38,000 *g*, 10 minutes) followed by passage of the supernatant through a plug of glass wool, held in a pastuer pipette (for further clarification). The entire procedure, including the final centrifugation step, was carried out in the one test tube (per sample) and was highly reproducible. For the determination of radioactivity in histone, a known volume

(0.1 ml) of the final supernatant was absorbed onto a glass fibre filter (GF/C, Whatman) for counting in a scintillation spectrometer (Packard Tri-Carb). For analysis on polyacrylamide gels, the supernatant was dialysed against water, freeze-dried and dissolved in 0.05 ml - 0.1 ml loading buffer for analysis on polyacrylamide gels (see p.21).

(c) Isolation of histones from non-erythroid tissues. Tissues used were calf thymus, chick embryo thymus, chick embryo feathers and chicken testis. Tissues were washed in the same way as for red blood cells, namely in isotonic buffer, then homogenised in isotonic sucrose (with 0.5% saponin) using a motor driven Potter-Elvejhem homogeniser (5 - 7 strokes). A crude nuclear pellet was obtained by centrifugation at 1,800 *g* for 10 minutes. This pellet was then washed as described for red blood cell nuclei (prior to the isolation of histones, see p.19). All buffers contained 0.05M sodium metabisulphite (pH of buffers re-adjusted with 4N NaOH). Histones were isolated using the same procedure used to isolate erythroid histones (see p.19).

(d) Analysis of histones on polyacrylamide gels. Polyacrylamide gel electrophoresis was carried out as described by Panyim & Chalkley (1969), with the minor modification that the loading buffer contained urea (1 M, pH readjusted to pH 2.8 with glacial acetic acid). The gels were stained with Amido black (Gurr's) - gels stained with an alternative dye, Coomasie brilliant blue (Mann), gave the same staining patterns. After destaining, the gels were scanned using a Densicord coupled to an Intergraph

unit (Photovolt Corp., New York City) fitted with a N 610 filter; the so-called densicord trace was thus obtained showing absorption at 610 nm, with the area under respective peaks of the trace intergrated in arbitrary units. For the quantitative data presented, the amounts of protein loaded (20 - 50 µg/gel) were such that for each band, the area under the respective peak was proportional to the amount of protein loaded. The Densicord could also operate on a logarithmic response and this was often used as it emphasised minor bands.

(e) Analysis of radioactive histones on polyacrylamide gels. To allow the solubilisation of gels, for the subsequent determination of radioactivity, ethylene diacrylate instead of bisacrylamide was used to cross-link gels (Cain & Pitney, 1968). The separation of histone components was identical in both polyacrylamide gel systems. Gels, stained and analysed on a densicord as described above, were frozen with finely crushed dry-ice and sliced into 1 mm sections using a gel slicer (Mickle Lab. Engineering Co., Comshal, Surrey, England). Each slice was placed in a scintillation vial, dissolved in 0.5N NH_4OH (0.5 ml, 5 hours, 60 - 80°) and a GF/C glass fibre filter (Whatman) placed in each vial to absorb the solution. After drying (110°, 10 hours) scintillation fluid was added and the radioactivity determined in a scintillation spectrometer.

(f) Determination of specific activities of lysine and arginine in histone and free in the cytoplasm. In experiments related to the labelling of histones by radioactive C^{14}-amino acid

precursors, the specific activity of amino acids in two cellular pools were of interest. Only the specific activities of lysine and arginine were required. The first of these pools was lysine and arginine free (or bound to t-RNA) in the cytoplasm (nuclei-free cell lysate) after incubation with an equal volume of 0.5M Tris-HCl, pH 8.9, at 37° for 45 minutes to de-acylate t-RNA (Mosteller, Culp & Hardesty, 1968). High molecular weight material was precipitated with picric acid (Beckman Model 120B Amino Acid Analyser Instruction Manual AI M-2, p.7-4) and the amino acids in the supernatant desalted by absorption to a Dowex 50-H^+ column (1 x 5 cm), equilibrated and washed with 0.02N HCl, followed by elution from the column with 10N NH_4OH. This eluate was dried and analysed for amino acid content as described on p.28. The total radioactivity in an aliquot of the sample analysed for amino acids was also determined - the proportion of radioactivity present in lysine and arginine being checked by paper electrophoresis at pH 6.5 (see p.29). The second pool of lysine and arginine which was of interest was that in histone bound to DNA. The specific activity of this pool was determined in two ways. In the first, the radioactive histone preparation was electrophoresed on polyacrylamide gels and the appropriately stained band cut out. The gel slice was dispersed in 6N HCl (using a hand homogeniser - dispersion occurs very readily if ethylene diacrylate is used to cross-link gels), hydrolysed for 22 hours at 110°, clarified by centrifugation (1,800 *g*, 10 minutes) and taken to dryness under vacuum. The sample was then dissolved in loading buffer, a portion taken for analysis on the

Beckman 120C amino acid analyser and further portions for the measurement of total radioactivity. The acrylamide material still present in this sample caused 50% quenching of C^{14}-radioactivity. Because this procedure involved analysis of very small amounts of amino acids (lysine plus arginine about 0.01 μmole) a second method was also used. The radioactivity in a total histone sample was determined and the proportion of the label attributable to f2c histone was obtained from acrylamide gel analyses (see above). Knowledge of the lysine plus arginine content of this total histone sample plus the distribution of lysine and arginine in avian erythroid histones, including f2c histone (Murray, Vidali & Neelin, 1968) and consideration of the quantitative distribution of avian histones (see Chapter 4) was then used to obtain a figure for the specific activity of f2c histone.

(3) Preparation and analysis of chromatin

(a) Preparation of chromatin. Nuclei were isolated and washed as described for the preparation of histones on a large scale (p.19). Generally 4 ml of packed cells were used. After the last wash in 0.147 N NaCl, isolated nuclei were lysed in 0.2 mM EDTA, pH 7.1 (1 ml of packed nuclei per 15 ml solution), by homogenisation in a Dounce homogeniser with a tight fitting pestle (30 strokes). The nuclear pellet collected by centrifugation at 38,000 *g* for 20 minutes was then resuspended in 0.01M Tris-HCl, pH 8.3 (9 ml), as described by Bonner *et al.* (1968_2). On the scale described here, 3 ml of resuspended chromatin were layered on 28 ml quantities of sucrose (top 1/3

of tube mixed to form a rough gradient) and centrifuged for 3 hours at 22,500 rpm in a SW 25.1 rotor (52,000 *g*). A gelatinous pellet of chromatin is obtained which is resuspended in 0.01M Tris-HCl, pH 8.3, using a Dounce homogeniser (30 strokes) and dialysed against 0.01M Tris-HCl, pH 8.3. After dialysis chromatin was re-homogenised (30 strokes) to ensure suspension of the chromatin. Nuclear membrane material is readily recovered from near the top of the sucrose 'gradient'; for analysis this was dialysed extensively against water, freeze-dried and taken up in a small volume of 0.01M Tris-HCl, pH 8.3 (1 - 4 ml).

(b) Preparation of DNA from chromatin. DNA was isolated from chromatin (prepared as described above), or washed nuclei, by the procedure of Marushige, Brutlag & Bonner (1968).

(c) Melting point determinations. Chromatin or DNA (deproteinised chromatin) were dissolved in 0.2mM EDTA, pH 7.1. Solutions were heated in an electrically heated block of a Unicam (Cambridge, England) SP500 spectrophotometer. After each temperature step (2^{o}) 10 minutes was allowed for equilibration.

(d) Sucrose gradient analyses of crude chromatin (washed nuclei). Washed nuclei suspended in 0.2mM EDTA, pH 7.1 were sheared using a Dounce homogeniser as previously described for the preparation of chromatin. For sucrose gradient analyses, however, nuclei were extensively sheared (for 1 hour) as described by Dingman & Sporn, 1964. This procedure solubilises the DNA-protein complex and the remaining nuclear debri is removed by centrifugation at 10,000 *g* for 10 minutes (using a

swinging bucket rotor). 3 ml of solution (OD_{260} approximately 7) were loaded on 28 ml sucrose gradients (5 - 20%, in 0.1mM phosphate buffer, pH 7.0). Centrifugation was carried out in a SW 25.1 rotor for 18 hours at 22,500 rpm. Fractionation of gradients was generally carried out by collection of drops from the bottom of the tube -alternatively fractionation was carried out by displacing the gradient with 40% sucrose and monitoring the OD_{260} profile in a Optika (United Kingdom) densitronic spectrophotometer flow cell system. Linearity of gradients was checked by determining the refractive index of fractions (using a refractometer from Bellingham & Stanley Ltd., London).

(e) Transcription of chromatin by E. coli RNA polymerase. *RNA polymerase reaction.* The *E. coli* RNA polymerase was purified as described by Burgess (1969) using the enzyme from a low salt glycerol gradient purification, for the transcription experiments with chromatin. The polymerase reaction was carried out at low ionic strengths as described by Bonner *et al.* (1968). The reaction mix (final volume 0.25 ml) contained Tris-HCl, 0.04M (pH 7.9), $MgCl_2$, 3mM, EDTA, 0.1mM, potassium phosphate, 0.2mM (pH 7.9), BSA, 0.5 mg/ml, ATP, GTP and CTP (dissolved in 0.2mM EDTA, pH 7), 0.133mM each, α-UTP^{32}, 0.08mM (specific activity 1.8 x 10^8 cpm/µmole), RNA polymerase, 3 - 5 units (as defined by Burgess, 1969), DNA (in the form of chromatin or deproteinised DNA), 5 - 50 µg.

When ATP (α- or γ-labelled phosphate) was used, no unlabelled ATP was added and unlabelled UTP was added to a final concentration of 0.133mM.

Incubations were carried out at 37°, usually for 10 minutes (assays linear for this time). Reactions were terminated by the addition of 1 ml of BSA (0.2 mg/ml) in 0.02M EDTA, pH 7. High molecular weight material was precipitated by the addition of 5% trichloroacetic acid, 0.01M sodium pyrophosphate, and collected by filtration on GF/C filters. Filters were washed with 4 x 9 ml of 5% trichloroacetic acid, 0.01 M sodium pyrophosphate followed by 2 ml of ether. After drying, radioactivity was determined in a scintillation spectrometer.

Preparation of labelled nucleotide triphosphates.

Nucleotide triphosphates labelled in the α- position were prepared by the method described by Symons (1969). Nucleotide triphosphates labelled in the γ-position were prepared enzymatically using the method described by Glynn & Chappell (1964) with triphosphates being chromatographically purified as in the method for preparing the α-labelled nucleotide triphosphates.

Culture of E. coli for the preparation of RNA polymerase.

E. coli B were kindly supplied by Dr. J.B. Egan. An overnight culture was grown in Medium A or B. This was subcultured at a $^{1}/_{10}$ dilution in the same medium and grown to the end of log phase (90 minutes) before collecting cells by centrifugation.

MEDIUM A: NH_4Cl, .1%, Na_2HPO_4, .3%, KH_2PO_4, .15%, NaCl, .15%, $MgCl_2.6H_2O$, .01%, Na_2SO_4, .013%, nutrient broth .4%, .5% glucose.
Yield 20 - 30 gm per 11 litres.

MEDIUM B: Peptone, 4%, yeast extract, 1.5%, K_2HPO_4, .3%, KH_2PO_4, 0.07%. Yield 40 - 50 gm per 11 litres.

(4) Other analytical procedures

(a) Protein estimations. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). BSA (Sigma) dissolved in water was used as a standard for all estimations ($A^{1\%}_{1cm}$ = 6.61 at 278.5 nm (Peters & Blumenstock, 1967).

(b) DNA estimations. DNA was estimated either by the diphenylamine reaction (Burton, 1956) or the p-nitro-phenylhydrazine reaction (Martin, 1970). Salmon DNA (Sigma) dissolved in standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate) was used as a standard for all estimations. For standard DNA, $A^{0.1\%}_{1cm}$ = 22.0 at 260 nm (Bonner *et al.*, 1968_2) or an extinction coefficient per mole DNA-phosphate at 260 nm of 6500 was assumed (Hirschman & Felsenfield, 1966). For the standardisation of DNA, optical densities were determined using a Zeiss PMQ II spectrophotometer.

(c) Amino acid analyses. Amino acid analyses were carried out on the analytical system of the Beckman 120C amino acid analyser. A 10 hour modified gradient system (Wells, 1967) developed through a 120 cm column of Chromobeads type A (Technicon Instruments Inc.) was used to separate all amino acids. Protein samples were hydrolysed *in vacuo* (6N HCl, 110°) for 22 hours; no corrections were made for hydrolytic losses of amino acids.

(d) Paper electrophoresis. Analyses of peptides or amino acids by paper electrophoresis were carried out in a Michl tank, usually at pH 6.5 (pyridine acetate buffer - pyridine : acetic acid : water = 100 : 4 : 896).

CHAPTER 3

CHARACTERISTICS OF AVIAN ERYTHROID CELL POPULATIONS AT DIFFERENT STAGES OF MATURATION

(a) Introduction

Detailed analyses of populations of erythroid cells have been published (Williams, 1971a). Three populations of cells were defined for the purpose of this work (see p.16 of literature survey), namely erythroblasts (dividing cells), polychromatic erythrocytes (cells active in RNA and protein synthesis) and erythrocytes (inactive in macromolecular synthesis).

(b) Characteristics of cell populations

Differential cell counts of the preparations used in this investigation are shown in Table I.

Erythroblasts were obtained in 2 ways; type 1 erythroblasts were from linear BSA gradients (2 experiments, for details of linear BSA gradients see Williams, 1971a) and contained fewer non-dividing polychromatic erythrocytes than the more easily obtained type 11 preparation from discontinuous BSA gradients. Both types contained some non-erythroid cells.

The polychromatic erythrocyte preparations were from a density fractionation of anaemic blood cells and contained almost solely mid- and late-polychromatic erythrocytes.

The virtually homogeneous mature erythrocyte preparations were from normal blood with low-density cells removed by centrifugation through BSA. The density steps used in all cases are described under Methods (p.17).

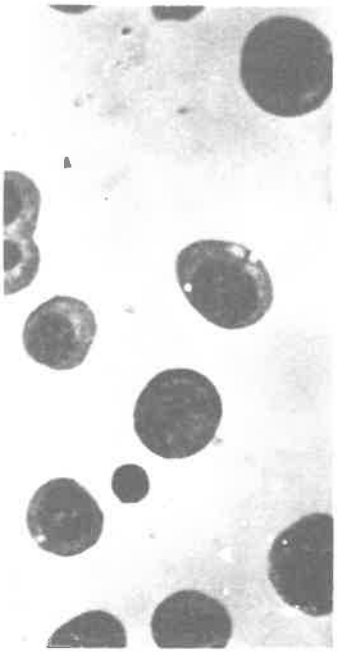
Photographs of the cell populations used in this investigation are shown in Fig. 1.

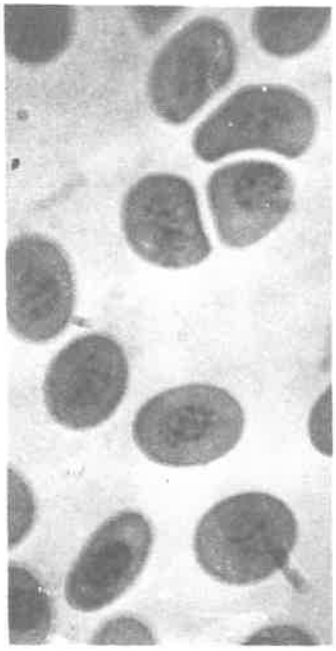
Fig. 1. Light microscopic examination of avian erythroid cell populations. Smears of cell suspensions on glass slides, stained with Leishman's stain (Lucas & Jamroz, 1961) were examined under oil immersion. Identification of cells: from left to right, mature erythrocytes, polychromatic erythrocytes and erythroblasts.

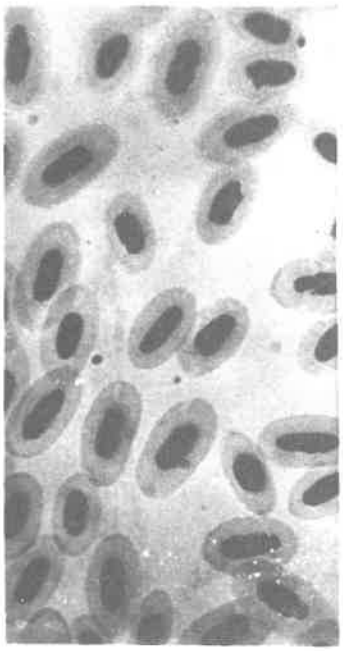
TABLE 1: CHARACTERISTICS OF CELL POPULATIONS WITHIN THE AVIAN ERYTHROID CELL SERIES

Cell fraction	Buoyant density range g/cm^3	1 % Erythro-blasts	2 % Poly-chromatic erythro-cytes	3 % Erythro-cytes	4 % Non-eryth-roid cells	5 % Cells in S phase
Erythroblasts I[a]	1.061-1.064	86	4.5	<1	9.5	63
Erythroblasts I[b]	1.059-1.071	83	10	<1	7	-*
Mid- and late-polychromatic erythrocytes[c]	1.071-1.083	<1	97	3	<1	<1
Erythrocytes[d]	>1.091	<1	<1	100	<1	<0.5 (Williams 1971)

After staining with Leishman's stain (Lucas & Jamroz, 1961), 7 fields with an average of 35 cells per field were counted to obtain the figures shown.
*Not carried out.
1, erythroblasts are not distinguished from immature lymphocytes.
2, in erythroblast fractions these were early polychromatic erythrocytes.
3, non-erythroid cells include mature lymphocytes, eosinophiles, basophiles and monocytes as defined by Lucas & Jamroz, 1961.
a, one population of cells from a linear BSA gradient.
b, average figures from 5 separate experiments.
c,d, average figures from 3 separate experiments.
b,c,d, cell populations prepared on discontinuous BSA gradients.







CHAPTER 4

CHARACTERISATION OF AVIAN ERYTHROID HISTONES

The contents of this chapter have been published:

Appels, R., Wells, J.R.E. & Williams, A.F. (1971). Characterisation of DNA-bound histone in the cells of the avian erythropoietic series. *J. Cell. Sci. 9, in press.*

(a) Introduction

The fact that chicken erythrocytes contain a unique histone (commonly called F V (Neelin, 1964) or f2c (Hnilica, 1964) as used in this thesis) has been known for many years (Neelin, 1964), and was an important point in the initial postulation of histones as specific gene repressors (Stedman & Stedman, 1950). In contrast to this suggestion, the function of histones within cells is at present regarded as one involving mainly structural organisation of nuclear chromosomal material (literature survey pp.10 and 13). Although tissue specific differences in histones have been found, there is insufficient information available at present for any detailed model relating histones to transcriptional controls within the nucleus.

The tissue specificity of f2c histone to nucleated erythrocytes of non-mammalian vertebrates has been well established (Dick & Johns, 1969; Johns & Diggle, 1969; Murray, Vidali & Neelin, 1968; Nelson and Yunis, 1969). At the same time the function of histone f2c remains unclear. One fundamental question is whether or not histone f2c histone is to be found in all cells of the erythroid series, or whether its synthesis is induced part way through the differentiating cell line. If the latter is correct it suggests a role for the histone in bringing about events in the cessation of macromolecular synthesis which characterise the terminal stages of avian erythropoiesis (Neelin, 1964; Johns, 1969).

Some authors (Purkayastha & Neelin, 1966) have suggested that erythroblasts do not contain histone f2c;

however, their study was carried out on unfractioned bone marrow cell populations and their results are thus difficult to interpret. In this chapter, histones from preparations of erythroblasts, polychromatic erythrocytes and mature erythrocytes have been characterised, with particular reference to the presence of f2c histone. We were also interested in the f1 histone fraction since Johns (1969) had suggested that histone f2c may replace f1 as erythropoiesis proceeds, and also because Panyim & Chalkley (1969) have suggested from studies with calf tissues that there may be f1 histone components specific to dividing and non-dividing cells respectively.

The results in this chapter show that f2c histone is found in the earliest erythroid cells available and that no clear-cut difference in the f1 histone exists between dividing and non-dividing cells.

(b) Histone:DNA ratios of avian erythroid cells

Prior to a study of fractionated histone preparations (protein soluble in 2N NaCl, 0.25N HCl, Dingman & Sporn, 1964), the histone:DNA ratios from the various cell populations were examined to characterise any gross differences. While it is clearly desirable to obtain all data from purified preparations of chromatin, washed nuclei were used to determine the histone:DNA ratios for erythroblasts (Table 2). Due to contaminating DNA'ase activity, purification of erythroblast chromatin through 1.7M sucrose has proved difficult (see Chapter 7 for details). To allow direct comparison of

nuclear histone:DNA ratios, nuclei from polychromatic erythrocytes (from the same animal) were determined.

TABLE 2. HISTONE:DNA RATIOS (W/W) FOR AVIAN ERYTHROID CELLS

Cell fraction	Histone:DNA (chromatin)	Histone:DNA (nuclei)
Erythrocytes	1.21 ± 0.15 (5)	-
Polychromatic erythrocytes	1.07 ± 0.13 (5)	0.83 ± 0.04 (3)
Erythroblasts	-	0.88 ± 0.08 (3)

The numbers in parentheses indicate the number of different cell populations examined. Washed nuclei or purified chromatin were analysed. Values are stated ± S.E.

No statistically significant differences in histone:DNA ratios could be established between mature and polychromatic erythrocytes and polychromatic erythrocytes and erythroblasts. Others have observed similar results for embryonic and mature erythroid cells (Dingman & Sporn, 1964).

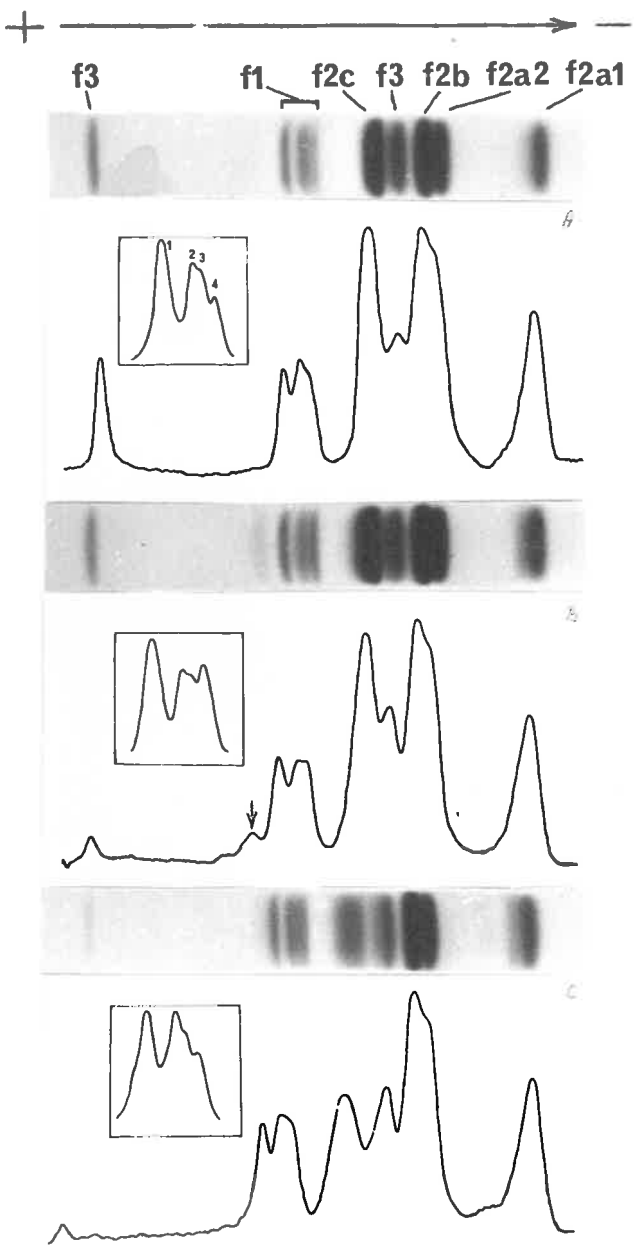
(c) Fractionation of histone preparations

Histone preparations from avian erythroid cells were further analysed on polyacrylamide gels to observe any differences in individual fractions. As pointed out above, chromatin could not be prepared from erythroblasts; thus histones were routinely prepared from washed nuclei. This appeared to be a valid procedure since histones isolated from

either washed nuclei or purified chromatin preparations from non-dividing erythroid cells were identical (see Chapter 6 for analyses). Sodium metabisulphite was added to solutions used to isolate and wash nuclei, to inhibit protease activity (Panyim & Chalkley, 1969); under the conditions used no components identified by electrophoresis could be shown to be due to degradation (Harlow & Wells, 1971).

The results of electrophoresis of erythroid histone preparations are shown in Fig. 2; all the histone components previously identified in erythrocytes by others (Murray *et al.*, 1968; Nelson & Yunis, 1969) were found (Fig. 2A). The f1 and f2c histones were identified by their behaviour in the procedures of Johns & Diggle, 1969. The remaining histones (including the aggregate f3 histone) were identified by comparison with published electrophoretic analyses of mature erythrocyte histones (Nelson & Yunis, 1969; Dick & Johns, 1969).

Occasionally some additional bands were identified in histone preparations (see arrow Fig. 2B and 4), but these were not routinely found and thus not classified as histones. It is unlikely that cytoplasmic proteins contaminating histone preparations contribute significantly to the histone components identified above since the electrophoretic patterns were highly reproducible whereas cytoplasmic proteins analysed on the same gel system showed extremely variable patterns. The one exception to this was found to be avian globin (prepared from the haemoglobin moiety purified by CM-cellulose chromatography, pH 6.3, Fantoni, de la Chapelle, Rifkind & Marks, 1968) which


+
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f3
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4

Fig. 2. Histone analyses from the avian erythroid cell series. About 30 µg of protein were loaded per gel and electrophoresed for 4 hr at 2 mA/gel. Gels were scanned with the Densicord set to a logarithmic response (see Methods, p.21). The insets are detailed analyses of the f1 regions on 25-cm gels. The protein component indicated by the arrow was not identified as a histone (see text).

A. Histones from mature erythrocytes.
B. Histones from polychromatic erythrocytes.
C. Histones from erythroblast type I cells. The f1 analyses (inset) were carried out on erythroblast type II cells.

was found reproducibly in cytoplasmic protein analyses. This protein did in fact co-electrophorese with peak I of the f1 histone group (see inset Fig. 2A for identification). Thus if cytoplasmic contamination occurs, only this particular histone moiety would be affected. This aspect of cytoplasmic contamination of histone preparations is discussed further in Chapter 5.

Fig. 2B,C show the histone components of immature erythroid cells. It can be seen that polychromatic erythrocytes and erythroblasts contain all the histones found in mature erythrocytes. This observation was confirmed by co-electrophoresis of samples of histones from the various cell types. The f1 and f2c histones could also be obtained from all cell types by the procedures of Johns & Diggle (1969). Amino acid analyses of the f2c histone fractions from all erythroid cell types were very similar, particularly with regard to the characteristically high content of lysine, serine, alanine and arginine (Table 3). One dimensional analyses of peptides obtained by thermolysin digestion were also very similar (Fig. 3).

As has been mentioned previously the f1 histones, as well as the f2c histone, of erythroid cells were of particular interest. To examine the heterogeneity known to exist in the f1 histone fraction (Sherod, Johnson & Chalkley, 1970), longer polyacrylamide gels were also run. Densicord traces of the f1 regions from such analyses are shown in the insets to Fig. 2A,B and C. A full separation of mature erythrocyte histones on

a 25-cm polyacrylamide gel is shown in Fig. 4. From the results of this study of the f1 region it was clear that, within the limits of resolution of the methods used, no qualitative differences were found between the species of f1 histones present in dividing and non-dividing erythroid cells.

TABLE 3. AMINO ACID ANALYSES OF f2c HISTONE FROM AVIAN ERYTHROID CELLS

Amino acid	Erythro-blast Type II cells	Poly-chromatic erythro-cytes	Erythro-cytes	Johns & Diggle (1969)	Murray *et al.* (1968)
Aspartic acid	3.0	1.9	2.2	2.1	1.8
Threonine	3.5	3.1	3.2	3.5	3.2
Serine	10.3	12.6	13.2	11.2	12.5
Proline	5.2	6.0	6.5	3.2	6.7
Glutamic acid	6.7	4.9	4.3	4.8	4.1
Glycine	7.5	5.3	5.3	5.3	4.4
Alanine	14.5	15.6	15.6	15.7	16.3
Valine	4.6	4.3	4.1	4.9	4.4
Methionine	0.7	0.8	0.0	0.5	0.5
Isoleucine	3.4	3.3	3.1	3.7	5.3
Leucine	4.9	4.3	4.3	4.9	4.3
Tyrosine	1.8	1.7	1.7	1.4	2.0
Phenylalanine	1.1	0.7	0.7	0.6	0.0
Lysine	21.1	23.4	23.1	24.9	22.7
Arginine	9.7	10.6	10.8	11.4	11.5
Histidine	1.9	1.7	1.7	1.8	1.8

Analyses are expressed as mol/100 mol amino acids recovered. The erythrocytes f2c histone analysis was similar to that reported by Johns & Diggle (1969) and Murray *et al.* (1968) for avian erythrocyte f2c histone preparations. The samples of f2c histone for analyses were shown to be one band by polyacrylamide gel electrophoresis.

Fig. 3 Thermolysin peptides from f2c histone preparations. Digestion of f2c histone preparations from polychromatic erythrocytes (A) and erythroblast type II cells (B) was carried out in 0.005M $CaCl_2$, 0.05N N-ethyl morpholine acetate, pH 8.0, for 4 hr at 37°, at A enzyme:substrate ratio of 1:30. Peptides were electrophoretically separated at pH 6.5 on Whatman 3 mm paper, (see also Methods p 29). Peptides were visualised by their reaction with ninhydrin.

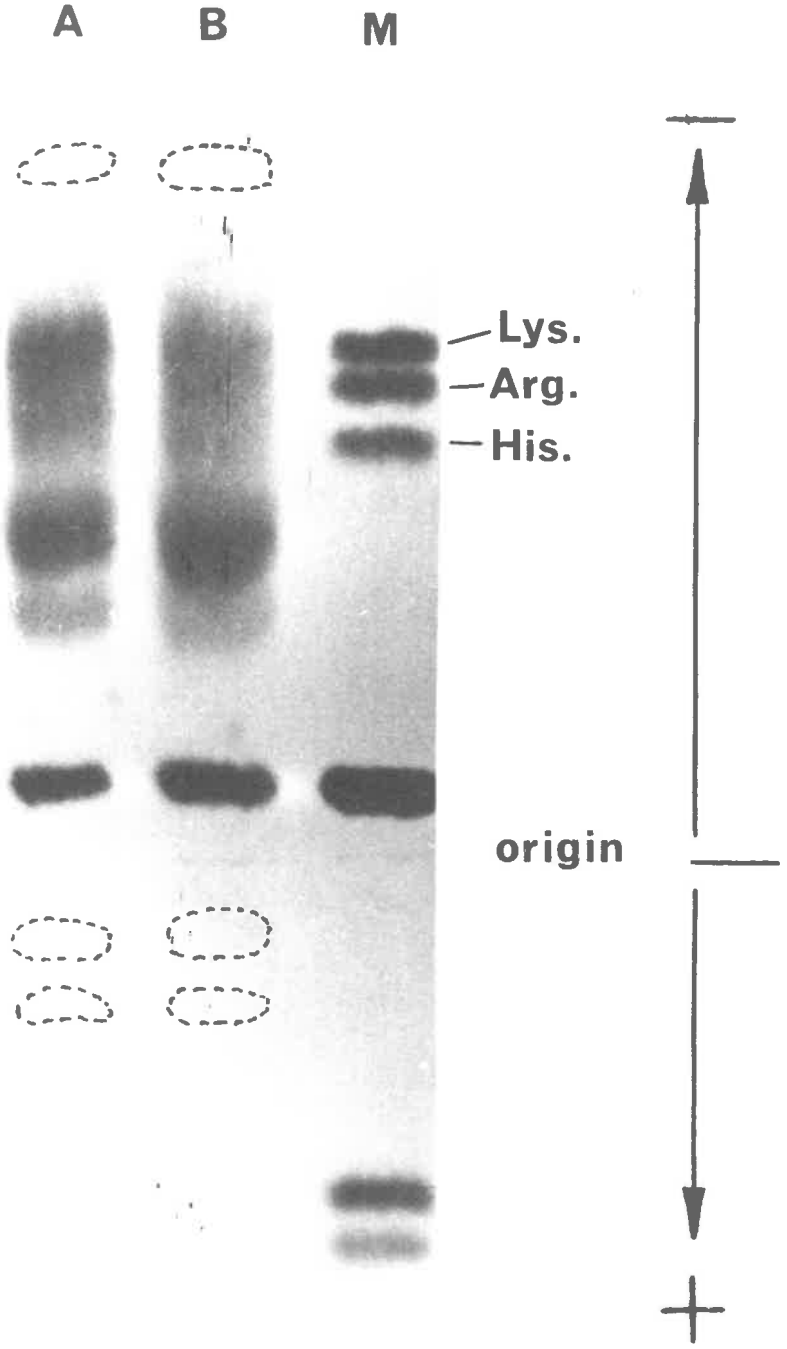
A
B
M
Lys.
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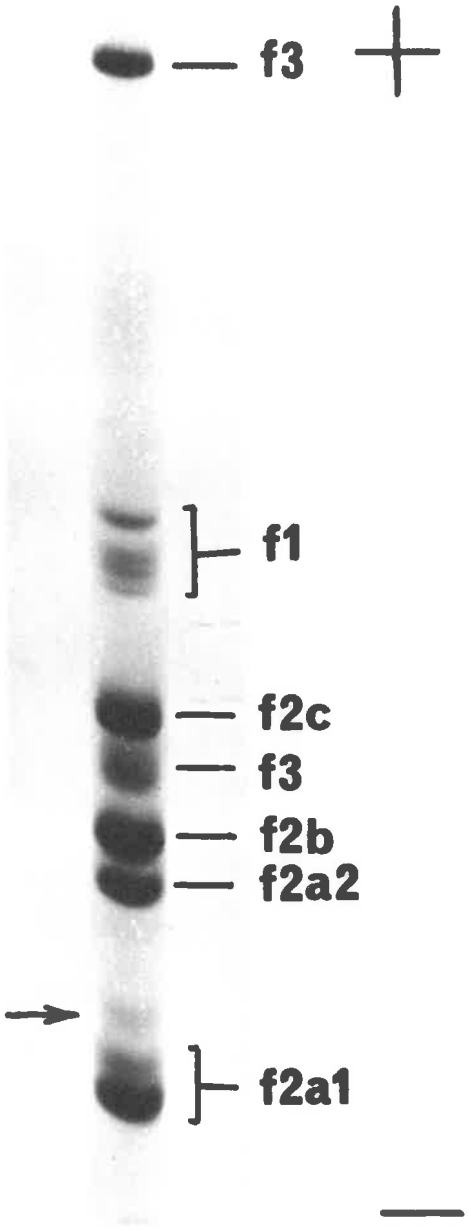
Fig. 4. Erythrocyte histones analysed on 25-cm polyacrylamide gels. About 40 - 50 µg of protein was loaded per polyacrylamide gel and electrophoresed for 16 hr at 2 mA/gel. The arrow indicates a band which was not defined as a histone (see text).


f3
+
f1
f2c
f3
f2b
f2a2
f2a1
−

(d) Quantitation of histone analyses

Although the results of the experiment shown in Fig. 2 suggested there was no clear-cut difference in the histone moieties from erythroid cells at various stages of erythropoiesis, there did appear to be some quantitative differences. The quantitation, after staining of protein bands, was carried out as indicated in the legends to the respective tables (Tables 4 and 5). This ignores the differential staining behaviour of individual proteins with a particular dye (in this case amido black); however, the procedure was considered sufficient to allow a comparison of the distribution of histones in the different cell types.

TABLE 4. DISTRIBUTION OF HISTONE COMPONENTS IN AVIAN ERYTHROID CELLS

Cell fraction	f1	f2c	f3	f2b+ f2a2	f2a1
Erythrocytes	10.0±2.3	24.5±1.4	16.0±1.1	34.0±1.1	14.7±1.3
Polychromatic erythrocytes	11.9±3.1	23.8±0.8	13.1±1.7	34.3±1.2	16.9±0.6
Erythroblasts	16.1±1.9	17.0±0.7	17.6±0.9	34.7±1.1	14.6±1.1

Three separate cell populations were analysed for each fraction; mean values are ± S.E. For erythroblasts, one histone preparation from erythroblast type I cells and 2 from erythroblasts type II cells were examined. To obtain the data shown, peak areas of respective histone groups were expressed as % of the total area analysed.

The data in Table 4 indicate that the f2c histone content of erythroblasts, relative to the remaining histones, was significantly lower when compared to the f2c content of polychromatic and mature erythrocytes ($P < 0.05$). Erythroblasts contain approximately 70% as much f2c histone as the non-dividing cells; after adjusting for the presence of non-erythroid cells in these preparations (Table 1) this figure becomes 78%. This is a minimum estimate and it is possible that this figure may be slightly higher when the difficulty in distinguishing erythroblasts from blast cells of the non-erythroid lines is considered (Williams, 1971).

An analysis of the other histone fraction to show a difference between cell types, the f1 histones, is shown in Table 5. In this case erythrocytes appear to contain

TABLE 5. ANALYSIS, ON 25-CM GELS, OF THE f1 GROUP OF HISTONES FROM AVIAN ERYTHROID CELLS

Cell fraction	Peak 1	Peak 2	Peak 3	Peak 4
Erythrocytes	32.6±2.0	26.0±0.5	24.4±2.3	17.1±1.0
Polychromatic erythrocytes	31.4±2.5	23.0±1.7	21.6±2.1	24.0±1.2
Erythroblasts	29.7±1.9	29.5 1.6	23.7±0.9	18.0±1.7

For identification of peaks see Fig. 1A (inset). Four separate cell populations were analysed for each fraction; mean values ± S.E. are shown. The data were obtained by measuring the peak heights and expressing respective peak heights as % of the total peak height measured. Peak

heights were used since the f1 components were not sufficiently resolved to allow integration of the total areas under each component.

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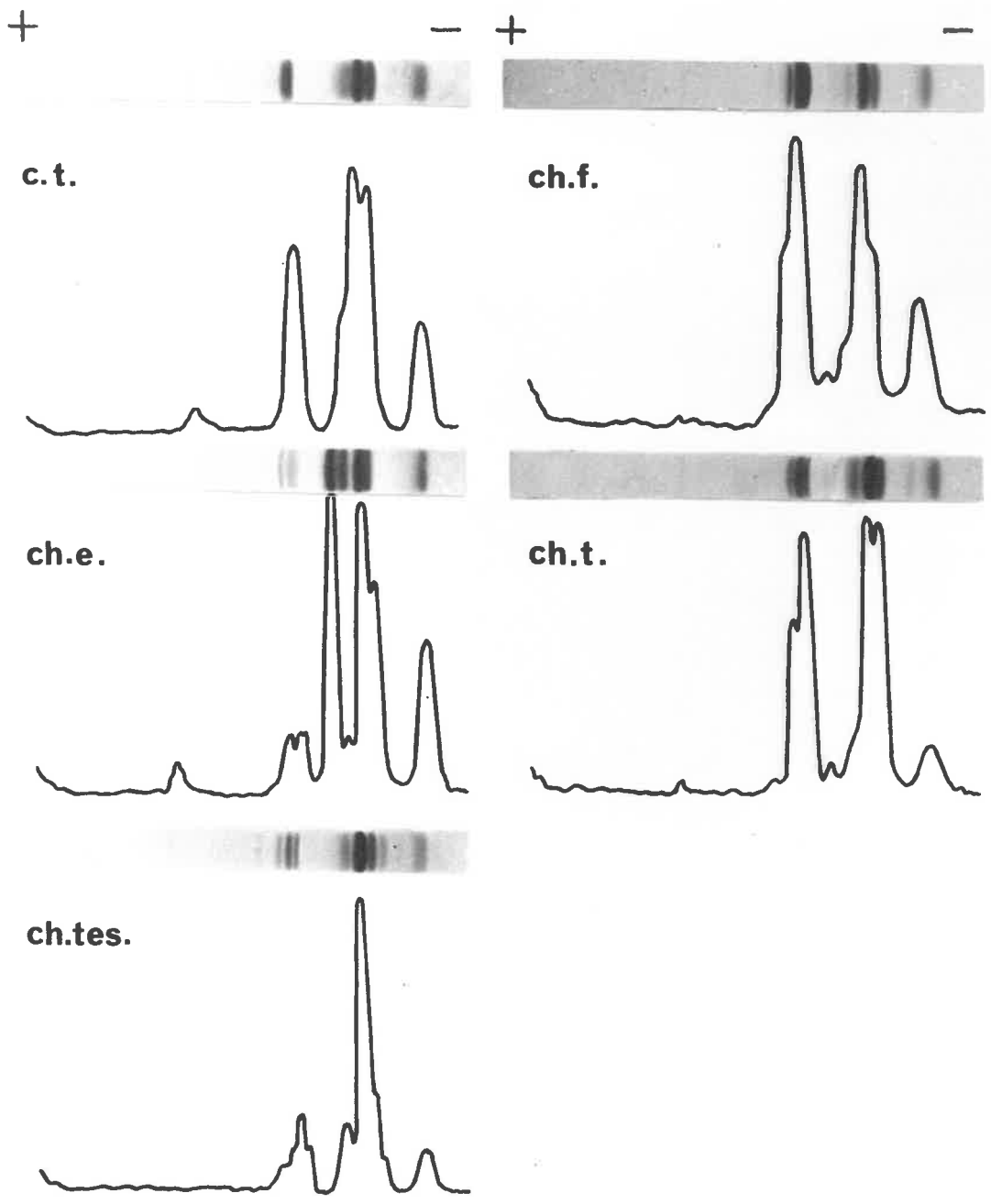
significantly lower amounts of the f1 histone - peak 4 (relative to the remaining components) than polychromatic erythrocytes; this difference was not found in erythroblasts. No other statistically significant differences could be established.

(e) Fractionation of histones from non-erythroid tissues

In this study it seemed necessary to examine histones from other tissues as well as erythroid cells to be quite sure our results were in accord with the work of others in the field. Therefore, histones were prepared from calf thymus, embryonic chick feathers (from 13-day embryos - this is the stage just prior to major onset of keratin synthesis, Malt & Bell (1965); Kemp, personal communication), embryonic chick thymus (from 20-day embryos - this is the stage at which the thymus is largest, Lillie, 1965) and chicken testis. Analyses of the preparations are shown in Fig. 5.

The calf thymus histone pattern was exactly as described by other workers (Panyim, Jensen & Chalkley, 1968; Panyim & Chalkley, 1969) with no trace of histone running at the mobility of avian f2c (Purkayastha & Neelin, 1966). The small amount of f2c in the embryonic chick feather and thymus histone preparations would be accounted for by 7 - 9%

Fig. 5. Analyses of histones from non-erythroid tissues. About 30 µg of protein was loaded per polyacrylamide gel and electrophoresed for 4 hr at 2 mA/gel. Histones were isolated as described in the Methods (p.21). Gels were scanned with the Densicord set to a linear response (see Methods, p.21). Symbols: c.t., calf thymus; ch.f., 13-day chick embryo feathers; ch.e., mature erythrocytes; ch.t., 20-day chick embryo thymus; ch.tes., adult chicken testis.


+
−
+
−
c.t.
ch.f.
ch.e.
ch.t.
ch.tes.

contamination of erythrocytes, which seems reasonable since the tissues were not subjected to cellular fractionation procedures.

(f) Discussion

Although much information on histone from different tissues is now available, our aim was to investigate the possible role of these proteins in a well-characterised system in which relatively pure populations of cells, of defined biochemical activity, could be obtained. Previous work related to histones in the avian erythroid system has been reported (Purkayastha & Neelin, 1966; Dick & Johns, 1969; Johns & Diggle, 1969) but we have extended studies to the dividing cells of the series.

The central findings in this study were that, as far as can be ascertained, cells at all stages of avian erythropoiesis contained the same total amount of histones (Table 2) and identical histone components (Fig. 2). In particular, dividing erythroblasts already contained histone f2c, unique to nucleated erythrocytes. In addition, no qualitative differences in individual f1 components were found between dividing and non-dividing cells within the erythropoietic series.

Although the results are somewhat different from earlier reports, it is unlikely that they are artifactual since defined cell populations were used and all possible precautions were taken to minimise degradation and contamination of histones

during their isolation. In addition, analyses of histone preparations, particularly from calf thymus (Fig. 5) agree well with published electrophoretic analyses of histone (Panyim & Chalkley, 1969). The suggestion from previous authors that f2c histone is absent from erythroblasts (Purkayastha & Neelin, 1966) probably resulted from lack of definition of cell populations, since unfractionated bone marrow preparations were used.

Our results are in agreement with recent studies on defined populations of HeLa cells (Sadgopal & Bonner, 1970) and *Physarum polycephalum* (Mohberg & Rusch, 1970) in that we find no new histone component in erythroid cells as they change in biochemical activity. In particular, the data presented rule out any simplistic role for histone f2c as suggested by some authors (Neelin, 1964; Johns, 1969). In the following chapter (Chapter 5) the function of f2c histone is considered in more detail. In addition any obvious possibility of an f1 histone fraction having a specific role within this cell series was also eliminated. This had also been suggested for dividing and non-dividing cells by Panyim & Chalkley (1969). A minor, reproducible, redistribution of f1 histone components - similar to that reported in other tissues of changing biochemical activity (Bustin & Cole, 1968; Kinkade, 1969) - was found (Table 5) but not investigated further. The reduced amount of f2c histone in erythroblasts (Table 4) was investigated further and the results of these studies are presented in the following chapter (Chapter 5).

The f2c histone was absent from a closely related tissue, the thymus, which is thought to arise from the same cells as bone marrow erythroid cells (Yoffey, 1966). Thus the appearance of f2c histone is closely correlated with commitment to the erythroid cell series. Whether, in fact, it constitutes a vital event in the postulated divergence of the 2 cell lines cannot be deduced from correlative evidence.

CHAPTER 5

AVIAN ERYTHROID HISTONE METABOLISM

The contents of this chapter have been submitted for publication:

Appels, R. & Wells, J.R.E. Synthesis and turnover of DNA-bound histone during maturation of red blood cells.

(a) Introduction

One cellular change which is particularly evident during the maturation of non-dividing avian erythroid cells is the heterochromatinisation of the nuclear material. Sadgopal & Kabat (1969) have suggested that concomitant with this heterochromatinisation there occurs an accumulation of histone (as well as other chromosomal protein) on the chromatin. In this chapter this problem of whether or not histone accumulates on the chromatin during maturation of avian red blood cells was examined.

Two observations made in the preceding chapter (Chapter 4) are relevant to this study. The first was that all histone species, including the f2c histone (tissue specific to avian erythroid cells) are present in all cells of the series. This observation is incompatible with the notion of a simple relationship between the presence or absence of a histone (in particular the f2c histone) and the cessation of macromolecular synthesis during maturation of these cells, as proposed by others (Purkayastha & Neelin, 1966; Johns, 1969). The second observation was that no significant change in the total histone to DNA ratio could be established within the cells of the erythroid series. Although this observation ruled out gross accumulation of histone on chromatin during maturation, we did observe that the relative amount of f2c histone present in erythroblasts was lower than that present in the non-dividing cells (f2c histone in erythroblasts/f2c histone in non-dividing cells = 0.8). This later observation therefore did not exclude

the possibility that f2c histone may accumulate on erythroid chromatin as cell maturation proceeds. Such a process could, in principle, result in gradual inactivation of the genome and may also give rise to the highly condensed chromatin seen in the mature erythrocyte. We sought a definitive answer to this possibility by studying the metabolism of histones in these cells. Our observation that f2c histone is the only histone which becomes labelled when non-dividing cells (polychromatic erythrocytes) are incubated with C^{14}-amino acids, enabled us to study the metabolism of this histone in conditions uncomplicated by requirements of histone for newly synthesised DNA (compare Gurley & Hardin, 1970). If the synthesis of f2c histone represented *accumulation* of this histone on chromatin, we predicted that in 'pulse-chase' experiments the radioactivity in f2c should be metabolically *stable*. Whereas radioactivity in cytoplasmic protein (mainly haemoglobin) is completely stable in such 'pulse-chase' experiments, we find the radioactivity in f2c histone to be *unstable*. The implications of these observations to avian erythroid cell maturation in particular, and to the potential of chromatin for transcription in general, are discussed.

(b) Incorporation of C^{14}-amino acids into histones.

The method of preparation of histones defines them as proteins attached to DNA. As a result of this definition, for C^{14}-amino acids to appear in histones, (after *in vitro* incubation), at least two cellular events must occur:

C^{14}-amino acids ⟶ C^{14}-histone ⟶ C^{14}-histone bound to DNA

protein synthesis, presumably in cytoplasm.

attachment to DNA (also requiring transport into the nucleus).

The rate limiting step in this process is not known. More importantly, in this work, conditions of the overall reaction do not change, as judged by the linear incorporation of radioactivity into histones (30 - 40 min. pulses, Fig. 6). The incorporation of radioactivity into histone is inhibited by puromycin (150 µg/ml), and cycloheximide (100 µg/ml), (Fig. 6).

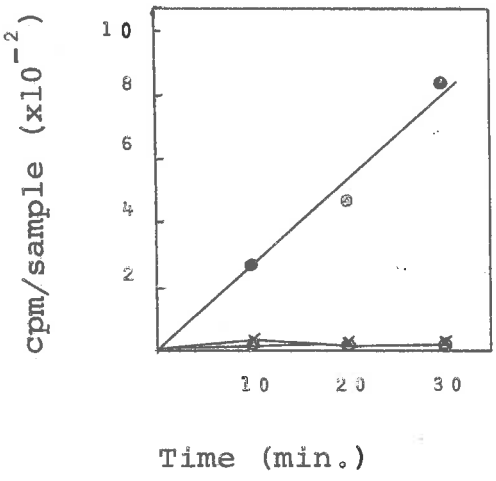


Fig. 6. Effect of inhibitors of protein synthesis on C^{14}-lysine incorporation into histones. Polychromatic erythrocytes were incubated with C^{14}-lysine (4 µC/ml, spec. act. 100 mC/m-mole) *in vitro*. Symbols: ⊙ —— ●, in the absence of inhibitors; x —— x , plus 150 µg/ml puromycin; o —— o , plus 100 µg/ml cycloheximide.

(c) Labelling pattern of erythroblast histones.

All histones were found to be labelled in erythroblasts, (Type II, see Table I, Chapter 4), after an *in vitro* incubation with C^{14}-amino acid (in this case lysine), as shown in Figure 7. The additional peak of radioactivity, not defined as a histone (arrow, Fig. 7) indicated that the histone preparation was contaminated with other proteins (discussed further in 'contamination of histone preparations' section (e)).

(d) Labelling pattern of polychromatic erythrocyte histones.

In contrast to erythroblasts, only the f2c histone (tissue specific to avian erythroid cells) was labelled in polychromatic erythrocytes incubated *in vitro* with C^{14}-amino acids (in this case lysine and arginine - Fig. 8). The additional, minor, peaks of radioactivity also seen in Figure 8 are discussed in the following section on the contamination of histone preparations (section (e)).

Confirmation that f2c histone and no other histone was labelled was obtained by two other procedures for fractionating avian erythroid histones (for details of results, see Appendix 3). First, a histone preparation from C^{14}-lysine labelled polychromatic erythrocytes was fractionated into 5% perchloric acid - soluble and insoluble fractions (Nelson & Yunis, 1969). The f1 and f2c histones are recovered in the 5% perchloric acid - soluble fraction (containing histones f3, f2b, f2a2 and f2a1) no label could be detected. The second procedure was that of Johns & Diggle (1969) in which pure f2c

Fig. 7. Analysis of C^{14}-histones isolated from erythroblasts. Erythroblast type II cells were incubated with C^{14}-lysine (4 µC/ml, spec. act. 100 mC/m-mole) *in vitro*, for 30 min. Histones isolated from these cells (see Methods, p.20) were analysed on polyacrylamide gels at a loading of 60 µg/gel. Symbols: ● —— ●, distribution of radioactivity in cpm per 1 mm gel slice; ———, densicord trace of the gel used to obtain the distribution of radioactivity. The arrow indicates a component which was not identified as a histone (see text).

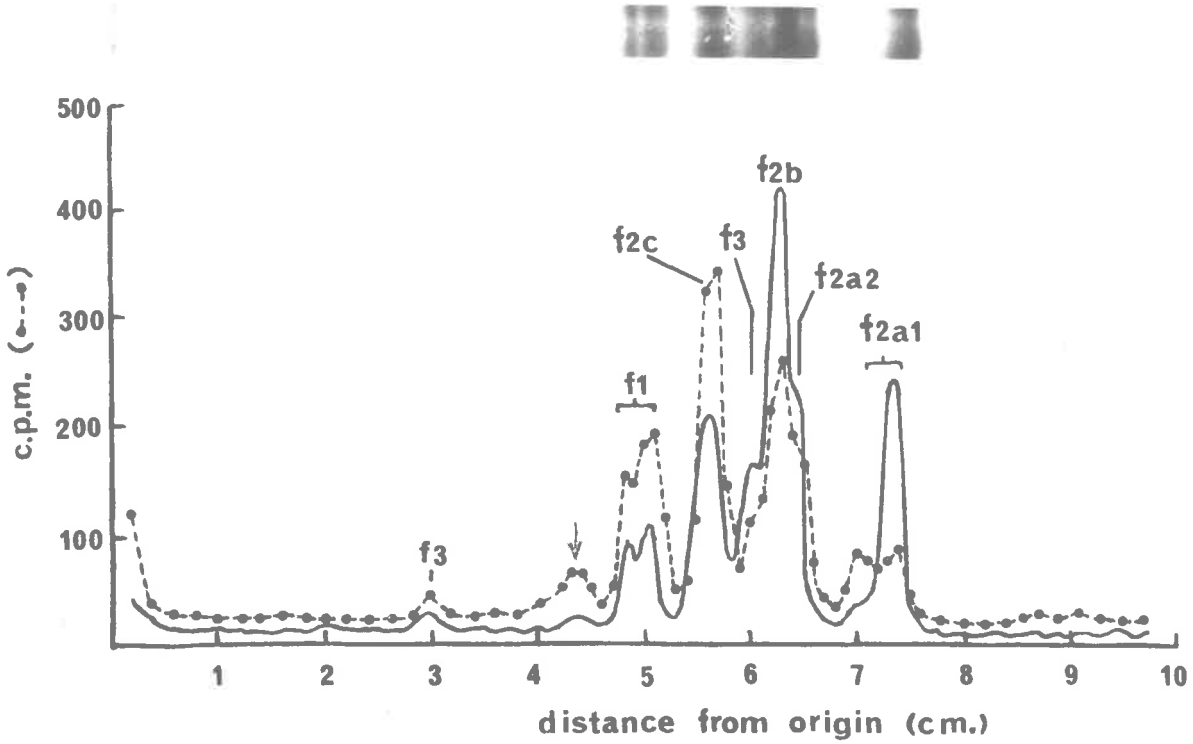
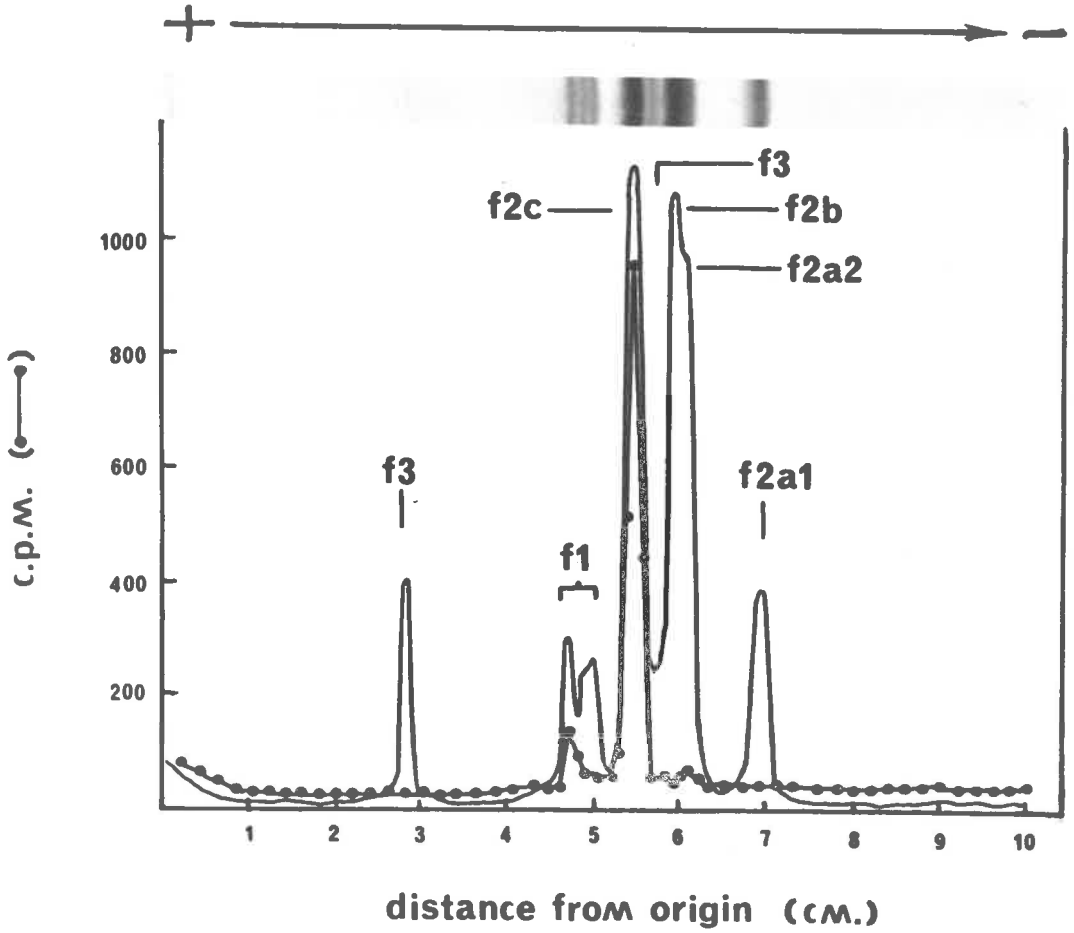

500
400
300
200
100
c.p.m. (•---•)
f2b
f2c
f3
f2a2
f2a1
f1
f3
1
2
3
4
5
6
7
8
9
10
distance from origin (cm.)

Fig. 8. Analysis of C^{14}-histones isolated from polychromatic erythrocytes. Polychromatic erythrocytes were incubated with C^{14}-lysine (20 µC/ml, spec. act. 210 mC/m-mole) plus C^{14}-arginine (10 µC/ml, spec. act. 198 mC/m-mole) *in vitro* for 30 min. Isolated histones were analysed on polyacrylamide gels as described in Methods (p.22), at a loading of 40 µg per gel. Symbols: ● —— ●, distribution of radioactivity in cpm per 1 mm gel slice; ——— densicord trace of the gel used to obtain the distribution of radioactivity.


+
−
f3
f2c
f2b
f2a2
f3
f1
f2a1
1000
800
600
400
200
c.p.m. (●—●)
1
2
3
4
5
6
7
8
9
10
distance from origin (cm.)

histone was prepared. Once again, examination of all fractions recovered from this purification indicated that f2c histone was the only histone containing radioactivity.

(e) Contamination of histone preparations.

Although, as outlined above, the major peak of radioactivity from histones labelled in polychromatic erythrocytes was found to be in f2c histone, some minor peaks were observed (Fig. 8). In addition, radioactivity has been found in some peaks not identified as histone (e.g., arrow, Fig. 7). These minor peaks deserved some further study since they may represent either a low level of histone synthesis (other than that described for f2c histone) or non-histone protein 'contaminating' the histone preparations as suggested by Stellwagen & Cole (1968_1). A general observation made during the course of this study was that radioactivity in these peaks was variable in position as well as prominence - this fact indicated that these peaks probably represent a contamination in histone preparations. As pointed out in the previous chapter (Chapter 4) bands corresponding to histone species on polyacrylamide gels are highly reproducible. When polychromatic erythrocytes were incubated with C^{14}-leucine rather than C^{14}-lysine or C^{14}-arginine, the minor peaks mentioned above were more readily detected (Fig. 9B). The shoulders on the f2c histone peak, in particular, were not detected when C^{14}-lysine or C^{14}-arginine were used as the source of radioactivity. This behaviour is consistent with the

proposal that the peaks of radioactivity seen in Figure 8, other than that attributable to f2c histone, are due to non-histone proteins (low in lysine and arginine, in contrast to histones which are relatively rich in these amino acids).

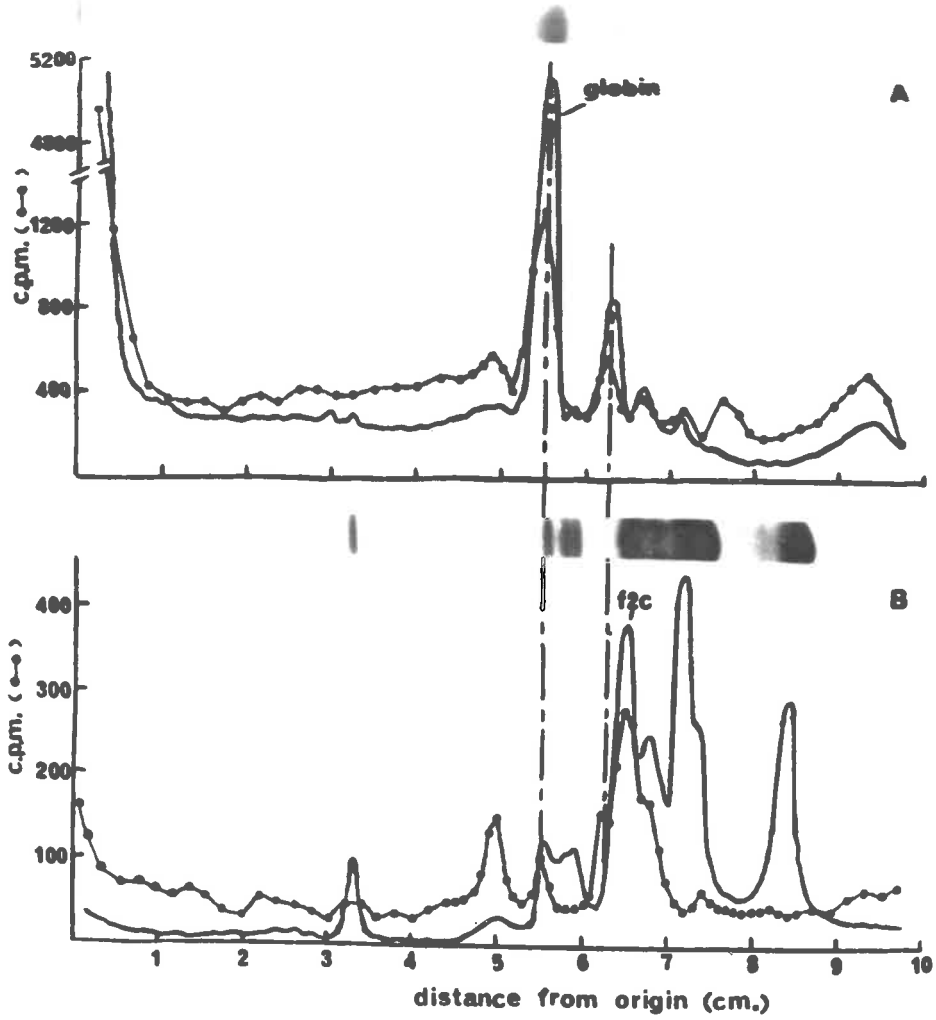
When preparations of cytoplasmic protein are analysed on the gel system used to analyse histones (Fig. 9A), major cytoplasmic proteins are seen in the positions of radioactivity shown in Fig. 9B - except for that of the f2c histone. Thus it is possible that the non-histone proteins in histone preparations are of cytoplasmic origin (as suggested by Stellwagen & Cole, 1968_1).

The results in this section, although rather indirect, would appear to validate the claim that f2c histone is in fact the only histone labelled in polychromatic erythrocytes.

(f) Stability of radioactivity found in f2c histone

The fact that f2c histone was the only histone which became labelled in polychromatic erythrocytes allowed an investigation of the metabolic stability of this histone in particular. The protocol for the experiment was to pulse cells with C^{14}-lysine and C^{14}-arginine, *in vitro*, and to measure the rate of labelling of f2c histone. To examine the stability of the label in f2c histone, the pulse of radioactivity was terminated and followed by a 'chase' period to measure the rate of loss of radioactivity (if it did in fact occur). To compare the rate of incorporation of radioactivity to the possible loss of radioactivity, the specific activities of two

Fig. 9. C^{14}-leucine incorporation into polychromatic erythrocyte histone and cytoplasmic protein. Polychromatic erythrocytes were incubated with C^{14}-leucine (4 µC/ml, spec. act. 311 mC/m-mole) *in vitro* for 30 min. Histone and cytoplasmic protein isolated from the same cells (see Methods, p.20), were analysed on the gel system routinely used to analyse histones.
A. Cytoplasmic protein fraction, 40 µg of protein loaded per gel. B. Histone fraction, 100 µg of protein loaded per gel to obtain the distribution of radioactivity.
Symbols: ● —— ●, distribution of radioactivity in cpm per 1 mm gel slice; ———, densicord trace of gel used to obtain the distribution of radioactivity (for B, this trace was from a gel run in parallel, with a lower amount, 30 µg, of the same histone sample loaded).
Identification of the globin peak in A. has been described in Chapter 4 (p.35). Precise alignment of the two analyses shown is described in Appendix 2.


5200
4800
1200
800
400
c.p.m. (•—•)
globin
A
400
300
200
100
c.p.m. (•—•)
f2c
B
1
2
3
4
5
6
7
8
9
10
distance from origin (cm.)

pools of amino acids were measured. One pool was that of free lysine and arginine (at the beginning of the pulse and giving rise to proteins) and the other was that of lysine and arginine in f2c histone (at the end of the pulse). The feasibility of this approach for detecting loss of radioactivity in the chase period, depends largely on the specific activity of the f2c histone obtained in the pulse. If this is too low, then even if the rate of loss of DNA-bound histone equals its rate of addition to DNA, the loss of radioactivity predicted may not be experimentally significant.

Polychromatic erythrocytes were pulsed for 40 minutes in the presence of C^{14}-lysine and C^{14}-arginine (total 30 µC/ml) as the only external source of these amino acids. The pulse of radioactivity was terminated either by the addition of puromycin or an excess of lysine and arginine (or both) and followed by a 'chase' of 4.5 hours. Under all conditions in which the 'chase' was carried out, the label appearing in f2c histone in the initial pulse was *not stable* (Fig. 10). In these experiments each sample of histone was analysed on polyacrylamide gels to determine the distribution of radioactivity (as in earlier section). The radioactivity present in f2c histone constituted 70 - 95% of the total radioactivity in the sample. The radioactivity in histone samples, shown in Fig. 10A, has been corrected for this variation. A useful control in these experiments is the radioactivity incorporated into cytoplasmic proteins (recovered from the same cells used to prepare histone samples - see Methods p.18). Figure 10B

shows the radioactivity in cytoplasmic protein was quite stable in the 'chase' period, under the conditions of the incubation.

The pulse-chase experiments were carried out under the various conditions of pulse termination described above, a total of five times. In every case, the pattern of C^{14}-amino acid incorporation into f2c histone, together with the subsequent loss of counts from this moiety in the 'chase' period was essentially identical. The measurement of specific activities of the various pools (discussed earlier) in one such experiment are as follows. The specific activity of the pool

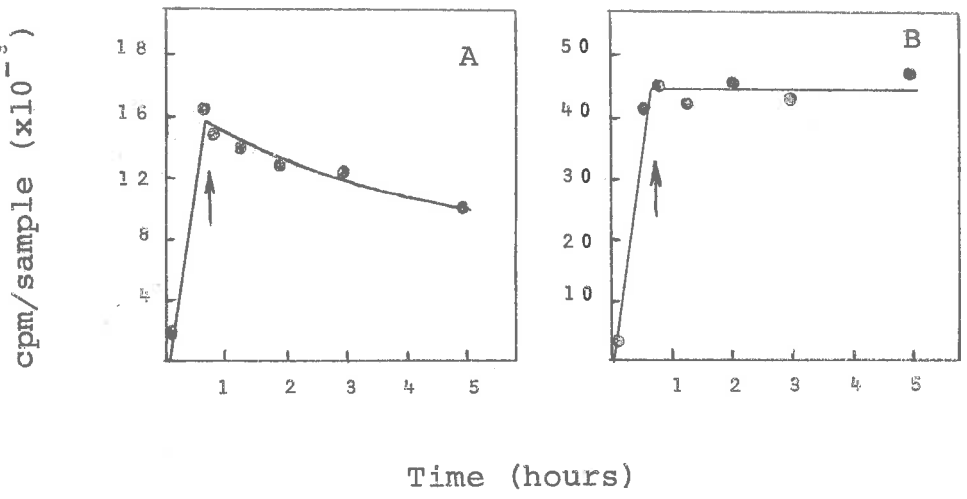


Fig. 10. Pulse-chase experiment with polychromatic erythrocytes. Polychromatic erythrocytes were incubated under the conditions described in the legend to Fig. 8, for 40 min. The arrow indicates termination of the pulse of radioactivity with either puromycin (150 µg/ml) or an excess of lysine plus arginine (0.5 mg/ml each).

A. Radioactivity in histone samples (discussed in text.

B. Radioactivity in samples of cytoplasmic protein from the same cells.

of amino acids from which protein was synthesised was 1.0×10^7 cts/min/μmole lysine plus arginine. The specific activity of f2c histone at the end of the pulse was determined by two procedures (see Methods p.22). The first gave a figure of 3.3×10^5 and the second 4.1×10^5 cts/min/μmole lysine plus arginine. These specific activities may now be used to normalise the rate of incorporation of radioactivity (16,000 cts/min/40 min) and the rate of loss of label (5,800 cts/min/270 min). When these rates are normalised to have occurred from an initial pool of radioactivity of specific activity 10^6 cts/min/μmole lysine plus arginine (an arbitrary figure), the rate of incorporation of radioactivity equals 40 cts/min/min, while the rate of loss of radioactivity becomes 67 cts/min/min and 53 cts/min/min respectively (depending on the figure used for the specific activity of f2c histone). The rate of synthesis and rate of loss of f2c histone are thus of the same order.

(g) Discussion

The central question investigated in this study was whether nett addition of histone to DNA occurred during maturation of non-dividing avian erythroid cells. In contrast to a previous study (described in Chapter 4) in which histone: DNA ratios were determined, this question was examined here by determining the metabolic stability of radioactivity appearing in histone bound to DNA in cells incubated in the presence of C^{14}-amino acids. Our prediction was that if histone is in fact

accumulating on DNA as the erythroid cells mature, radioactivity incorporated into histone should be metabolically stable in cells undergoing maturation. The polychromatic erythrocyte was the cell type used for these studies and is well along the path toward terminal differentiation to the mature erthyrocyte

In the dividing avian erythroid cells (erythroblasts), radioactivity was incorporated into all histone species (Fig. 7). Since new histones would be required for complexing with newly synthesised DNA, this result was expected and has been reported for a number of dividing tissues (for review see Stellwagen & Cole, 1969). However, our histone labelling studies with non-dividing cells (polychromatic erythrocytes) consistently showed incorporation of C^{14}-amino acids into f2c histone only (Fig. 8), even when conditions of incubation were changed (e.g., medium used, number of cells per incubation - C. Mundy, personal communication). This result, in cells that do not incorporate thymidine (Williams, 1971_1; Table I in Chapter 3) allowed the study of histone metabolism uncomplicated by the requirements for newly synthesised DNA.

Sadgopal & Kabat (1969) have previously examined histone synthesis in non-dividing avian erythroid cells. These authors did not specifically identify the f2c histone, but found radioactivity incorporated into the f3 and/or f2a1 histone regions of eluant from cation exchange columns. These particular regions, however, are also very susceptible to contamination by non-histone proteins (Stellwagen & Cole, 1968_1), rendering interpretation of the results ambiguous.

Our finding of radioactivity appearing only in f2c histone was verified by additional fractionation procedures (see Appendix 3). The further claim of Sadgopal & Kabat that low level of histone labelling in polychromatic erythrocytes implies that there is accumulation of histone on the chromatin as cells mature to erythrocytes does not take into account possible removal of histone from DNA.

When puromycin was added to polychromatic erythrocytes, after a pulse of radioactivity, label was seen to decay from the histone pool at a rate which was of the same order as the rate at which label was initially incorporated (Fig. 10). This was contrary to that predicted if histone was accumulating on chromatin during erythroid cell maturation. To check for possible artefacts introduced by the use of puromycin, the pulse of radioactivity was also terminated by dilution with an excess of unlabelled amino acids; it had been shown in independent experiments that the addition of an excess of lysine, for example, did not affect the linear incorporation (with time) of H^3-leucine into polychromatic erythrocytes. The results obtained, when the pulse of radioactivity was terminated with either puromycin or excess amino acids were identical. In all experiments, the radioactivity in cytoplasmic proteins (mainly haemoglobin) was completely stable during the entire 'chase' period. The fact that the metabolic instability of labelled histone was detected in experiments in which only puromycin was used to terminate the pulse of radioactivity indicates that histone released from chromatin is

not immediately (if at all) available for re-addition to DNA. There may be a number of reasons for this. *Firstly*, the histone lost from chromatin may be degraded (or, degradation may be required for removal of histone from chromatin). *Secondly*, it is possible that chemical modification of histone is required for its removal from chromatin as suggested by Candido & Dixon (1971). Thus chemical modification may effectively remove histone released from the pool of histone being added to chromatin. *Finally*, if the process of addition of histone to chromatin is closely linked with histone synthesis on polysomes, then histone released from chromatin may be virtually excluded from being added back to the chromatin. To date no definitive experiments have been carried out to distinguish between these alternatives.

Regarding the function of f2c histone, Johns (1969) has suggested that this histone is implicated in the inactivation of chromatin which occurs as avian erythroid cells mature. From the results presented in Chapter 4 it is apparent that there is no simple model in which f2c histone appears at a certain stage in development. The metabolic instability of radioactivity in f2c histone as found here suggests nett addition of the histone to DNA during maturation may not occur. We suggest therefore that in the mature erythrocytes, it is the final loss of the capacity for synthesis *and turnover* of histone which allows chromatin to attain its highly condensed, inactive form. (The involvement of only the f2c histone in this stabilisation, at least in terminal stages, is presumably

part of the highly specialised nature of avian erythrocytes.) We suggest that it is the dynamic state of the histones in earlier cells which relates to a greater potential of the chromatin for RNA synthesis; this allows for the f2c histone to be present in the earliest cells of the erythroid series where the machinery for its synthesis and removal from DNA would also be present. This dynamic situation could then allow finer control elements to bear upon specific transcription. Similar, more general, models have been considered by others (Spalding, Kajiwara & Mueller,1966; Stellwagen & Cole, 1969; Gurley & Hardin, 1970).

The metabolism of f2c histone in non-dividing red blood cells may be a manifestation of a more general phenomenon for other histones [in particular the f1 histones, which exhibit some tissue specificity and specific response to hormones (for review see Stellwagen & Cole, 1969; Hohmann & Cole, 1971) and have been suggested to be closely related to f2c histone (Greenaway & Murray, 1971)]. The important feature relating histones to the overall potential of chromatin for transcription is not the static level of histones, but rather the dynamic state of their synthesis and release from DNA.

CHAPTER 6

A COMPARISON OF WASHED NUCLEI AND CHROMATIN AS SOURCES OF HISTONES FROM AVIAN ERYTHROID CELLS

(a) Introduction.

The overall aim of this thesis has been to correlate differences in the chromosomal components of chromatins isolated from purified cell populations (of one cell line) with the biochemical activity of the cells from which they were prepared. While it is desirable that such studies be carried out on purified chromatin from the respective cell populations, for the studies on the distribution (Chapter 4) and the metabolism (Chapter 5) of avian erythroid histones, washed nuclei were used as a source of histones. This latter procedure was adopted to overcome two technical problems associated with these studies. (I) It allowed rapid isolation of histones from the time of cell lysis - thus minimising the possibility of histone degradation. Hydrolytic enzymes are particularly active in erythroblasts (Harlow & Wells, 1971; Chapter 7, this thesis). (2) It allowed the quantitative isolation of histones from small quantities of cells as required for the study of histone metabolism (Chapter 5).

Chromatin is normally prepared from washed nuclei by shearing the nuclei and removing the membrane on a sucrose density gradient (see Methods, p.24). In essence the work reported in this chapter examines whether washed nuclei rather than purified chromatin can be used as a valid source of histones. In particular, it is shown that histones are quantitatively localised in chromatin and that the presence of membrane (particularly nuclear membrane) does not introduce artefacts into histone preparations. It is also shown that this situation

does not necessarily apply to other nuclear proteins. Membrane material from different cell types contains variable amounts of protein which may contribute to the so-called non-histone complement of chromatin.

(b) Cells used.

Due to the instability of the nuclear material from erythroblasts (see Chapter 7), the studies in this chapter were confined to polychromatic and mature erythrocytes. In addition, cell populations used were often not purified on BSA gradients because of the large scale of the experiments (particularly for the analysis of the membrane from washed nuclei). Cell populations used were, however, checked by staining with Leishman's stain and the micro-density gradient technique of Williams, 1971_2 (see also Appendix 1). For polychromatic erythrocyte cell populations in particular there was no more than 10 - 15% contamination by mature erythrocytes. In this respect the histone marker established for purified cell populations, namely, the reduced amount of f1 histone-peak 4 in mature erythrocytes has proven useful in confirming the nature of the cell populations analysed (Table 5, Chapter 4, p.39).

(c) Electron microscopic examination of nuclei.

Washing isolated nuclei (see Methods, p.19) in preparation for the isolation of chromatin, removes cytoplasmic material and causes the clumping of the nuclear material as

judged by electron microscopic examination (Fig. 11). Additional washing to that specified in the Methods does not alter the protein:DNA ratio of the chromatin isolated from the respective nuclei, from that shown in Table 6 (discussed below). The clumping of the nuclear material occurs only during the last wash of the nuclei with 0.147 M NaCl and is analogous to a phenomenon described for isolated chromatin, namely, extensive aggregation in 0.15 M NaCl (Smart & Bonner, 1971). Membranous material remains associated with the washed nuclei, as is clearly seen from the electron micrographs (Fig. 11, E_2 and PE_2).

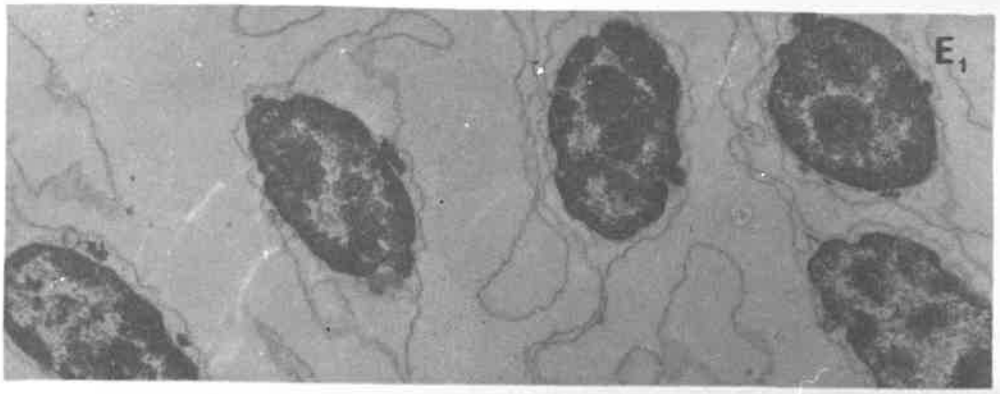
(d) Analyses of membrane and chromatin fractions from washed nuclei.

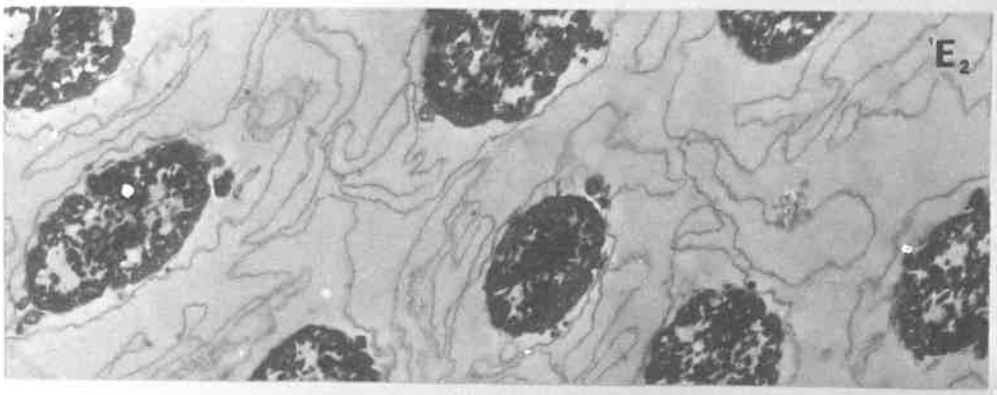
Fractionation of washed nuclei as described for the preparation of chromatin (see Methods p.24) separates the bulk of the membranous material (mainly nuclear in origin - electron micrograph, Fig. 12) from chromatin. Some membrane remains attached to the chromatin, however, the amount has not been quantitated.

To check whether the presence of the excess of membranous material present in washed nuclei could introduce artefacts into analyses of histones, the latter were isolated from chromatin and washed nuclei of the same cell populations. In addition the small amounts of histone recoverable from membrane preparations were also analysed (for quantitative yield of histone see Table 6, discussed below). Analyses of histones from chromatin and washed nuclei (from either

Fig. 11. *Electron microscopic examination of avian erythroid nuclei.*

Electron microscopic examinations of nuclei were kindly carried out by Miss P.Y. Dyer, using a Siemens Elmiskop I. Nuclei were fixed for 30 min. in 2% glutaraldehyde, 1% $CaCl_2$, 0.1M cacodylate buffer, pH 7.2, followed by washing with 0.18M sucrose, 0.1M cacodylate buffer, pH 7.2. Finally, the nuclei were treated with 1% osmic acid, 1% $CaCl_2$, for 30 min. Dehydration was carried out using acetone, starting at 70%, in 10% steps. Nuclei were then embedded in araldite, sections cut (LKB microtome, glass knife) and placed on carbon coated grids. Sections were positively stained with 1% uranyl acetate (15 min.) followed by lead citrate (4mM, pH 12). Examination of sections was at an aperture of 50 mμ, at 80 Kv.


E1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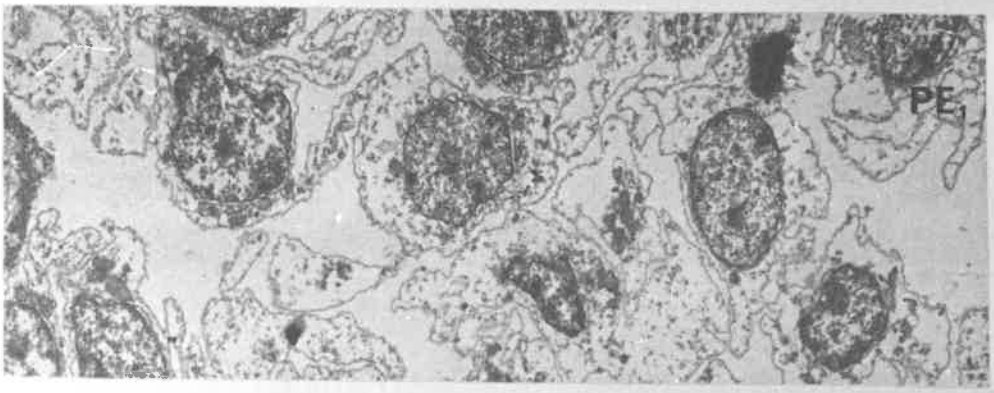
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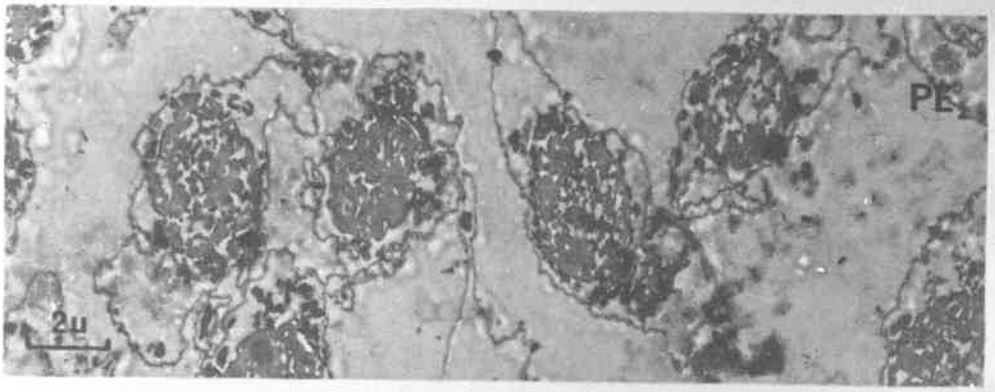
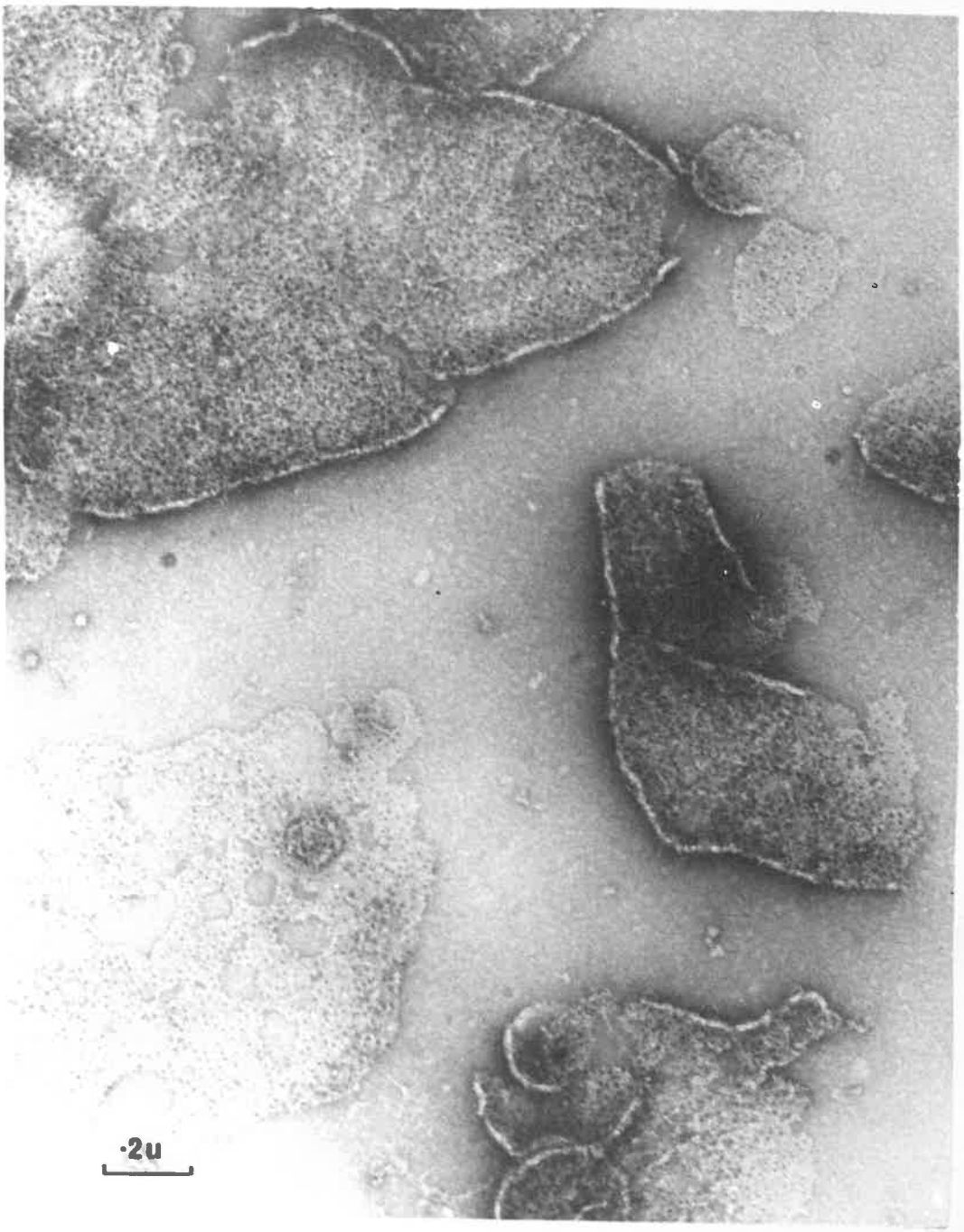

PE2
2u

Fig. 12 Electron micrograph of membranous material separated from chromatin in the fractionation of washed nuclei. One drop of membrane material, directly from the sucrose density gradient fractionation of washed nuclei, was placed on a carbon coated grid for electron microscopy. Negative staining was carried out by the addition of a drop of 2% uranyl acetate. Observation was carried by Miss P. Dyer, see legend to Fig. 11 for details.


·2u

polychromatic or mature erythrocytes) were identical (Fig. 13). This indicates that within the limits of resolution used, the additional amount of membrane present in washed nuclei does not affect the extraction of histones.

Quantitative analyses carried out on chromatin and membrane, nuclear fractions are summarised in Table 6. For both polychromatic and mature erythrocytes the membrane fraction contained little DNA or histone relative to the total protein content - virtually all the DNA and histone moved down through 1.7M sucrose with the fraction identified as chromatin. However, this does not apply to other nuclear proteins. For polychromatic erythrocyte nuclei, 29.8% of the total protein remained in the

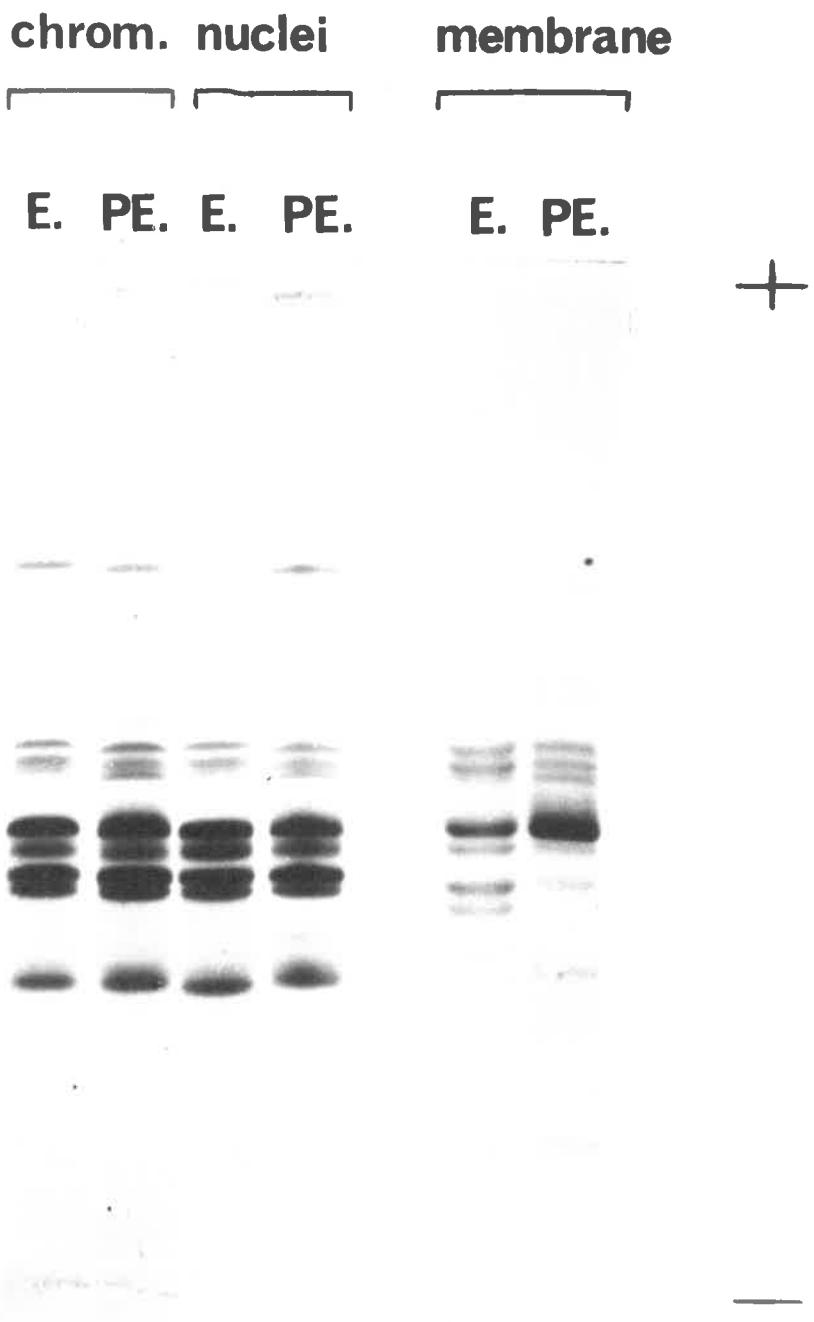
TABLE 6. ANALYSIS OF AVIAN ERYTHROID NUCLEI

	chromatin		yield of membrane*		
	protein: DNA (9)	% protein as histone (5)	protein (3)	DNA (3)	histone (3)
Polychromatic erythrocyte	1.56±0.08	72.1±1.52	29.8±1.9	2.4±0.6	1.0±0.5
Erythrocyte	1.48±0.10	78.9±3.49	8.4±1.4	1.1±0.6	0.6±0.5

*Figures show the recovery of the respective component as a % of the amount of that component in the whole washed nuclear preparation.

All figures are shown ± S.E. Numbers in parentheses indicate the number of separate experiments in which estimations were made.

Fig. 13. Avian erythroid histones from chromatin, washed nuclei and membrane material prepared from polychromatic erythrocytes (PE.) and mature erythrocytes (E.). Chromatin and membrane material were prepared from washed nuclei as described in the Methods (p.24). Histones were isolated as described in Methods (p.19). 30 - 40 µg of protein were loaded per polyacrylamide gel and electrophoresed for 4 hr at 2 mA/gel. For identification of histone bands see Chapter 4.


chrom.
nuclei
membrane
E.
PE.
E.
PE.
E.
PE.
+
—

membranous fraction. In contrast to this, for mature erythrocyte nuclei only 8.4% of the total protein remained in the membranous fraction - it is unlikely that in this case more membrane moved down with the chromatin fraction since protein: DNA ratios of polychromatic and mature erythrocyte chromatins were the same. The quantitative analyses verify that chromatin is the only source of histone since in chromatin, histone comprises approximately 75% of the chromatin protein whereas in the membrane fraction it comprises only 2-3%. (Latter figure derived from data used to prepare Table 6.) Furthermore, the histone present in the membrane fraction can be accounted for by chromatin bound to the membrane - there is a small amount of DNA in the membrane fraction. The presence of DNA in nuclear membrane preparations from mature avian erythrocytes has been reported (Zentgraf *et al.*, 1971).

It is interesting to note that not all the histone species are present in the histone preparations from membranes - in particular f2a2, f2b and f2a1 are low or not present at all (Fig. 13). Further investigation of this feature of histones isolated from membranes indicated this may in fact be due to an artefact introduced as a result of the relatively large amount of membrane material present. Addition of chromatin, containing a known amount of histone, to membrane preparations prior to histone isolation indicated only a 50% recovery of histones. Histones f2a2, f2b and f2a1 appeared to be preferentially lost. The mechanism of this phenomenon is not clear and does not appear to be an important factor in comparing

the histones isolated from chromatin and washed nuclei.

(e) Discussion.

The important finding in this chapter is that washed nuclei provide a valid source of chromatin for the isolation of histones. The work provides an experimental basis for assuming that the histones characterised in Chapters 4 and 5 are actually bound to DNA as part of the chromatin complex. This fact, which is usually assumed in publications related to histones, allows changes in histones to be correlated with changes in chromatin within the avian erythroid cell series.

Although the study of components other than histones was not extensive, the quantitative data in Table 6 does reveal an important difference between polychromatic and mature erythrocytes regarding the so-called non-histone component of chromatin. The yield of membrane protein (non-histone in character) from polychromatic erythrocyte nuclei was almost four times that from mature erythrocyte nuclei. It is thus clear that unless care is taken to establish the origin of non-histone components of chromatin, in particular, ambiguous correlations between these components and cell activity can arise. Thus several authors have correlated chromatin protein:DNA ratios with the level of cellular gene activity (Dingman & Sporn, 1964; Gershey & Kleinsmith, 1969). While histone:DNA ratios remain unaltered, chromatin protein:DNA ratios have been suggested to increase with increasing gene activity. This, however, was not found

to be the case in our investigation. Protein:DNA ratios of polychromatic and mature erythrocyte chromatins were not significantly different (Table 6). In view of the greater yield of membrane protein from polychromatic erythrocyte nuclei, however, it seems possible that previous correlations may have been due to membrane attached to the 'chromatin' since the membranous material *per se* was not removed.

CHAPTER 7

CHARACTERISATION OF CHROMATIN FROM AVIAN ERYTHROID CELLS

(a) Introduction.

To date little information has been documented regarding the chromatin complex from cells within the avian erythroid series. While mature erythrocyte chromatin has been a favored subject for physical studies on a DNA-protein complex, no such study has been carried out to compare its properties to chromatin from earlier cells. The relevance of such a study to the work in this thesis is that it examines the properties of isolated chromatin which alter on maturation of avian erythroid cells. These changes may then be correlated with the changes in the histone component, in particular, discussed in the previous chapters.

The parameters of chromatin examined included sucrose density gradient analyses, ultraviolet absorption spectra, melting profiles, dye binding properties, precipitability with Mg^{++} and template activities with added *E. coli* RNA polymerase. The studies were mostly limited to chromatin from polychromatic and mature erythrocytes. The instability of erythroblast chromatin, demonstrated in this chapter, has made its routine preparation difficult; inhibition of hydrolytic enzymes associated with erythyroblast chromatin (and presumably responsible for its instability) has been the subject of additional research (Harlow & Wells, 1971).

The important fact established in this chapter is that chromatin isolated from polychromatic and mature erythrocytes can be distinguished at least on the basis of its template activity with added RNA polymerase. The relevance of

these results to utilising the avian erythroid cell series as a model system for studying changes in chromatin are discussed.

(b) Sucrose density gradient analyses of 'chromatin'.

The 'chromatin' examined here was prepared as described

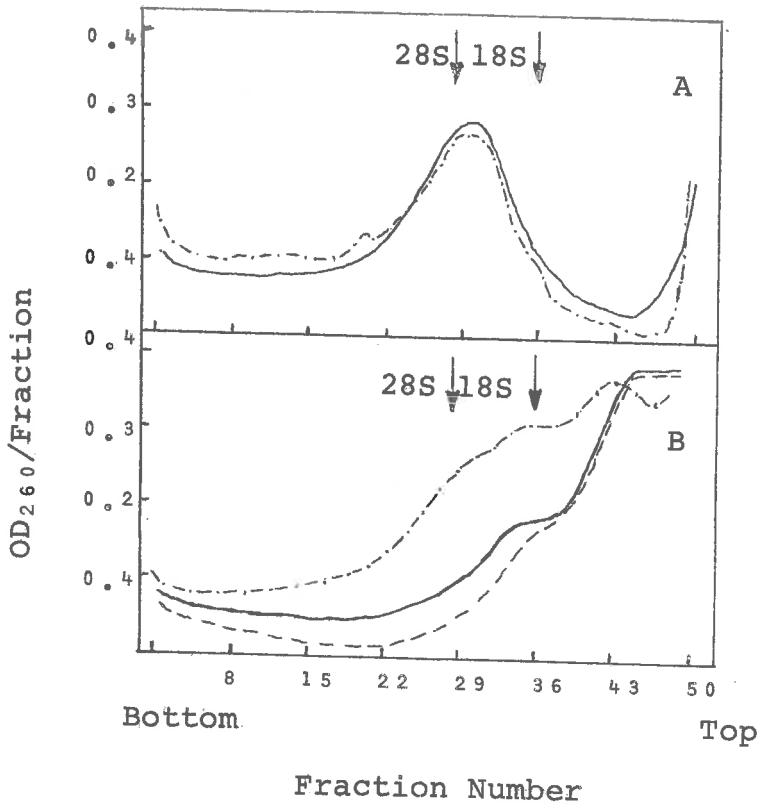


Fig. 14. *Sucrose density gradient analyses of sheared nuclei.*

Nuclear complexes as isolated in 0.2mM EDTA, pH 7.0, were loaded on 5 - 20% sucrose gradients and centrifuged for 18 hr at 22,000 rpm in a SW 25 rotor. Pea ribosome markers were kindly supplied by Mr. D.R. Hewish. 0.7 ml fractions were collected.

A. Polychromatic erythrocyte nuclear complex (mature erythrocyte material was identical). Symbols -.-.-, unincubated; ——— , incubated for 4 hr at 37°.

B. Erythroblast nuclear complex. Symbols: -.-.-, unincubated; ——— incubated for 1 hr; -----, incubated for 2 hr at 37°.

by Dingman & Sporn, 1964; the method of preparation involved shearing washed nuclei to solubilise a nuclear DNA complex which was defined as chromatin. This differs from the routine preparation of chromatin described on p.24 (Methods) in that the nuclear membrane *per se* is not removed. For polychromatic and mature erythrocytes, the material solubilised showed a sedimentation coefficient of 20-30 S (as expected from the work of Dingman & Sporn, 1964) which was stable to incubation at 37° (Fig. 14A). In contrast to this the 'chromatin' from erythroblasts did not sediment as a single peak and was unstable to incubation at 37° (Fig. 14B). The levels of hydrolytic enzymes such as DNA'ase II and proteases have been found to be much higher in washed nuclear preparations from erythroblasts than from polychromatic or mature erythrocytes (Harlow & Wells, 1971). Any one of these enzymes (or combination) may be responsible for the observed instability of erythroblast nuclear material.

(c) Physical characterisation of chromatins from polychromatic and mature erythrocytes.

(i) Ultraviolet absorption spectra. Polychromatic and mature erythrocyte chromatins (prepared as described in Methods, p.24) were found to have identical ultraviolet absorption spectra (Fig. 15, polychromatic erythrocyte chromatin spectrum is shown). The spectra demonstrated the features typical of chromatin preparations (Bonner *et al.*, 1968_2), namely, a trough at 240 nm, a peak at 258 nm and

virtually no absorbance at 320 nm.

(ii) Melting profiles. The DNA in both polychromatic and mature erythrocyte chromatins was more stable to thermal denaturation than deproteinised DNA (Fig. 16). Both chromatins showed a biphasic melting profile which is often observed for DNA complexed ionically with other molecules (Jordan*, personal communication). The differences between the melting profiles shown in Fig. 16 (between polychromatic and mature erythrocyte chromatins) could not be established

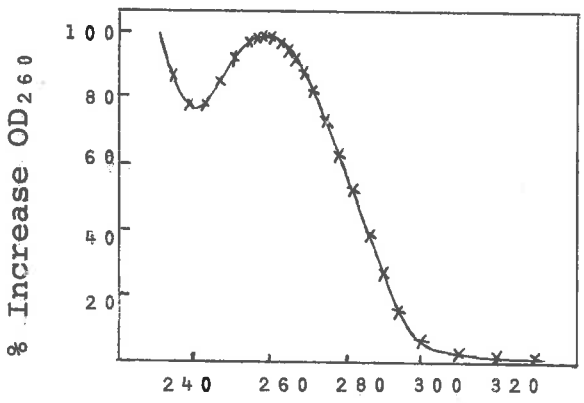


Fig. 15. Wavelength (nm)

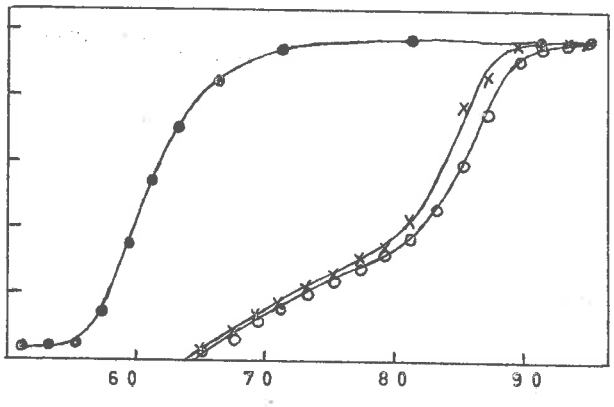


Fig. 16. Temperature (C°)

Fig. 15. *Ultraviolet absorption spectrum of polychromatic erythrocyte chromatin.*
Chromatin analysed was dialysed against 0.2mM EDTA, pH 7.0.

Fig. 16. *Melting profiles of chromatins and DNA.*
All preparations were dialysed against 0.2 mM EDTA, pH 7.0. For method of determination of melting profiles see Methods, p.25. Symbols: ● —— ●, avian DNA; x —— x, polychromatic erythrocyte chromatin; o —— o, mature erythrocyte chromatin.

*Professor D.O. Jordan, Department of Physical and Inorganic Chemistry, Adelaide University, South Australia.

as being significant.

(iii) Dye binding properties. The ability of DNA to bind ethidium bromide (Olins, 1969) was used in an attempt to localise differences between polychroamtic and mature erythrocyte chromatins. While binding curves could be readily (and reproducibly) established for deproteinised DNA (Fig. 17), this was not the case for chromatin preparations. Precipitation of chromatin in the presence of even a small amount of dye, prevented repeatable dye binding curves from being obtained. This problem of precipitation could not be overcome by changing the ionic environment of the chromatin (0.01M Tris-HCl, pH 8.0 or 0.2mM EDTA, pH 7.0) or extensive shearing of the chromatin.

(iv) Precipitation by Mg^{++}. Cations such as Mg^{++} have been shown to precipitate chromatin from solution (Fredericq, 1971; Jackson, Earnhardt & Chalkley, 1968). While the mechanism of this precipitation is not clear, it seems to be related to the proteins attached to DNA in chromatin, since deproteined DNA is not precipitated under similar conditions. As shown in Fig. 18 the chromatin preparations from polychromatic and mature erythrocytes could not be distinguished by their precipitation behaviour. It is interesting to note that both chromatins are almost completely aggregated at 1.5mM Mg^{++} (i.e., removed from solution by centrifugation at 1,800 g for 20 min.). Since the template activities of the

respective chromatins (described below) are carried out at 3 mM Mg^{++}, under these conditions both chromatins are in the aggregated form.

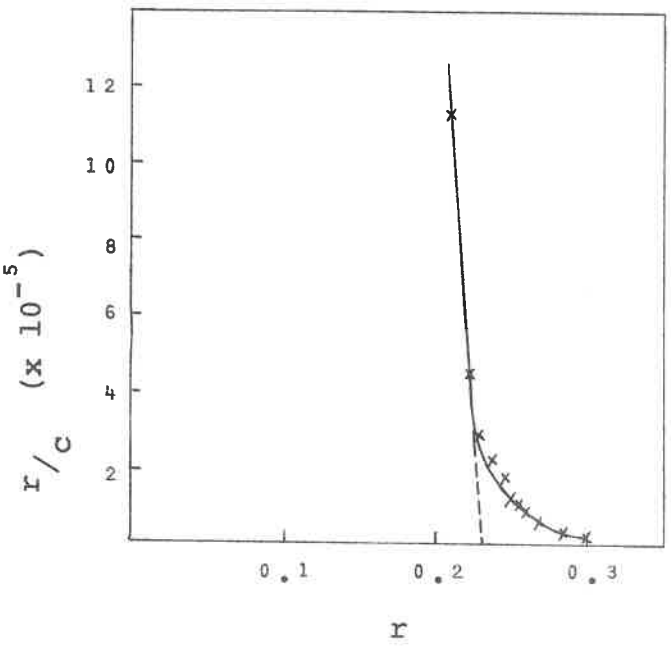


Fig. 17. *Dye binding curve for deproteinised DNA.*

For explanation of symbols r/c and r (and also their calculation), see Appendix 4. 10 ml of a solution of DNA (10^{-4} M in DNA phosphate, in 0.01M Tris-HCl, pH 8.0) in a 4 cm cuvette was titrated with 0.01 or 0.02 ml aliquots of ethidium bromide (Boots, 1.13 x 10^{-3}M). OD_{460} changes were monitored in a Zeiss PMQ II spectrophotometer. The value of 1/n (number of nucleotides per dye binding site, obtained by extrapolation as indicated by dotted line - see also Appendix 4) was 4.4 which agrees well with the value of 4.1 for calf thymus DNA (Olins, 1969).

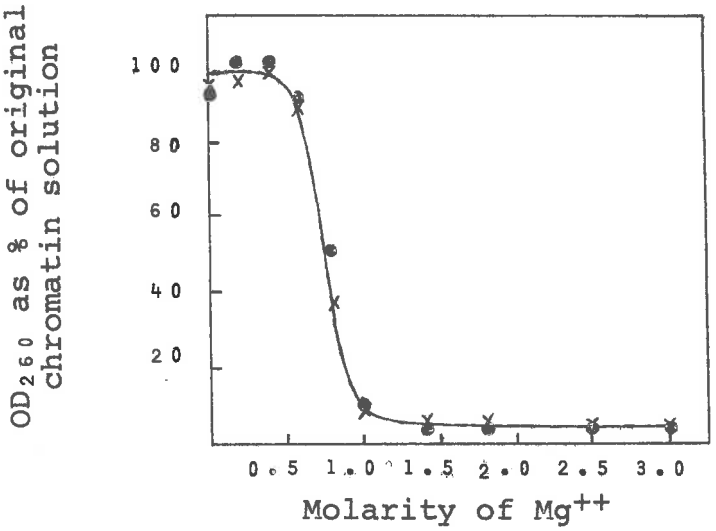


Fig. 18. *Mg^{++} precipitation of chromatin.*

2 ml quantities of chromatin (dialysed against 0.2mM EDTA, pH 7.0) were made to the respective molarities of Mg^{++} by the addition of 0.1 ml quantities of a more concentrated $MgSO_4$ solution. Vigorous stirring during the addition of Mg^{++} was required to prevent local concentrations of the cation. After standing for 20 minutes, aggregated material was removed by centrifugation at 1,800 g, 20 min. The OD_{260} of the supernatant was expressed as a % of the absorption of the *original* chromatin solution (adjusted to approximately 1.0, prior to the addition of Mg^{++}).

(d) Template activities of polychromatic and mature erythrocyte chromatins.

The *template activity* of a sample of chromatin may be defined as the number of mμ-moles of nucleotide monophosphate incorporated into acid insoluble material/10 min./μg chromatin-DNA, under defined conditions of temperature of incubation and amount of RNA polymerase added. In the results presented, incorporations were carried out at 37° and were linear to 10 min. The absolute amount of RNA polymerase added need not be defined (provided the same amount is added to chromatins being compared) since the binding of the polymerase to DNA is essentially irreversible while RNA synthesis is occurring - a detailed discussion on the use of *E. coli* RNA polymerase for measuring the template activities of chromatins is given by Shih & Bonner, 1970).

Preliminary experiments (Table 7) verified that the polymerase reaction was dependent on added DNA, inhibited by actinomycin-D and produced a product that was sensitive to RNA'ase. Chromatin preparations without added polymerase did not incorporate labelled nucleotide triphosphates into acid insoluble material.

Fig. 19 compares the template activities of polychromatic and mature erythrocyte chromatins. The fact that polychromatic erythrocyte chromatin was a better template than mature erythrocyte chromatin was shown in 4 separate experiments, using either [α-P^{32}]UTP or [α-P^{32}]ATP as the labelled substrate for the polymerase reaction. The DNA's

isolated from the respective chromatins used in the 'template activity' experiments, were virtually indistinguishable in their ability to provide a template for RNA polymerase. The results, shown in Fig. 19, thus argue against differential affects on the DNA occurring during the isolation of chromatin which could account for the observed difference in template activity of the chromatins. As expected, deproteinised DNA provided a much better template for added RNA polymerase. Another control carried out was the assay of RNA'ase activity in the two chromatins. Dati & Maurer (1971) have suggested that differences in template activity such as described above may be accounted for by differences in RNA'ase activity of the respective chromatin preparations.

TABLE 7. *CHARACTERISTICS OF RNA POLYMERASE REACTION.*

Conditions	[P^{32}]UMP incorporated (relative to standard assay)
Standard assay	1.00
minus DNA	0.00
plus actinomycin-D (5 µg/ml)	0.08
Standard assay followed by ribonuclease (0.4 µg/ml) digestion for 30 min. at 37°	0.00
Standard assay minus polymerase and DNA, with polychromatic erythrocyte chromatin added (40 µg DNA)	0.00

Incorporation of [P^{32}]UMP from [α-P^{32}]UTP into acid insoluble material is shown as a fraction of the incorporation of the standard assay. The standard assay was as described in Methods, p.26, with 30 µg calf thymus DNA; the spec. act. of [α-P^{32}]UTP was 3.4×10^7 cpm)µ-mole.

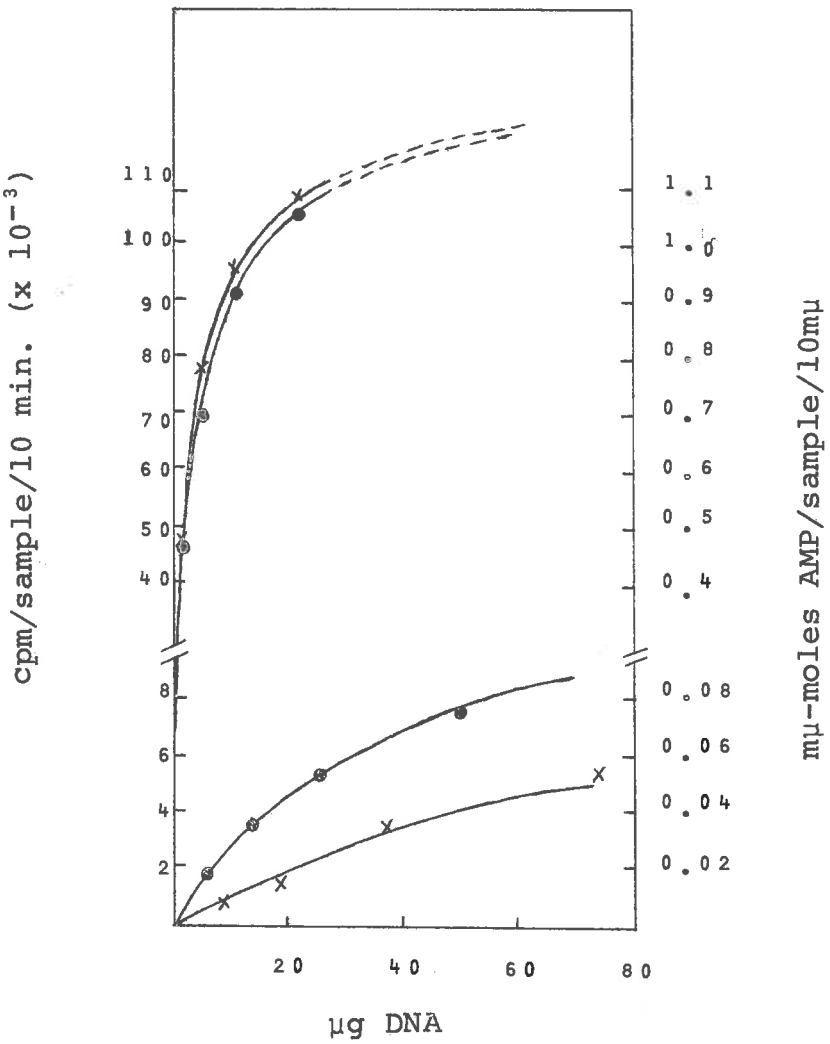


Fig. 19. *Template activities of chromatin and DNA.*

Respective chromatins and DNA preparations were dialysed against 0.01M Tris-HCl, pH 8.0. The reaction with RNA polymerase was carried out as described in the Methods, p.26 with [α-P^{32}]ATP, spec. act., 1 x 10^8 cpm/μmole, as labelled substrate. Symbols: x —— x, mature erythrocyte, chromatin (lower scale) or deproteinised DNA (upper scale); ● —— ●, polychromatic erythrocyte, chromatin (lower scale) or deproteinised DNA (upper scale).

Using the assay of these authors (Dati & Maurer), mature erythrocyte chromatin could not be shown to be more active in RNA'ase activity than polychromatic erythrocyte chromatin preparations (Fig. 20). This observation discounts RNA'ase levels as an explanation of the differences in template activities

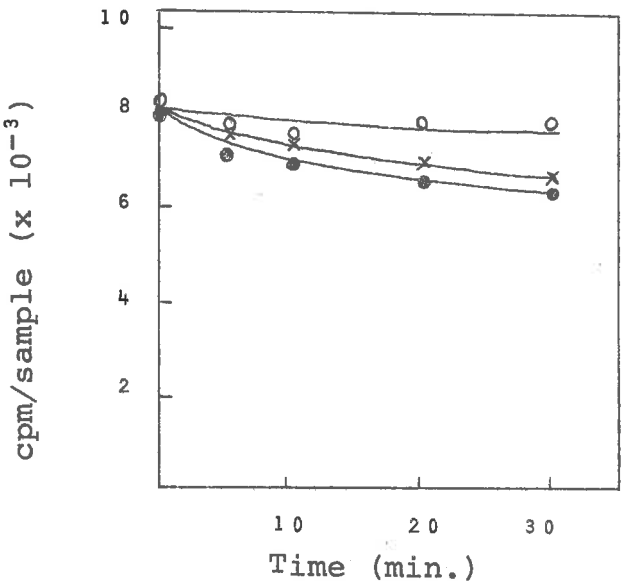


Fig. 20. *RNA'ase activities of chromatin.*
The assay used was that described by Dati & Maurer (1971). The RNA polymerase reaction was carried out as described in Methods, p.26, using calf thymus DNA (30 µg) as a template. After 10 min. of incubation at 37° the reaction was effectively terminated by the addition of ATP (0.1 m-moles/ml - [α-P^{32}]ATP, spec. act. 1 x 10^8 cpm/µ-mole, was used as the labelled substrate). 0.2 ml quantities of the reaction were then incubated with 0.05 ml quantities of the respective chromatin preparation (55 µg chromatin-DNA), or 0.05 ml Tris-HCl, pH 8.0 (control). Loss of radioactivity from acid insoluble material was monitored for the assay. Symbols: o —— o, Control; x —— x, mature erythrocyte chromatin; ● —— ●, polychromatic erythrocyte chromatin.

observed. Additional assays using the RNA'ase assay described by Crestfield, Smith & Allen (1955) (detecting release of 5% perchloric acid soluble, OD_{260} absorbing, material from a substrate of yeast ribosomal RNA) failed to detect RNA'ase activity in chromatin preparations. More detailed analyses of the RNA'ase activity in chromatin preparations have been carried out by Harlow (1971, personal communication) and are in general agreement with the above results.

(e) Discussion.

During the maturation of polychromatic erythrocytes to mature erythrocytes, heterochromatinisation of the nuclear material occurs. One important question which has remained unanswered to date is whether this heterochromatinisation occurs as a result of changes in the actual chromatin complex or whether it is a response of the complex to a change in (for example) the ionic content of the cell. The evidence relating to the template activities of chromatin from polychromatic and mature erythrocytes indicates that a change occurs in the physical state of the chromatin. The fact that polychromatic erythrocyte chromatin is a better template for *E. coli* RNA polymerase than mature erythrocyte chromatin argues against the *in vivo* change being merely a response to a change in ionic environment. This is elaborated on further in the next chapter (Final Discussion, p. 85). The template activity of the chromatins was the only parameter examined which could distinguish between the polychromatic and mature

erythrocyte chromatins.

Some preliminary experiments have been undertaken to determine why mature erythrocyte chromatin was not as good a template for RNA polymerase as polychromatic erythrocyte chromatin. The question posed was whether inactivation occurred to reduce the number of initiation sites for the polymerase or reduced the extent to which a given number of polymerase molecules could travel along and transcribe chromatin-DNA. An experiment of the type described in Fig. 19 but with [γ-P^{32}]ATP (to detect only initiation of RNA chains, Maitra, 1966) as the only source of labelled substrate instead of [α-P^{32}] nucleotide triphosphate was carried out. The results indicated that for polychromatic erythrocyte chromatin there are more sites at which the polymerase can initiate RNA synthesis than in mature erythrocyte chromatin. If this observation is correct it may explain the difference in template activities between the chromatins. Furthermore, if the length of the RNA chains synthesised from both types of chromatin (determined as described by Koslov & Georgiev, 1971) are the *same*, it implies the presence of specific elements reducing only the *number* of initiation sites for RNA polymerase in mature erythrocyte chromatin. Alternatively, if the RNA chains synthesised from mature erythrocyte chromatin are shorter than those from polychromatic erythrocyte chromatin this would be consistent with a postulate of maturation of erythroid cells involving structural changes which act at a coarse level of control (see also Literature Survey, p.13).

Regarding specific elements of control, the template activity parameter of chromatin would be a useful one to examine using erythroblast chromatin as a template. In this system, which may require the avian erythroid RNA polymerase for correct transcription, it may be possible to obtain the formation of globin messenger RNA. Erythroblasts still synthesise globin messenger RNA and an *in vitro*, cell-free system would provide a useful tool in examining the controls involved in forming this messenger RNA.

CHAPTER 8

FINAL DISCUSSION

(a) Introduction.

In this chapter an attempt is made to orientate the work presented in this thesis within the general framework of current theories on control of transcription from DNA and the research carried out on this topic. The discussion is limited to changes in DNA as a template for RNA polymerase - as discussed in the Literature Survey (p.3), other modes of control are possible. The following aspects are discussed here: approaches to the study of DNA transcription controls - a summary; criticism of previous correlative studies on isolated chromatin; changes in the chromatin of avian erythroid cells; models for the control of DNA transcription and the avian erythroid cell series; future studies.

(b) Approaches to the study of DNA transcription controls.

(i) Genetic analyses.

Changes in bacteria such as induction and repression of certain enzymes and 'phage infection of bacteria (particularly infection by λ 'phage) have been found to be very amenable to genetic studies. It is in fact the predictions from genetic analyses of such systems which has led to the elucidation of transcriptional controls in prokaryotic systems by biochemical means (discussed below, see also Literature Survey, p.3). The lack of a suitable eukaryotic system which can be readily analysed both genetically and biochemically (as well as the greater complexity of eukaryotes), has limited the understanding of control of transcriptional events in such systems.

While Drosophila has been well characterised genetically (for review see Schneiderman & Bryant, 1971; Ashburner, 1970), biochemical analyses of specific cells or tissues has been very limited (Pelling, 1970; Daneholt *et al.*, 1970).

The classical example of the power of genetics in predicting the nature of cellular controls is shown in the elucidation of control of the *lac* enzymes of *E. coli*. Two individual control systems (a specific negative control and a more general positive control) have been shown to be operative.

Negative control. For the inducible *lac* enzymes of *E. coli*, Jacob & Monod (1961) have described a class of pleiotropic mutants, i^-, which were constitutive for all the *lac* enzymes. The wild type, i^+ phenotype (*lac* enzymes normally inducible), was found to be *trans*-dominant to the i^- phenotype. This class of mutant together with i^s type mutants (no synthesis of *lac* enzymes, even when inducer is present), where the i^s phenotype was *trans*-dominant to i^+, developed the concept of a diffusable cytoplasmic component which affected the flow of information from *lac* genes. The further characterisation of *cis*-acting o^c mutations (constitutive for *lac* enzymes) with the o^c phenotype being dominant to i^s phenotypes, argued for the simplest interpretation of the available data, i.e., that the i gene product is a repressor molecule which binds to an operator region (o) to prevent the expression of the *lac* genes. The inducer was then postulated

to act by antagonising the action of the repressor, thus allowing expression of the *lac* genes. Biochemical characterisation of bacterial messenger RNA later showed it to be unstable, suggesting the repressor molecule functioned by binding to DNA to control transcription. Subsequently, i^q mutants (synthesising an excessive amount of repressor) facilitated the purification of the repressor molecule (Müller-Hill, Crapo & Gilbert, 1968). The repressor was shown to be a protein which bound to DNA in a highly specific manner; this binding reaction was inhibited by the presence of inducer, as predicted from the model proposed by Jacob & Monod. Detailed analyses of the binding reaction of repressor to DNA (Riggs, Bourgeois & Cohn, 1971) have argued for the repressor recognising double stranded DNA *per se*.

Positive control. Pastan & Perlman (1970) have reviewed the biochemical evidence which correlated the presence of cyclic AMP with the ability of *E. coli* to synthesise many of its inducible enzymes (when inducer is present). While these correlations suggested cyclic AMP was required for the expression of inducible enzymes (acting as a general positive control system), it was only after genetic studies that its mode of action became clear. Pleiotropic mutants, unable to induce a number of normally inducible enzymes (including the *lac* enzymes), were characterised (Schwartz & Beckwith, 1970). One class of these mutants could be corrected by the additon of cyclic AMP (i.e., deficient in cyclic AMP synthesis). Other mutants, however, could not be corrected

and were shown to be deficient in some factor required for cyclic AMP action (Zubay, Schwartz & Beckwith, 1970; Emmer, de Crombrugghe, Pastan & Perlman, 1970). These studies characterised the CAP protein, which was postulated to bind cyclic AMP to produce a moiety which was required for the expression of inducible enzymes such as *lac* enzymes. This was confirmed in the elegant studies of Chen *et al.* (1971) in which a cell-free system synthesising β-galactosidase from *lac* DNA, was constructed; the system required CAP protein and cyclic AMP for the complete *in vivo* characteristics of control of β-galactosidase synthesis by repressor and inducer.

(ii) Biochemical analyses of chromatin.

In eukaryotic systems readily amenable to biochemical analyses two distinct, although closely related, approaches have been used to elaborate on the transcriptional controls operating in such systems. The first approach relates to studies on a single source of chromatin (e.g., calf thymus). Here dissociation and reassociation of chromosomal components is used to search for components which have properties with the *potential* for mediating changes in the template properties of chromatin, for an RNA polymerase. The second approach correlates differences between two or more chromatin preparations with their *in vivo* properties in the respective cells from which they were isolated. It is the comparison of chromatin preparations which is important here and can identify components which may be important in the *in vivo* control of DNA

transcription. A combination of the two approaches can conceivably localise biologically important interactions affecting the template properties of chromatin.

The difficulty in these approaches lies in the complexity of eukaryotic chromatin. Conditions for the isolation of chromatin (or selective removal of certain components) may introduce contaminants, or remove components which are present *in vivo*, or affect the arrangement and mode of binding of components (Olins & Olins, 1971; Clarke & Felsenfield, 1971; Hoare & Johns, 1971). Related to this is the problem of an adequate assay for the template properties of the chromatin prepared. To date only the reaction of chromatin with bacterial RNA polymerase to produce RNA has been used (see Literature Survey, p.12). Gene products such as ribosomal RNA or specific messenger RNA (which may be translated into identifiable protein products) would provide useful markers.

Despite obvious shortcomings, the above approach (in particular the second approach discussed) was used in this thesis to study the changes in the chromatin of avian erythroid cells.

(c) Criticism of previous correlative studies on isolated chromatins.

Comparative studies on chromatin isolated from different tissues (or a given tissue subjected to different stimuli) have indicated; (1) that tissue specific RNA products are transcribed from the respective chromatins by *E. coli* RNA

polymerase (Literature Survey, p.12); (2) a quantitative and qualitative tissue specificity of histones (Panyim & Chalkley, 1969); (3) a quantitative and qualitative tissue specificity of non-histones (Loeb & Creuzet, 1969; MacGillivray, Carroll & Paul, 1971). Interpretation of these studies in terms of correlating differences in histones and non-histones with the different RNA's formed from the chromatin (or with the fact that the chromatin was isolated from a tissue actively synthesising DNA and/or RNA) have been attempted but are somewhat ambiguous. The reasons are as follows.

Firstly, tissues generally contain a mixture of different cell types, when a given tissue is subjected to a stimulus, interpretation of changes in chromosomal components is complicated by the fact that the proportions of the various cells present may change (Stellwagen & Cole, 1968_2). Thus the over-all change in chromosomal components may merely reflect the fact that the different cell types have different chromosomal components and that one of the cell types predominates under the new conditions. The change observed does not necessarily relate *directly* to a change in the chromatin *in vivo*.

Secondly, when chromatin from a certain tissue is prepared, it is isolated from cells which are fully differentiated. The cells, therefore, may contain proteins not present in the cells of other tissues. Cytoplasmic and nuclear membrane proteins are particularly important here (the nuclear membrane proteins are mentioned since commitment to a given cell line may involve changes in the nuclear membrane relating to different species of RNA being allowed to pass out of the

nucleus - see Literature Survey, p.2). Since both cytoplasmic and nuclear membrane proteins must be considered important sources of 'contamination' of chromatin, this fact clearly makes tissue specific differences in chromosomal components rather difficult to interpret. Unless analyses of other cellular aspects such as cytoplasmic and membrane components have been carried out, tissue specific differences in chromosomal components may not relate *directly* to the *in vivo* transcriptional activity of the respective chromatins.

Finally, because the cells from a given tissue are fully differentiated they may contain real chromosomal components which are not necessarily related to the actual transcriptional activity of chromatin. This is best illustrated by an example. The f2c histone is tissue specific to erythrocytes in chickens - the characteristic feature of erythrocytes being a highly condensed, inactive nucleus. However, as shown in Chapter 4 of this thesis, the mere presence of this histone does not correlate with the transcriptional activity of chromatin within this cell line.

The work in this thesis has attempted to minimise the problems discussed above by confining correlative studies to *one* cell line from which clearly defined populations of cells could be obtained (see Table 1, Chapter 3, p.31). In addition, analyses of other cellular components, besides chromatin, were carried out to clarify any complicating factors related to defining the origins of the components of isolated chromatin (Chapter 4, p.35; Chapter 5, p.48 ; Chapter 6; Appendix 2).

(d) Changes in the chromatin of avian erythroid cells.

In vivo changes in the chromatin of avian erythroid cells, particularly in the maturation of polychromatic erythrocytes to mature erythrocytes, are clearly evident from light and electron microscopic observations (Lucas & Jamroz, 1961; T. Douglas, unpublished observations). In this phase of maturation, marked heterochromatinisation occurs. Concomitant with this heterochromatinisation, the number of dye binding sites on nuclear DNA decreases and its thermal stability increases (Kernell, Bolund & Ringertz, 1971). Harris (1970) has suggested such changes could be due to changes in the ion content of the cells; although no analyses have been carried out on avian erythroid cells, in mammalian reticulocytes the cellular water content, potassium and calcium (decrease), and magnesium, zinc and copper (increase) levels change on maturation (Rowley & Morris, 1966; Valberg *et al.*, 1967). It is thus significant that in this study *isolated* nuclei retain the appearance characteristic of the cell from which they were obtained (Fig. 11 , Chapter 6, p. 57). The *in vivo* appearance is retained even after several washings with 0.08M NaCl, 0.02M EDTA, pH 6.3 - only after washing the nuclei with 0.147M NaCl is this characteristic appearance masked by severe clumping of the nuclear material (a similar phenomenon has been observed with isolated chromatin which aggregated extensively in 0.15M NaCl, Smart & Bonner, 1971). It thus appears that changes in the actual chromatin complex occur during maturation which may be correlated with the observed

heterochromatinisation. Consistent with this statement is the fact that polychromatic erythrocyte chromatin was found to be a better template for *E. coli* RNA polymerase than mature erythrocyte chromatin (Fig. 19, Chapter 7, p. 72).

Changes in chromosomal components which *may* correlate with the above physical changes in avian erythroid chromatin, include several non-histone components present in polychromatic erythrocytes but not in mature erythrocytes (Shelton & Neelin, 1971) and an increased content of phospho-protein (and rate of phosphorylation) in polychromatic erythrocytes compared to mature erythrocytes (Gershey & Kleinsmith, 1969). Unfortunately, these authors equated nuclei with chromatin, an assumption which clearly cannot be made for non-histone proteins from the simple quantitative data in Table 6 (Chapter 6, p. 59). The data shows almost a 4-fold increase in yield of membrane protein from polychromatic erythrocyte nuclei compared to that from mature erythrocyte nuclei. Thus no conclusions can yet be drawn from these studies. Regarding histones, Dick & Johns (1969) did provide some evidence that histones from polychromatic and mature erythrocytes were the same. More detailed studies have shown that in fact the distribution of f1 components in mature erythrocytes is reproducibly different from that of the polychromatic erythrocytes (Table 5, Chapter 4, p.39). In addition, the f2c histone (tissue specific to avian erythroid cells) is the only histone which is actively added to, and removed from the chromatin complex in polychromatic erythrocytes

(Chapter 5). Thus in polychromatic erythrocytes the f3, f2b, f2a2 and f2a1 histones may be considered stable, unchanging parts of the chromatin complex, as was deduced for all histones from the data of Dick & Johns (1969). The f1 and f2c histones cannot, however, be included in this category. In this regard it is interesting that Greenaway & Murray (1971), have suggested these two histones to be closely related - this point is developed further in the following section. It is also interesting to note, in this resumé of the work carried out on avian erythroid histones, that the slightly lower amount of f2c histone in erythroblasts (Table 4, Chapter 4, p.38) is possibly the result of this histone 'turning-over' more rapidly than the other histones. This could then result in preferential losses on cell lysis and isolation of nuclei. The effect would not be as marked in polychromatic erythrocytes (and mature erythrocytes) since the 'turn-over' is probably much lower than in erythroblasts.

(e) Models for the control of DNA transcription and the avian erythroid cell series.

Considering the size of a DNA molecule and the space into which it is packed in the cell, the actual conformation of the nuclear DNA complex must be a basic consideration in cellular control of DNA transcription (see also DuPraw, 1970; Bradbury & Crane-Robinson, 1971). The function of all the DNA in eukaryotes is at present not clear (Britten & Davidson, 1970; Crick, 1971). However, it seems a reasonable hypothesis

that changes in the conformation of the chromatin complex could constitute a broad level of transcriptional control (Hearst & Botcham, 1970; Literature Survey, p.13). The working hypothesis we have considered is that conformational changes in chromatin control large blocks of genes and may be pre-requisites for finer control elements to be functional. It is in a study of the changes in conformation which can occur in the nuclear DNA complex that the avian erythroid cell series provides a useful model system. Since the series terminates in a cell which is completely inactive in transcribing its DNA, all that need be carried out, *in vivo*, during maturation to achieve this state is gross structural changes to make the DNA inaccessible (rather than changes in finer control elements such as the addition of specific repressors). Evidence in this thesis indicates this process is achieved by changes in the physical nature of the nuclear DNA complex.

The properties of histones (Literature Survey, pp.6, 10,13) must make them an important consideration in the maintenance of structure of the nuclear DNA complex (Literature Survey, p.13). For the f2a1 and f2b histones it seems likely that one half of the molecule (NH_2-terminal portion) interacts with DNA, while the other half interacts with other histones and/or cellular information molecules. This possibly applies to the f3 and f2a2 histones as well to allow these histones to form the DNA into supercoiled structures (favoured from electron microscopic and X-ray studies, DuPraw, 1970; Bradbury & Crane-Robinson, 1971). To form the highly compacted form of

chromatin seen in heterochromatin additional, extensive, cross-linking between supercoils has been postulated (Bradbury & Crane-Robinson, 1971). The f1 and f2c histones in particular are implicated in this cross-linking of chromatin. The evidence for the latter postulate is at best indirect, being related to the more exposed nature of f1 histone in the chromatin complex. [The f2c histone is included in this category because it is similar to f1 (Greenaway & Murray, 1971).] The evidence has been summarised by Smart & Bonner (1971) and includes, (a) the higher susceptibility of f1 histone in chromatin to proteolytic enzymes, (b) the greater susceptibility of f1 histone in chromatin to removal by ionic dissociating reagents (removed at lowest ionic strength), (c) the greater contribution f1 histone in chromatin makes to the state of aggregation of the chromatin*, when compared to the other histones. The f1 histone also appears to differ from the f2a1 and f2b histones

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*The evidence here pertains to the solubility of chromatin after the removal of given histone fractions from the chromatin preparation. Thus when sodium deoxycholate is used to dissociate histones from the chromatin, the residual chromatin is seen to become particularly soluble after the removal of f1 histone (removed at the highest concentration of sodium deoxycholate). There is thus *no linear* relationship between the absolute amount of whole histone removed and the solubility of the remaining chromatin. It seems significant, however, that this same relationship does not hold when a similar experiment is carried out using sodium chloride to remove histones (in this case the f1 is removed at the lowest concentration of sodium chloride). The validity of point (c) is thus not clear at present.

in that it does not appear to interact with DNA with a specific portion of its structure (Boublik *et al.*, 1971). The f1 histone, it should be noted does exhibit a marked assymetric distribution of basic amino acids (Bustin, Rall, Stellwagen & Cole, 1969); in addition the basic amino acid rich portion of the molecule (cleaved from the remainder of the molecule) alters the structure of DNA (on binding) more markedly than the non-basic part of the molecule (Fasman, Valenzuela & Adler, 1971). At present there is little information on the structure of f2c histone (Greenaway, 1971) to correlate with its postulated cross-linking function. The postulate, however, is an attractive one, in relation to the avian erythroid cell series, for the following reasons.

(1) One can postulate that in mature erythrocytes the f2c histone can set up favorable interactions to form the highly condensed chromatin. This form of the chromatin is the most stable form of chromatin with that particular complement of histones. The greater stability of the erythrocyte chromatin compared to that of the early cells of the series is indicated by the greater stability of the respective chromatin-DNA to thermal denaturation (Kernell, Bolund & Ringertz, 1971).

(2) In the early cells of the series, with the *same* complement of histones as mature erythrocytes the interactions in (1) are postulated to be reduced by fluctuation of the f2c histone on and off the chromatin complex this may involve chemical modification of the histone reactions, see Discussion, Chapter 5, p. 54 .

If these assumptions are correct the heterochromatinisation occurring as polychromatic erythrocytes become mature erythrocytes is accounted for by random decay of the enzymes involved in the synthesis of f2c histone as well as those for its removal from chromatin. [Such a decay is seen for general RNA and protein synthesis, (Williams, 1971_1) and loss of enzymes such as DNA and RNA polymerase (Williams, 1971_2; Appels & Williams, 1970.] Possibly the respective enzymes are labile and are not resynthesised in cells after the cessation of division (and/or their sensitivity to erythropoietin) as postulated for DNA polymerase by Williams, 1971_2. The prediction from the above hypothesis is that a prerequisite for transcription of higher cell DNA is the metabolism of certain histone fractions (see also Chapter 5, discussion). The histone fractions particularly involved here would be f1 and/or f2c histones (depending on which one predominates in the chromatin). Systems required to test this hypothesis would preferably be limited to those displaying a change in transcription of DNA, not complicated by replication of DNA occurring as well (see also Chapter 5, discussion). Such systems include polytene chromosome puffing and reactivation of hen erythrocyte nuclei (by cell fusion), prior to the onset of DNA synthesis.

More general models, similar to the one outlined above have been considered by others (Gurley & Hardin, 1970; Stellwagen & Cole, 1969). The models correlate well with the observed tissue specificity of f1 and f2c histones in that this may

represent a broad level of control on transcription of DNA.

It seems likely that in situations other than of the all or none type (as seen in the transition from interphase to metaphase chromatin or in terminal differentiation of avian erythroid cells) similar mechanisms could operate differentially in conjugation with other protein or RNA components of chromatin. In this respect the sites of attachment of chromatin to the inner nuclear envelope may be of considerable importance. To date this has been considered in regard to control of DNA synthesis (Mizuno, Stoops & Peiffer, 1971; Hearst & Botcham, 1970), but not in relation to control of DNA transcription.

(f) Future studies.

The work in this thesis has emphasised a number of general and specific questions which deserve further study. These are summarised below.

(1) The work predicts a characteristic metabolic flux of f1 histones (and/or f2c histone if it is present), particularly in systems where only the transcription of DNA is altered. Such systems would include polytene chromosome puffing and reactivation of hen erythrocyte nuclei by cell fusion. Some evidence related to this point has been obtained by Gurley & Hardin (1970) in Chinese hamster cell culture.

(2) Related to the above point is the possibility of a non-uniform distribution of histones along

the DNA (and/or differential rates of 'turnover' of a histone fraction depending on its position). The point of attachment of the chromatin to the nuclear membrane may be a region of particular interest.

(3) An investigation of finer control elements operating to control DNA transcription virtually demands an *in vitro*, cell-free system of isolated chromatin transcribed by added RNA polymerase. Erythroblast chromatin would be expected to have genes for globin in a transcribable state which would thus provide a useful marker for a fully functional system (since the RNA synthesised may be translated into a well-defined protein product). Success of the system would presumably depend on proper transcription of DNA, processing of the RNA formed and its consequent correct translation into globin subunits. The usefulness of such an approach is demonstrated by the studies of Chen *et al.* (1971) on the *in vitro*, cell-free transcription of *lac* operon DNA. A major problem in such a study is the high content of degradative enzymes in erythroblasts (Chapter 7, p.64 ; Harlow & Wells, 1971).

(4) Limited mutational studies such as an investigation of chickens defective in the formation of mature

erythrocytes might provide an alternative approach to the control mechanisms operative in the avian erythroid cell series. The micro-density gradient technique for rapid analysis of blood samples (Williams, 1971; see also Appendix 1) would be useful in the scanning of large numbers of chickens. The initial requirement of the mutants of interest would be a high content of polychromatic erythrocytes in the circulation.

(5) Physical studies on histone f2c, such as its primary sequence and mode of interaction with DNA are essential for further elucidation of the function of this interesting histone species. Related to this point is the need for information on the nature and site of synthesis of histone f2c.

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APPENDIX 1

Induction of anaemia

Anaemia was induced as described by Williams (1971_1), namely by daily, subcutaneous injections of phenylhydrazine (a 2.5% solution in 47.5% ethanol). The course of anaemia was followed by daily measurement of blood haematocrit (before administration of phenylhydrazine). In this way the blood of the animal could be accurately titrated so that 43 - 50% of the cells originally present, remained in the circulation (Fig. 21). This *absolute* amount of phenylhydrazine injected depends entirely upon the effectiveness of the chemical to lyse cells (found to decay on storage) and on the ability of the animal to respond and produce more red blood cells as required. The first two days are generally found to show the greatest drop in blood haematocrit (Fig. 21). It is in this period that the animal is most likely to die of anoxia unless the third dose of phenylhydrazine is lowered considerably. During the period of injection (7 days) the population of red blood cells in enriched for polychromatic erythrocytes (Fig. 22), as previously described by Williams (1971). Polychromatic erythrocytes are characterised by a lower buoyant density, Williams (1971) - for details see Table I, Chapter 3, p.30.

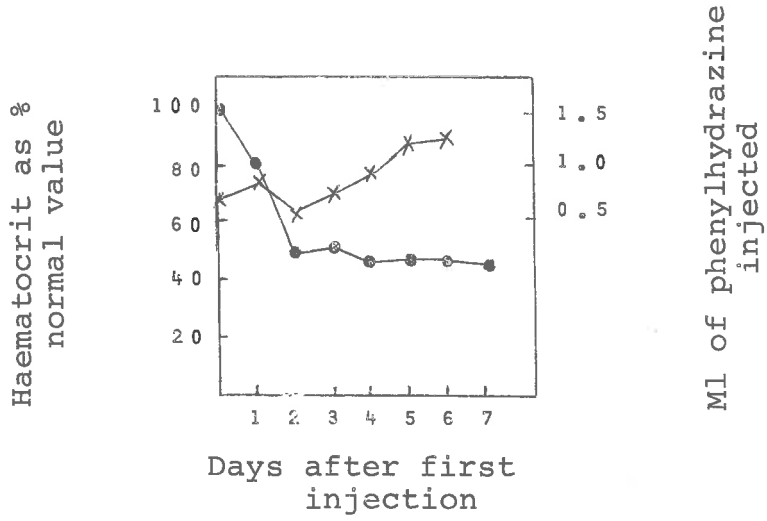


Fig. 21. *Induction of Anaemia*

Symbols: ⊙ —— ⊙, blood haematocrit as % of the normal healthy value (packed cell volume/total blood volume = 35 ± 2); x —— x ml of phenylhydrazine injected.

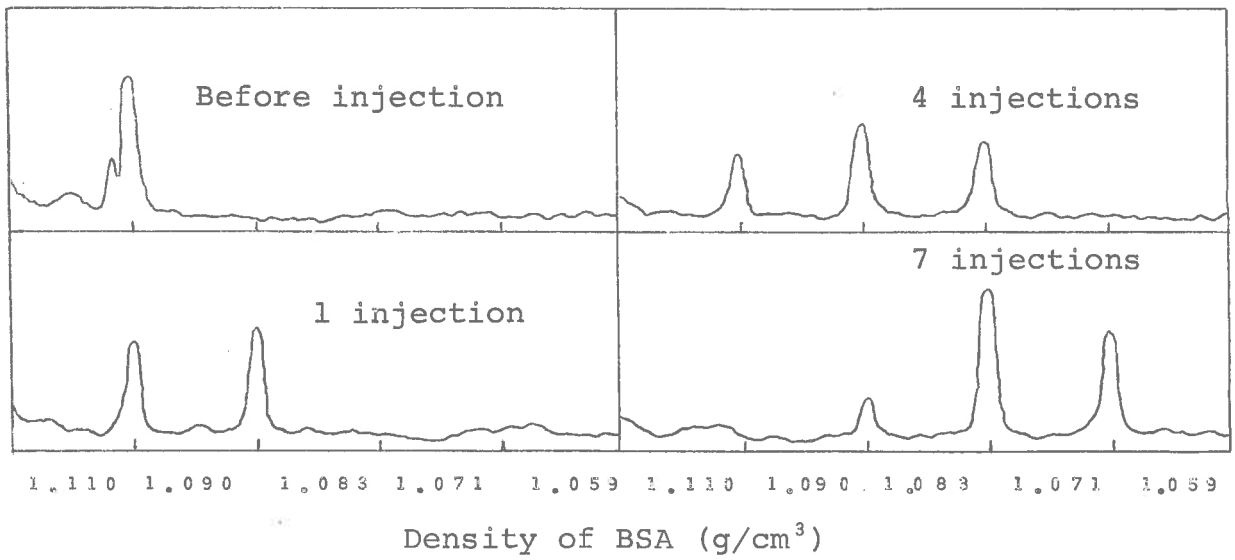


Fig. 22. *Cell Populations During Induction of Anaemia.*

Densicord traces of micro-gradients (discontinuous gradients of BSA in capillary tubes) showing analyses of red blood cells as described by Williams (1971). Blood analysed was taken at various stages during induction of anaemia.

APPENDIX 2

Co-electrophoresis of protein samples - precise alignment of different protein analyses on polyacrylamide gels.

Precise alignment of components (separated by polyacrylamide gel electrophoresis) from two different samples of protein was often required during the course of the work presented in this thesis. The alignment was carried out by mixing the two protein samples to be compared and electrophoresing them on the same polyacrylamide gel. An example is shown in Fig. 23, where cytoplasmic proteins were aligned with histones prepared from the same cell type. Although the resulting pattern is complicated, by careful examination of the respective analyses unambiguous alignment of respective components can be obtained.

It should be noted that the cytoplasmic protein analysis shown here is somewhat different from that shown in Chapter 5 (Fig. 9A, p.48). Such variability in cytoplasmic protein analyses (particularly in the region of electrophoresis of the f3-aggregate histone) was often obtained.

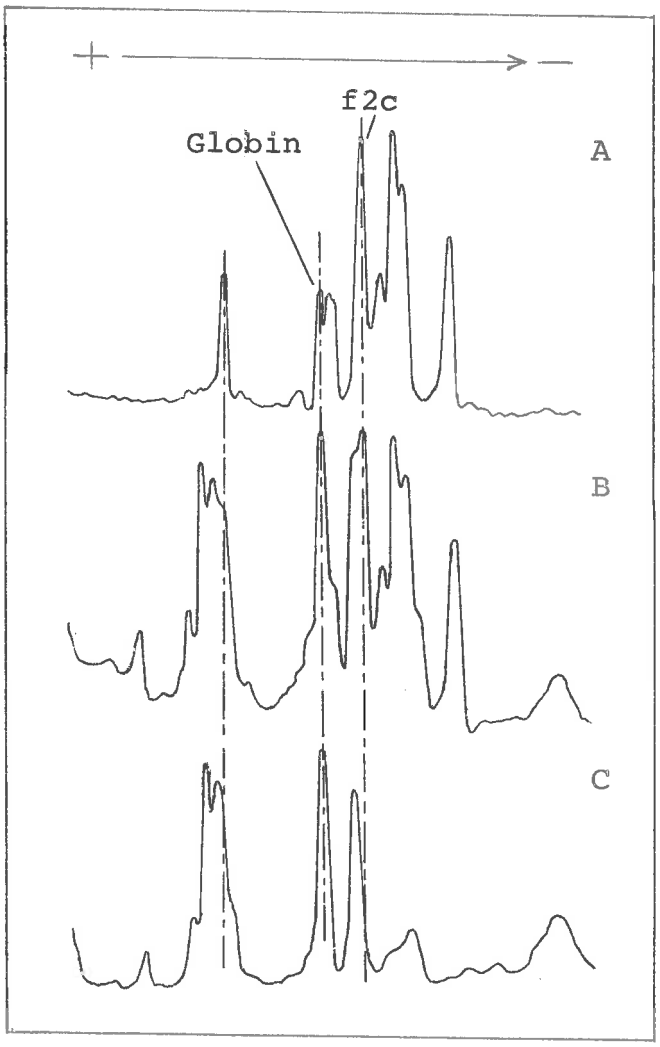


Fig. 23. *Co-electrophoresis of Protein Samples.*

Histone and cytoplasmic protein (prepared as described in Methods, pp. 19 and 18 respectively) samples were obtained from mature erythrocytes. The positions of electrophoresis of globin and f2c histone only are shown - for complete identification of histones see Chapter 4.

A. Histone sample - 30 µg loaded per gel.
B. Histone plus cytoplasmic protein samples - total of 60 µg protein loaded per gel.
C. Cytoplasmic protein sample - 30 µg loaded per gel.

Electrophoresis in each case was for 4 hr at 2 mA/gel, on the polyacrylamide gels routinely used to analyse histones (see Methods, p.21).

APPENDIX 3

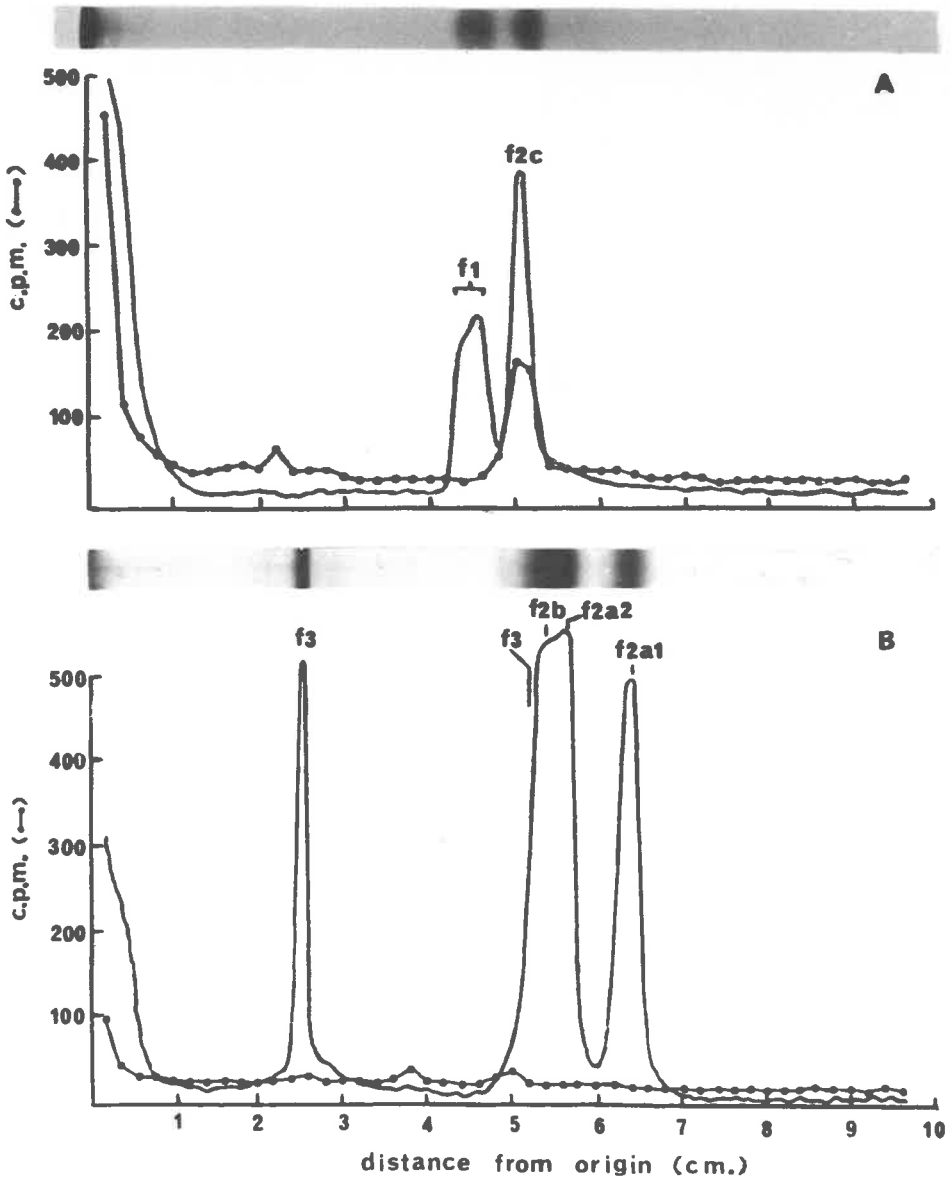
Fractionation of radioactive histones.

The results of two fractionations of radioactive histones are presented. The procedures have been published and were utilised here to verify the distribution of radioactivity in polychromatic erythrocyte histones (see Chapter 5 for details).

Fig. 24 shows C^{14}-lysine labelled polychromatic erythrocyte histones fractionated into 5% perchloric acid-soluble and insoluble fractions, as described by Nelson & Yunis (1969). The 5% perchloric acid-soluble fraction contained f1 and f2c histones and analysis for radioactivity showed only the f2c histone was labelled (Fig. 24A). The aggregated material at the origin of the polyacrylamide gel probably contains f2c histone since the colour yield of f2c histone in Fig. 24A was low compared to that expected from the colour yields of the other histones (shown in Fig. 24B). The 5% perchloric acid-insoluble fraction contained the f3, f2b, f2a2 and f2a1 histones, and none of these were found to be labelled.

Fig. 25 shows C^{14}-lysine labelled f2c histone from polychromatic erythrocytes. This was isolated from washed nuclei by the procedure of Johns & Diggle (1969). The fractionation verified f2c histone was labelled under the conditions of incubation of polychromatic erythrocytes used (see Chapter 5 for details). The other histone fractions recovered from this fractionation procedure, and analysed on polyacrylamide gels were not labelled.

Fig. 24. Fractionation of C^{14}-lysine labelled histones from polychromatic erythrocytes. Polychromatic erythrocytes were labelled with C^{14}-lysine as described in the legend to Fig. 7. Histones, routinely isolated (see Methods, p.20) were fractionated into 5% perchloric acid-soluble and insoluble fractions, as described by Nelson & Yunis (1969).
A. 5% perchloric acid-soluble histones.
B. 5% perchloric acid-insoluble histones.
For each analysis 60 µg of protein were loaded per gel and electrophoresed for 4 hr at 2 mA/gel. Symbols: ● —— ●, distribution of radioactivity in cpm per 1 mm gel slice; ———, densicord trace of the gel used to obtain the distribution of radioactivity.


A
f1
f2c
c.p.m. (⟷)
500
400
300
200
100
B
f3
f3
f2b
f2a2
f2a1
1
2
3
4
5
6
7
8
9
10
distance from origin (cm.)

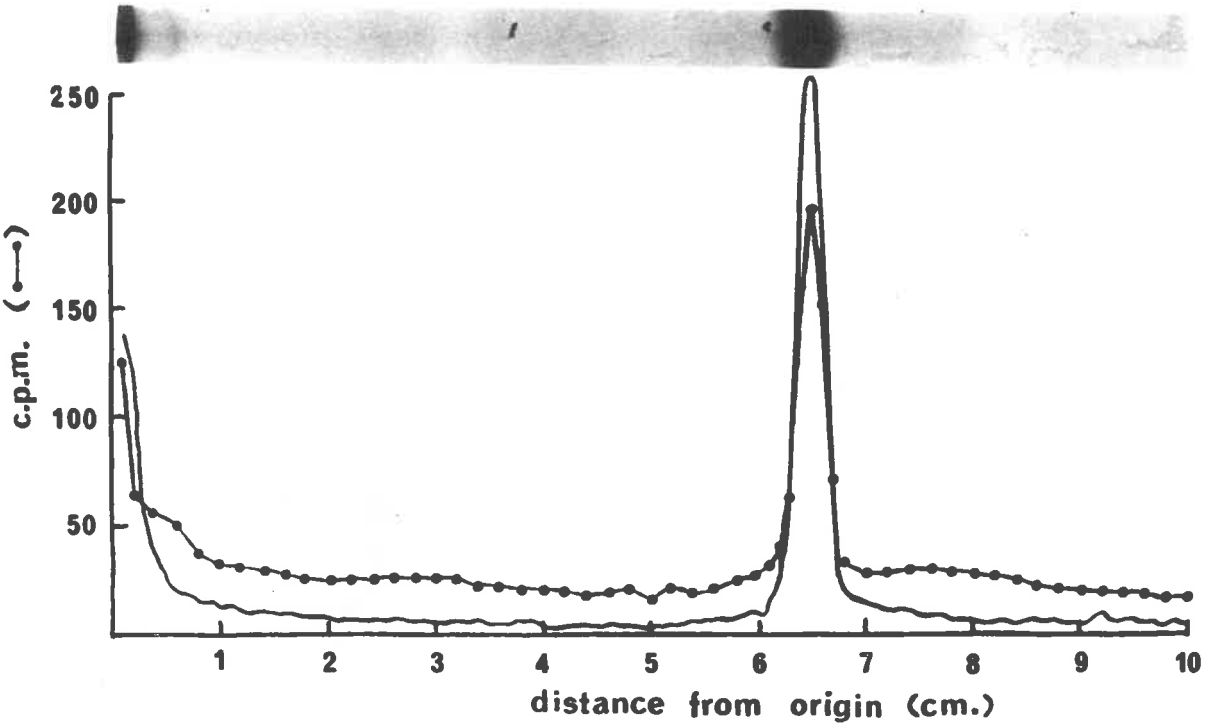


Fig. 25 Preparation of pure f2c histone. Polychromatic erythrocytes were labelled with C^{14}-lysine as described in the legend to Fig. 7. Washed nuclei isolated from these cells were treated as described by Johns & Diggle (1969) for the purification of f2c histone. The f2c histone isolated in 5% perchloric acid was dialysed against 0.9% acetic acid, freeze-dried and dissolved in loading buffer for analysis on polyacrylamide gels at a concentration of 1 mg/ml. Approximately 30 µg of protein was loaded per gel and electrophoresed for 4 hr at 2mA/gel.
Symbols : ●—● , distribution of radioactivity in cpm per 1 mm gel slice ; ——— , densicord trace of the gel used to obtain the distribution of radio-activity.

APPENDIX 4

Calculation of the number of dye binding sites on nucleic acids.

For a detailed discussion of the theory and method of spectrophotometric titrations see Peacock and Skerrett (1956) (in relation to the use of ethidium bromide, see Waring, 1965, and Olins, 1969).

For the binding of dyes to DNA, the average number of dye molecules bound per DNA-nucleotide is given by the relation:

$$r = \alpha \frac{D}{P}$$

where D is the molarity of total dye (bound + free); P is the molarity of DNA-phosphate; and α is the fraction of the total dye molecules bound. The value of α, after the addition of a certain amount of dye to DNA is given by

$$\alpha = \frac{\varepsilon_f^{460} - \varepsilon_t^{460}}{\varepsilon_f^{460} - \varepsilon_b^{460}}$$

where ε_f^{460} and ε_b^{460} are the molar extinction coefficients of free and bound dye, respectively (at 460 nm). ε_t^{460} is the apparent molar extinction coefficient calculated after each addition of dye. This is monitored at 460 nm since at this wavelength absorption of free and bound dye molecules show maximal differences. For ethidium bromide $\varepsilon_f^{460} = 4.88 \times 10^3$ (Waring, 1965); the value of ε^{460} was measured in each experiment by averaging the apparent molar extinction coefficients in the region of dye titration where $\alpha = 1$ (i.e., all the dye molecules are bound and $\varepsilon_t^{460} = \varepsilon_b^{460}$). The value of ε_b^{460} was $1.3 - 1.4 \times 10^3$ which agrees well with the value quoted by Waring (1965,

$\varepsilon_b^{460} = 1.46 \times 10^3$).

Finally, to calculate the binding parameters of ethidium bromide to DNA, the concentration (c) of free dye present after each addition of dye is calculated from:

$$c = (1 - \alpha).D$$

After each addition of dye to DNA the fraction of occupied binding sites/unoccupied is:

$$\frac{r}{n - r} = Kc$$

where n is the total number of dye binding sites per DNA-nucleotide; K is the equilibrium constant. For the Scatchard (1949) plot this equation becomes:

$$\frac{r}{c} = Kn - Kr$$

thus a r/c *versus* r plot gives n as the X-intercept (and the slope equals K). The curve generally deviates from linearity at high value of c due to secondary binding effects - to obtain n, the linear portion of the curve at low c values is extrapolated. The reciprocal 1/n, is quoted as the number of DNA-nucleotides per dye binding site (see Fig. 18 , Chapter 7, p. 69).