THE BIOSYNTHESIS OF CHICK FEATHER KERATIN MESSENGER RNA.

A thesis submitted to

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for the degree of Doctor of Philosophy, by

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SUMMARY.

The work described concerns the synthesis of embryonic chick feather keratin messenger RNA, and two distinct aspects of the biosynthesis were investigated. Firstly, the temporal relationship between the onset of keratin synthesis and the synthesis of keratin mRNA was examined. Coupled with this, a study was made of total RNA and DNA levels in the feather during development. Secondly, the question of whether the messenger RNA was synthesised as a high molecular weight nuclear precursor was considered.

The following findings were made:

1. From the levels of DNA in pooled feather tissue or individual feathers, it was concluded that DNA synthesis is completed before the onset of keratin synthesis, and that DNA is lost from the cells late in development. Enzyme activities responsible for the degradation process were identified.

2. The number of genes for keratin is the same in embryonic feather nuclei as it is in adult chicken erythrocyte nuclei.

3. The level of RNA in the feather cells is greatest at the stage where the rate of keratin synthesis is maximal, and declines rapidly in the later

stages of development. This decrease is marked by a parallel increase in the activities of two feather ribonucleases.

4. Keratin messenger RNA is not present in significant quantities in the feather cell prior to the onset of keratin synthesis. The level of keratin mRNA rises to a maximum value at the stage where the rate of keratin synthesis is also at a maximum. At this stage of development, most of the messenger RNA sequences in the cell are located in the cytoplasm, and are associated with polysomes. The estimated levels of mRNA in the cells throughout development are adequate to account for the observed synthesis of keratin.

5. High molecular weight species of RNA containing messenger RNA sequences could not be detected in the steady-state population of RNA, either in isolated nuclei which were free of observable contamination with cytoplasmic materials, or in the total RNA or poly(A)-containing RNA fractions of feathers from young embryos. It was concluded that either the mRNA is not synthesised as a high molecular weight species or that such a species has a transient existence in the cell and is not detectable under the conditions used.

STATEMENT.

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

Signed:

PETER E. M. GIBBS.

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ABBREVIATIONS.

The following abbreviations have been used in this thesis:

BPB :	bromophenol blue.
BSA :	bovine serum albumin.
C :	curie.
cDNA :	synthetic DNA complementary to messenger RNA.
DNA :	deoxyribonucleic acid.
DNase :	deoxyribonuclease.
EDTA :	ethylenediaminetetracetic acid.
EGTA :	ethyleneglycol-bis (β-aminoethyl ether)-N,N'-tetra-acetic acid.
hnRNA :	heterogeneous nuclear RNA.
mRNA :	messenger ribonucleic acid.
NP-40 :	Nonidet P-40
oligo(dT) :	oligodeoxythymidylic acid.
poly(A) :	polyadenylic acid.
RNA :	ribonucleic acid.
RNase :	ribonuclease.
rRNA :	ribosomal RNA
SDS :	sodium dodecyl sulphate.
s.e.m. :	standard error of mean
TCA :	trichloroacetic acid.
tRNA :	transfer RNA

CHAPTER 1

INTRODUCTION

A. GENERAL INTRODUCTION

The terminal differentiation of the embryonic chick down feather is characterised by an initial period of growth, after which the cells become filled with the fibrous protein, keratin, resulting in eventual cell death. Work in this laboratory has been directed toward an understanding of the molecular events which occur during feather differentiation. This thesis is concerned primarily with investigation of the synthesis of keratin messenger RNA, and the roles which the timing of mRNA synthesis, and any intermediates which may occur during synthesis, may play in the control of the process of keratinisation.

The following discussion is intended to provide relevant background information on feather development, and on the synthesis of eukaryote mRNA.

B. FEATHER KERATINS

Keratins are fibrous, insoluble, intracellular proteins of high cystine content, which are found in the epidermis and in tissues derived from it :- the feathers, scales, claws and beaks of birds, the hair and claws of animals, etc. In the cells the proteins are aggregated into fibrillar masses, the structure of which has been extensively studied (for review, see Fraser *et al.*, 1972). The protein aggregates are stabilised by disulphide bonding (Goddard and Michaelis, 1934), so that study of the proteins

requires breakage of the disulphide bonding, generally by reducing agents, followed by alkylation to form stable derivatives, e.g. carboxymethylation (Harrap and Woods, 1964a; Kemp and Rogers, 1972).

The S-carboxymethyl derivatives of chicken feather keratins appear to be homogeneous in molecular weight, the estimates varying between 10500 and 11500 daltons (Harrap and Woods, 1964b; Walker and Rogers, 1976a); reduced, non-alkylated keratin was shown to have a molecular weight of 10,000 daltons (Woodin, 1954). However, the proteins display heterogeneity within distinct keratinised tissues, as revealed by ion exchange chromatography and by electrophoresis (Harrap and Woods, 1964a; Kemp and Rogers, 1972; Walker and Rogers, 1976a). Woods (1971) was able to resolve some ten electrophoretically distinguishable species from each different morphological part of adult feathers, and Walker and Rogers (1976a) have extended this work to claim that there are a minimum of twenty two protein chains in the embryonic feather. Sequence analysis of the proteins has revealed that each chain is likely to be the product of a separate gene, but that all of the proteins are closely related in primary sequence (Walker and Rogers, 1976b).

Although each of the different parts of the adult feather, and the embryonic feathers, contain an assembly of closely related protein chains, each tissue has its distinct spectrum of protein species (Harrap and Woods, 1964a; Kemp and Rogers, 1972). Furthermore, the proteins of feather

appear to be fundamentally different to those of other keratinised tissues, such as scale (Kemp and Rogers, 1972; Walker and Bridgen, 1976).

The avian keratins appear, however, to have evolved from common ancestral proteins. The patterns of proteins observed in different species of birds reveal similar degrees of heterogeneity, and also display tissue specificity, although the patterns for a given tissue show some variation between species (Harrap and Woods, 1967; Woods, 1971; O'Donnell, 1973a). Primary sequence analysis of chains from the adult feather calamus of the emu (O'Donnell, 1973b) and gull (O'Donnell and Inglis, 1974), from chick embryonic feather (Walker and Rogers, 1976b) and from chick scale (Walker and Bridgen, 1976) reveal the presence of major regions of sequence homology between the proteins from different tissues.

C. EMBRYONIC FEATHER MORPHOGENESIS AND DIFFERENTIATION

(i) Structure of the down feather.

The down feather of the newly hatched chicken has been described in detail by several authors (e.g.Watterson, 1942; Romanoff, 1960; Rawles, 1972). The feather is attached to the skin *via* a short basal calamus, to which are attached some 10 to 15 barbs. Each barb consists of a core of medulla cells, overlain by flattened cortical cells, and has, attached to the basal two-thirds of its length, two rows of barbules. Each barbule is a row of single cells joined end to end. At hatching, each of these

cell types is dead, dehydrated and filled with keratin.

(ii) Development and Keratinisation.

The events involved in the morphogenesis of the feather have been studied extensively, and reviews of this work have been published by Romanoff (1960), Lillie (1965) and Voitkevich (1966). The data below have been taken mainly from two studies, at the light (Watterson, 1942) and electron microscope levels (Matulionis, 1970).

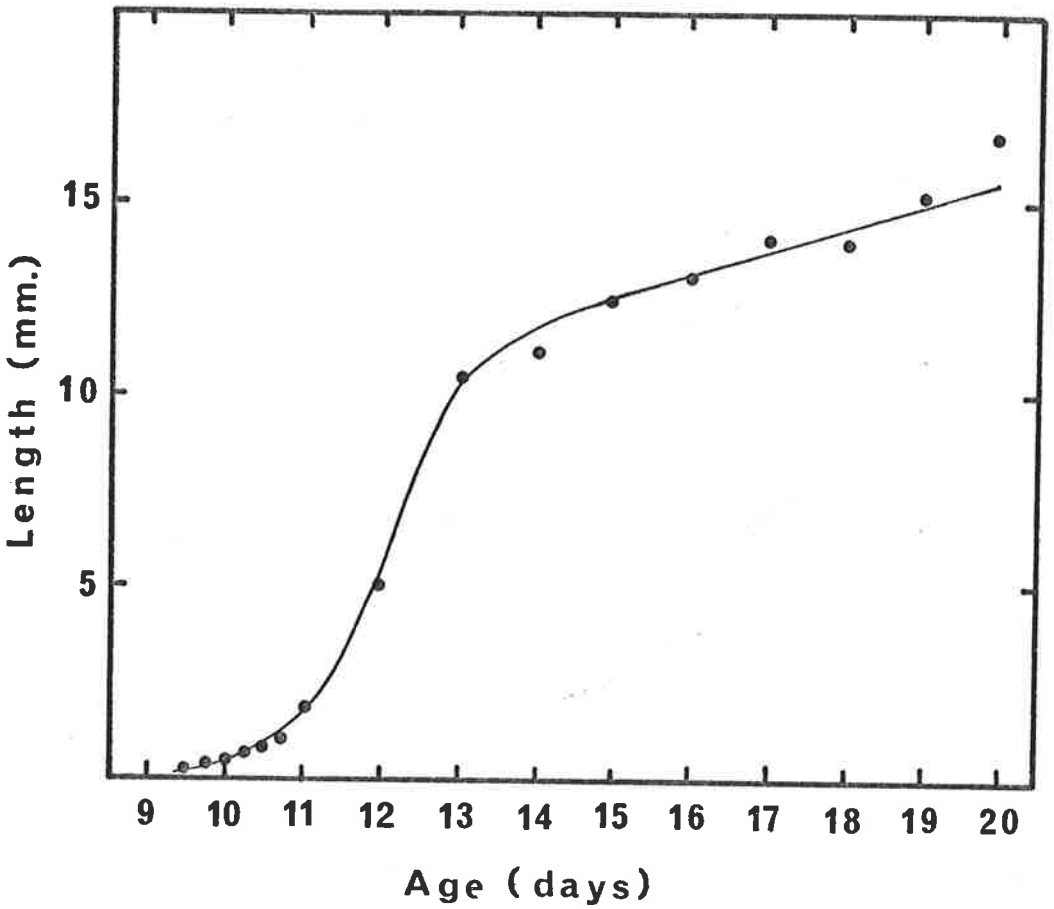
At five days of incubation, the skin of the chick embryo consists of a layer of cuboidal epithelial cells, overlain by flattened peridermal cells and underlain by mesoderm. Shortly afterwards, the mesoderm condenses into regions of high cell density, and the epithelial cells begin to proliferate, so that by eight days the feather germs are visible as epidermal thickenings, in defined tracts. By ten days the feather germs consist of cylindrical lumps of epidermal cells, with mesodermal cores.

Early on the eleventh day the rapidly dividing epidermal cells become organised into discrete sectors, the barb ridges, which are destined to become the barbs and barbules of the final down feather. Several cell types, all derived from the epidermal cells become evident at this stage. By twelve days the feather consists of a cylindrical arrangement of barb ridges. These are surrounded externally by the cells of the sheath, and by barb-vane-ridge cells, and internally by supportive cylinder cells. The core of the feather is occupied by a few pulp

cells of mesodermal origin, and by two capillary vessels. The bulk of the cells are the barb and barbule cells of the barb ridges.

Between 11 and 13 days the feather undergoes a rapid elongation (Figure 1.1), followed by a slower rate of growth over the later stages of development. The later growth has been attributed to cell growth and movement, rather than further cell division.

Keratin synthesis begins at about the twelfth day, as judged by the appearance of keratin fibrils in the cells (Matulionis, 1970) and by the appearance of keratin proteins on acrylamide gels of S-carboxymethylated tissue proteins (Kemp et al., 1974a). The keratin is first detected at the tip of the feather, in the sheath cells, and differentiation of the tissue proceeds downwards and inwards (Matulionis, 1970), thereby ensuring that no cells are isolated from the nutrient source of the capillary vessels. Early studies using gross techniques such as X-ray diffraction of whole tissue (Bell and Thathachari, 1963) or the sulphur content of total cellular protein (Malt and Bell, 1965) suggested that keratin synthesis took place in two stages, whereby the synthesis of a low-sulphur, fibrillar protein preceded that of a sulphur-rich species. Available evidence does not support the concept of high- and low-sulphur proteins (Harrap and Woods, 1964a). It was also noted, from labelling studies and gel electrophoresis of proteins, that the keratin proteins are co-ordinately


Length (mm.)
15
10
5
9
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12
13
14
15
16
17
18
19
20
Age (days)

synthesised in the tissue (Kemp et al., 1974a).

Keratinisation of the tissue is eventually completed by about 18 days of development. As the tissue matures, the barb-vane-ridge cells and cylinder cells atrophy, and the pulp is retracted.

The chick hatches at 21 days. The feathers dry out soon after hatching, the sheath splits open and is discarded, and the feathers adopt their normal fluffy appearance.

(iii) Factors affecting keratinocyte development.

Several factors have been implicated in the growth and development of keratinising tissues; these are discussed briefly below. A more extensive review may be found in Fraser et al. (1972).

(a) The role of the dermis.

Experiments performed using tissue culture of skin have suggested that the dermis plays a major role in epidermal differentiation. When the dermis and epidermis are taken from different regions of the embryo, and are recombined and allowed to develop in culture, the epidermis develops into the type normally associated with the dermis used (Sengel, 1957, 1971; Rawles, 1963, 1965; Wessells, 1962, 1965). Thus if presumptive scale dermis and presumptive feather epidermis, at appropriate stages of development, are recombined, the epidermis differentiates to form scales. These effects exerted by the dermis only occur at specific stages in development of the tissues

(Rawles, 1963, 1965), which are well in advance of the onset of keratinisation in these tissues determined by other workers (Matulionis, 1970; Kemp et al., 1974a; Beckingham Smith, 1973a).

The exact nature of the signals passing between the cells is not known, but it has been suggested (Wessells, 1968) that a specific inducer molecule is not needed. The effects could be mediated by factors such as changes in mesodermal cell density or by extracellular substances from the mesoderm which may influence the environment of the epidermal cells. The role of the dermis may be merely supportive, as it has been shown (Dodson, 1963) that dermis killed by freeze-thawing is able to fulfil the functions of maintaining the mitosis, orientation and spreading of the epidermis.

It is apparent that a major role of the dermis is to control the mitosis of the epidermal cells, thereby controlling the epidermal morphology. The dermis of one species of bird is capable of exerting this control over the epidermis from another species - recombination of duck dermis and chicken epidermis gives feathers which resemble those of the duck (Dhouailly, 1967). It is not clear, however, whether the dermis specifies the genes which areexpressed in the differentiating tissue. Different keratin genes are expressed in feather and scale (Kemp and Rogers, 1972), but it is not yet known whether the keratins synthesised in a recombination of

"scale" dermis and "feather" epidermis are of the scale or feather variety.

(b) Vitamin A.

It has been shown that if immature (Fell and Mellanby, 1953) or highly differentiated (Fell, 1957) embryonic chick skin is cultured in the presence of excess Vitamin A, keratinisation is inhibited, and the cells undergo a mucous metaplasia. Goblet cells, which secrete mucin, typical of secretory epithelia are formed. The synthesis of keratin proteins is completely repressed under these conditions (Beckingham Smith,1973b). Fitton-Jackson and Fell (1963) demonstrated that only the post-mitotic population of cells in older tissue was affected in the older tissue, and further showed that the effects were reversible on removal of vitamin A. It was noted that only the new generation of post-mitotic cells would then synthesise keratin.

(c) Hormones.

Several hormones have been implicated in the development of avian keratinocytes. The role of the pituitary and thyroid glands has been discussed at some length by Voitkevich (1966), but few studies have been made at the molecular level of these phenomena.

Yatvin (1966a,b) observed that an increase in the level of polysomes associated with the onset of keratin synthesis in the feather could be prevented by hypophysectomy, and that the administration of pituitary

extract would partly restore the polysome profile. It has been suggested (Kemp et al., 1976) that the effect of hypophysectomy is to merely delay the onset of keratin synthesis by about two days - the entire development of the embryo appeared to be retarded by a similar period.

In vivo (Bartels, 1943) and in vitro experiments (Wessells, 1961; Kitano and Kuroda, 1967) have indicated that thyroxine accelerates epidermal keratinisation. The thyroid gland reaches maximum thyroxine secretion (Shain et al., 1972) at about the same time as the onset of keratin synthesis in the feather. The onset of scale keratin synthesis, however, occurs somewhat later.

Hydrocortisone also hastens keratin synthesis in skin cultures (Fell, 1962; Sugimoto and Endo, 1969), and causes feather germs to abort. The presence of hydrocortisone in the medium stimulates the synthesis of one class of scale epidermal proteins, although another class is not synthesised (Sugimoto et al., 1974).

(d) Epidermal chalone.

Bullough (1967) has described the proporties of a highly tissue-specific inhibitor of mitosis, the epidermal chalone. This material appears to be a glycoprotein, and may also require the presence of adrenalin to express its full effect. The chalone has been shown to be ineffective against hair follicles, and it has been suggested that the chalone effect may only be observed on tissue which maintains a constant

relationship between the rate of cell division and tissue volume. It may not, therefore, affect feather development.

(e) Epidermal Growth Factor.

Cohen (1962) isolated a polypeptide from the submaxillary gland of the male mouse which caused precocious epidermal development when administered *in vivo* or in *in vitro* organ culture (Cohen, 1965). The growth factor caused an increase in RNA and protein synthesis (Hoober and Cohen, 1967) and accompanying this the proportion of the ribosomes associated with polysomes increased (Cohen and Stastny, 1968). The material isolated from mouse is effective against skin tissue from a variety of animal sources, including the chicken, and a similar material has recently been isolated from man (Cohen and Carpenter, 1975) suggesting it may be widely distributed in nature. An *in vivo* requirement for epidermal growth factor has not, however, been demonstrated.

(iv) *DNA synthesis and mitosis*.

It seems from the above discussion that there can be no simple mechanism whereby keratin synthesis is initiated in feather or skin cells. It is notable that many of the factors discussed appear to affect mitosis, and that some effects of differentiation (or pseudo-differentiation with vitamin A) are observed in post-mitotic cell populations. It has been shown that DNA

synthesis in skin (Rothberg and Ekel, 1971) and DNA polymerase activity in feather (Kischer and Furlong, 1967) are at maximum levels immediately prior to the onset of keratinisation of the tissues, and decrease significantly by the time keratin synthesis is established.

It is possible that the cells are committed to keratin synthesis quite early in development, as has been observed for myogenic cells (for example, Holtzer et al., 1972, 1973). These cells, although not actively synthesising muscle proteins, undergo several rounds of DNA synthesis and mitosis, until after a "quantal" mitosis, the cells become fully differentiated. Epidermal cells show a certain degree of developmental plasticity, depending upon the environment in which they are located; perturbations of that environment cause the cells to follow a different path of development. Nonetheless, the cells could be initially constrained to follow a limited series of fates, and that association with a given mesenchyme would lead to the cells being finally committed to one path. Thus, with the appropriate dermal stimulus, the cells could undergo a programmed pattern of cell division, culminating in a quantal mitosis and keratinisation. After the quantal mitosis the cells would be incapable of responding to external stimuli, but prior to it, major changes in environment, could lead to a different set of genes being expressed, as in, for example, abortion of feather development induced by precocious

keratinisation in the presence of hydrocortisone (Fell, 1962), or the mucous metaplasia induced by vitamin A (Fell, 1957). This scheme, however, still leaves the question of the influences on the dermis which cause it to specify a particular fate for the epidermis.

D. STRUCTURE OF FEATHER KERATIN mRNA.

Studies on feather keratin synthesis have been aided by the isolation of feather keratin mRNA (Partington et al., 1973; Kemp et al., 1974b). The mRNA codes for keratin synthesis in cell-free systems prepared from rabbit reticulocytes (Partington et al., 1973) and wheat embryos (Kemp et al., 1974c). In the wheat embryo system the only proteins synthesised were keratins.

The mRNA is a heterogeneous mixture of species, as judged from the kinetics of hybridisation of keratin mRNA with a synthetic cDNA, prepared using avian myeloblastosis virus RNA-dependent DNA polymerase (Kemp, 1975). The number of mRNA species estimated from hybridisation kinetics was 25 - 35, in good agreement with the number of protein chains in the tissue (Walker and Rogers, 1976a). Each mRNA species contains a sequence which is unique to it, and a sequence homologous to the other keratin mRNAs in the mixture. The mRNA has an extensive untranslated portion (see below), and on the basis of the extensive homology between the protein chains, it does not seem unreasonable to suggest that the

homologous RNA sequences correspond to the coding portion of the mRNA molecule.

The RNA sediments insucrose gradients at 12S (Partington, et al., 1973; Kemp et al., 1974b). Gel electrophoresis under denaturing conditions, in the presence of formamide, yielded a molecular weight of 2.5×10^5 daltons, or 760 nucleotides (Kemp et al., 1974b). This is much longer than is necessary to code for keratin synthesis (100 amino acid residues, or 300 nucleotides), implying that a large proportion of the mRNA is not translated. The molecular weights of globin (Gould and Hamlyn, 1973) and ovalbumin (Woo et al., 1975) mRNAs are also larger than is required for the synthesis of the protein, so that in each a major portion of the mRNA is untranslated.

The ability of the keratin mRNA to bind to cellulose (Partington et al., 1973; Kemp et al., 1974b) suggests that it contains a 3'-poly (A) sequence (Kitos et al., 1972; Schutz et al., 1972; DeLarco and Guroff,1973). This is a feature common to most other messenger RNAs studied (see, for example, Greenberg, 1975), with the notable exception of histone mRNA (Adesnik and Darnell, 1972). The length of the poly (A) sequence has been determined in the steady state mRNA population to be about 25 - 35 nucleotides (C.P. Morris, personal communication).

It has been shown recently that the 5'-terminus

of animal (Adams and Cory, 1975; Furuichi *et al.*,1975a; Perry *et al.*, 1975a) and viral mRNAs (Furuichi *et al.*, 1975b; Wei and Moss, 1975) possesses a structure having the sequence $m^7G5'ppp5'X^mpY^{(m)}pZp...$, where m^7G is 7-methylguanosine which is coupled via a triphosphate linkage to the next nucleotide in the RNA chain (X), which bears a 2'-O-methyl group. The third nucleotide (Y) is also occasionally methylated at the 2'-position. Evidence has recently been obtained suggesting the presence of 7-methylguanosine at the 5'-terminus, which is coupled by a triphosphate linkage to the penultimate residue (C.P. Morris, personal communication). The sequence of the adjacent residues could not be determined, as it is necessary to label the mRNA *in vitro* in order to detect the 5'-terminal structure (c.f. Symons, 1975).

Keratin mRNA therefore shows many features which are common to eukaryotic mRNAs. However, the sequence complexity indicates that keratin mRNA is a mixture of closely related sequences, presumably equivalent to the observed heterogeneity of the keratin proteins.

E. BIOGENESIS OF EUKARYOTIC MESSENGER RNA.

The following discussion is centred on the nature of the products of transcription of structural genes, rather than the mechanism of transcription *per se*. A significant feature of eukaryotic RNA synthesis is that the site of synthesis is the nucleus whilst the site of action in the

cytoplasm is a separate subcellular compartment. Thus there will be in the nucleus RNA species which are functional precursors of cytoplasmic RNAs.

The proof that a precursor-product relationship exists between two RNA molecules, or populations of molecules, requires that they both possess a common sequence, and that one is converted to the other *in vivo*. This has been clearly demonstrated for ribosomal and transfer RNA synthesis (for a recent review, see Perry, 1976), where the cytoplasm species can be shown to be derived by specific nucleolytic cleavage of species of higher molecular weight. Processing of these RNAs also requires modification, for example by methylation, of specific nucleosides.

Studies on mRNA synthesis in eukaryotes are complicated by the complexity of the mRNA population. (Bishop *et al.*, 1974a; Galau *et al.*, 1974; Levy W. and McCarthy, 1975), even in cells where a limited number of proteins dominate the protein synthesis of the cell (Axel *et al.*, 1976; Monahan *et al.*, 1976). The question of whether precursors analogous to those for rRNA or tRNA occur for mRNAs has been examined using these heterogeneous mRNA populations, and occasionally using mRNAs of defined function where these have been purified.

Early experiments (Warner *et al.*, 1966; Soeiro *et al.*, 1966; Attardi *et al.*, 1966) demonstrated the presence of a class of RNA, located in the nucleus,

which was rapidly labelled with radioactive RNA precursors. This RNA was of apparently high molecular weight, and was heterogeneous in size. The base composition was different to that of rRNA, and its precursors, and resembled that of the mRNA, and DNA, of the cells used. This RNA was found to be metabolically unstable, and it was suggested that it may function as a precursor to the cytoplasmic mRNA.

Attempts to prove a precursor-product relationship between the heterogeneous nuclear RNA (hnRNA) and messenger RNA are complicated by the observation that only a small fraction, perhaps 10%, of radioactively labelled hnRNA ever appears in the cytoplasm (Soeiro et al., 1968). Furthermore, even where a cell synthesises predominantly one or two mRNA species, the nuclear RNA shows a highly heterogeneous distribution of molecular weights (Attardi et al., 1966; Scherrer et al., 1966).

If hnRNA represents a precursor to messenger RNA, it should contain a significant proportion of primary transcription products, which would be characterised by a 5'-terminal triphosphate group. It is known that eukaryotic mRNA, with the exception of a limited number of viral mRNAs (e.g. polio virus; Hewlett et al., 1976; Nomoto et al., 1976) have highly modified 5'-terminal sequences (Adams and Cory, 1975; Furuichi et al.,1975b; Perry et al., 1975a), suggesting extensive post-transcriptional modification. Nuclear RNA molecules contain similar modified 5'-termini (Perry et al., 1975b;

Salditt-Georgieff et al., 1976; Schibler and Perry, 1976) but also contain some 5'-terminal triphosphorylated nucleosides (Ryskov and Georgiev, 1970; Georgiev et al., 1972; Schibler and Perry, 1976; Schminke et al., 1976), which suggests that hnRNA contains both primary transcription products and partially processed molecules. Analysis of the 5'-terminal nucleotides of both types of hnRNA molecules (Schibler and Perry, 1976) reveals that in at least some cases the modified 5'-termini are found on molecules from which a 5'-terminal nucleotide sequence has been removed by enzymic cleavage.

From this finding, it seems that primary transcription products are frequently longer than the processed molecules. Defining the size of hnRNA has proved to be a complex problem. While early studies (such as those described above) suggested that hnRNA could have species of very high molecular weight included within it, Bramwell (1972) proposed that these species could arise as a consequence of aggregation of smaller molecules. The apparent size of hnRNA may also vary because of conformational effects (Bramwell, 1974). When the size of hnRNA is investigated under denaturing conditions to suppress spurious aggregation, it is found that it contains species of higher molecular weight than mRNA - the hnRNA may vary from 20 - 25% longer than mRNA as in the slime mould Dictyostelium discoideum (Firtel and Lodish, 1973) to 3-5 x larger in higher eukaryotes

(Holmes and Bonner, 1973; Bramwell, 1974; Kung, 1974). Although it is possible that the lower molecular weight estimates obtained after denaturation could be due to exposure of hidden breaks in regions of high secondary structure, it has been found that some primary transcripts also contain a 3'-terminal poly(A) sequence (Schminke et al., 1976). These molecules are relatively short after denaturation, which suggests that denaturation destroys aggregates of molecules rather than exposing hidden breaks.

If nuclear RNA is to contain mRNA precursors, it will therefore contain RNA sequences normally associated with the mRNA. A substantial proportion of the cellular mRNA has a sequence of polyadenylic acid (poly(A)) associated with it (for review, see Greenberg, 1975). Some of the mRNA, specifically histone mRNA (Adesnik and Darnell, 1972) but also other species (Greenberg, 1976) does not have a poly(A) tract, although these species constitute a minority of the mRNA. Poly(A) addition is a post-transcriptional event - there are no polythymidylate sequences in the genome which are long enough to account for its synthesis by transcription (Birnboim et al., 1973; Bishop et al., 1974b), and the response of poly(A) synthesis to transcription inhibitors is indicative of a post-transcriptional origin (Darnell et al., 1971; Nakazato et al., 1974). Nuclear RNA has been shown to contain poly(A) (Edmonds et al., 1971; Greenberg and Perry, 1972) although not all molecules are

polyadenylated (Greenberg and Perry, 1972; Nakazato et al., 1973). The proportion of polyadenylated molecules in hnRNA is considerably less than that in mRNA.

Most of the poly(A) synthesis occurs in the nucleus (Jelinek et al., 1973a), although some cytoplasmic synthesis may also take place (Diez and Brawerman, 1974). Attempts to demonstrate a precursor-product relationship between the nuclear and cytoplasmic poly(A) containing RNAs have not yielded clear-cut results (Jelinek et al., 1973a; Perry et al., 1974: Puckett et al., 1975: Brandhorst and McConkey, 1975), as the data obtained are consistent with several models. The different models arise from difficulties in assessing different kinetic parameters; some technical problems are also apparent. It is possible that not all of the polyadenylated RNA in the nucleus ever reaches the cytoplasm (Perry et al., 1974: Brandhorst and McConkey, 1975): a similar conclusion has been drawn from a different approach (see below). One study (Price et al., 1974) relied on the isolation of a small subclass of polyadenylated hnRNA, which had characteristics of being a precursor to mRNA. This RNA was able to be chased into the cytoplasm, and showed simpler behaviour, in terms of kinetics, than bulk hnRNA.

It was thought until recently that if mRNA sequences were present in hnRNA, they would be located at or near the 3'-terminus, where the poly(A) is found in

both types of RNA (Nakazato *et al.*, 1973). However, the presence of modified 5'-termini on large hnRNA molecules (Salditt-Georgieff *et al.*, 1976; Schibler and Perry, 1976) would suggest that in some cases mRNA derives from internal regions of the molecule. It is conceivable that different mRNA sequences could be located at different sites in their respective precursors.

Some sequences have been detected in hnRNA which do not appear to be conserved in mRNA. Molloy *et al.*, (1972) reported that hnRNA contains an oligouridylate sequence in very large hnRNA molecules, and subsequently claimed that this sequence was located near the 5'-terminus of the large molecules (Molloy *et al.*, 1974). Nakazato *et al.* (1974) were able to describe two classes of poly(A) sequence, the 3'-terminal poly(A) whose properties have been discussed above, and a shorter internal oligo(A) sequence. The sensitivity of the latter to RNA synthesis inhibitors, such as actinomycin D, suggests that they are transcribed directly from the genome. The oligo(A) sequence was not detected in mRNA. It is possible, however, that it could serve as a primer for poly(A) synthesis after cleavage of an mRNA sequence from within the hnRNA molecule.

Double-stranded RNA sequences have also been reported (Jelinek and Darnell, 1972; Ryskov *et al.*, 1973b). These structures, which are restricted to the

nuclear RNA population, appear to be transcribed from repeated sequences within the genome (Ryskov et al., 1973a; Jelinek et al., 1974). Double-stranded regions would be ideal markers for processing enzymes. There is some evidence to suggest that sequences associated with mRNA are complementary to one strand of the double stranded sequence (Ryskov et al., 1976).

More direct approaches have been made to the question of a precursor-product relationship, and to the nature of the mRNA sequences conserved, or discarded, on transport to the cytoplasm. With heterogeneous mRNA populations, this is best done by examination of the sequence complexity of the hnRNA and mRNA populations. Estimates of the sequence complexity of hnRNA from sea urchin embryos (Hough et al., 1975) or mouse cells (Getz et al., 1975) reveals that the complexity of the nuclear RNA is higher, by some 5 - 10 fold than that of the cytoplasmic mRNA fractions. This suggests the presence in nuclear RNA of a large number of sequences, perhaps 80 - 90% of those expressed in the cell, which do not eventually appear as cytoplasmic messenger. Similarly, although it is found that there is extensive homology between nuclear poly(A) containing RNA and the polysomal mRNA of Drosophila cells (Levy W. and McCarthy, 1976) and Xenopus liver (Ryffel, 1976), 30% of the nuclear sequences are not observed in the cytoplasmic fraction - in the latter example, this amounts to some 10,000 different

sequences. These studies clearly demonstrate that hnRNA contains two types of RNA sequence, one of which is transported to the cytoplasm, and one which is degraded. The studies do not give an indication of whether the nuclear precursor species are longer molecules than the cytoplasmic ones.

Several mRNAs are well characterised species, which can be readily assayed. Thus, for example, viral mRNA can be detected by hybridisation to the virus DNA. Examination of the synthesis of viral mRNAs has led to the identification of high molecular weight RNA molecules which contain the sequences of SV-40 mRNA (Lindberg and Darnell, 1970), and adenovirus messengers (Wall et al., 1973; Bachenheimer and Darnell, 1975; Craig and Raskas, 1976). Such high molecular weight species may serve as precursors to the cytoplasmic mRNAs.

For eukaryotic cellular mRNAs, precursor-product relationships are not clearly defined. Neither ovalbumin (McKnight and Schimke, 1974) nor silk fibroin (Lizardi, 1976) mRNAs appear to be synthesised as species of higher molecular weight. Thus, for some mRNA, the transcript may serve as the mRNA, or processing by enzyme cleavage may be minimal. Similarly, the RNA transcribed from the Balbiani rings of Chironimus tentans is a very high molecular weight species, which is not processed to a smaller molecule prior to transport to the cytoplasm (Lambert and Edstrom, 1974). A large fraction of the

molecules are not, however, transported at all (Egyhazi, 1976).

Several studies have been made on globin mRNA biosynthesis, which have yielded somewhat conflicting results. Some experiments, employing either translation of mRNA sequence (Williamson et al., 1973; Ruiz-Carrillo et al., 1973; Knochel et al., 1975) or hybridisation with DNA complementary to the globin mRNA (Imaizumi et al., 1973; Spohr et al., 1974) suggested that globin mRNA precursors were of very high molecular weight. On the other hand, several workers have claimed that the mRNA precursor may not be more than 3-fold larger (Macnaughton et al., 1974; Hendrick and de Kloet, 1975; Ross, 1976). Another study suggested that nuclear globin mRNA sequences, of comparable size to the polysomal mRNA, were located in the non-polyadenylated nuclear RNA fraction (Spohr et al., 1976).

The finding of mRNA sequence in a high molecular weight species of RNA does not necessarily imply that the longer molecule is a precursor to the shorter. The studies of Ross (1976) were carried out using pulse-labelled RNA; the "precursor" molecule was approximately twice the size of mRNA, and it was found that label incorporated into the larger species could be chased into the final mRNA. This suggests that the species described is a functional precursor of globin mRNA.

It is clear from the foregoing discussion that

there can be no simple model for a precursor to mRNA, as derived from the experiments performed on heterodisperse RNA. Clearly, if the mRNA sequence can be located at any position in the primary transcript, it is difficult to assign those parts of the transcript which are lost during processing as sequences related to control elements in the DNA, of the type postulated by Britten and Davidson (1969). It is apparent that some polyadenylated sequences do not reach the cytoplasm (Levy W. and McCarthy, 1976; Ryffel, 1976) but whether these are mRNA sequences which would normally be transported to the cytoplasm in another cell type, and are subject to regulation by processes of the type suggested initially by Scherrer and Marcaud (1968), or whether they have a purely nuclear function is not known. Finally, it should be noted that hnRNA is normally associated with protein (e.g. Pederson, 1974), but it is not clear to what extent such proteins are involved in the processing of hnRNA, or in selecting which molecules are transported to the cytoplasm.

F. AIMS OF THE THESIS.

At the time at which this work was begin, there was little data available on the synthesis of keratin mRNA, in relation to the onset of keratin synthesis. The initial aims of the work were, therefore:

1. To define the levels of keratin mRNA in feather cells over the period of keratinisation of the

feather, and thus to examine to what extent keratin synthesis is controlled at a post-transcriptional level.

2. In the course of the above work, to examine the levels of total RNA and DNA in the feather, to characterise any role which they may play in keratinisation.

Furthermore, the mode of synthesis of keratin mRNA was to be examined, with a view to locating keratin mRNA containing molecules of higher molecular weight, and to determining whether or not these species could serve as precursors to the cytoplasmic mRNA.

CHAPTER 2

MATERIALS AND GENERAL METHODS.

A. MATERIALS

(i) Proteins and enzymes.

Bovine Serum Albumin: Fraction V. Sigma Chemical Co., St. Louis, Missouri.

Chicken erythrocyte histone: prepared by the method of Murray et al., (1968): gift from Dr. R. Harlow of this department.

Deoxyribonuclease I: Worthington Biochemical Corpn., Freehold, New Jersey.

Pancreatic ribonuclease: Type III, Sigma.

Proteinase K: E. Merck, Darmstadt, W. Germany.

RNA dependent DMA polymerase: gift of Dr. J.R.E. Wells, prepared from avian myeloblastosis virus donated by Dr. J. W. Beard and the NIH Cancer Program.

S-carboxymethylkeratin: predared by the method of Kemp et al., (1974a) from newly-hatched chicken feathers.

Trypsin, Difco 1:250 : Difco Laboratories, Detroit, Michigan.

(ii) Radiochemicals.

[^{3}H]-deoxycytidine triphosphate (26.2 C/m mol): the Radiochemical Centre, Amersham, Buckinghamshire, England.

α-[^{32}P]-deoxycytidine triphosphate (initial specific activity 39 C/mmol): prepared by Dr. R.H. Symons of this department.

5, 6-[^{3}H]-uridine (40 C/mmol): Schwarz-Mann, Orangeburg, New York.

(iii) Chemicals for specific procedures.

(a) polyacrylamide gel electrophoresis.

Acrylamide: Merck, twice recrystallised from $CHCl_3$

N,N'-methylenebisacrylamide: BDH, recrystallised from $CHCl_3$

N,N,N',N'-tetramethylethylenediamine: Eastman Organic Chemicals, Rochester, New York.

Formamide: BDH, deionised as described by Pinder et al., (1974).

Amido Black (Naphthalene Black): George T. Gurr, London, England.

Coomassie Brilliant Blue R: I.C.I. Ltd., England.

Stains-all (1-ethyl-2-[3-(1-ethylnaphtho[1,2d]thiazolin-2-ylidene)-2-methylpropenyl]-naphtho[1,2d] thiazolium bromide): Eastman.

Toluidine Blue: Gurr's.

(b) Complimentary DNA synthesis.

Actinomycin D: Gift of Merck, Sharpe and Dohme.

Deoxyribonucleoside triphosphates: Sigma.

Dithiothreitol: Sigma.

Oligodeoxythymidylic acid, free acid:

P. L. Biochemicals Inc., Milwaukee, Wisconsin.

(c) Nucleic acid estimations.

Deoxyribonucleic acid, from calf thymus (Type V): Sigma.

Deoxyribonucleic acid, from soft roe of coalfish (Type V1): Sigma.

Diphenylamine: Merck.

Ethidium bromide: Sigma.

Orcinol: BDH

Ribonucleic acid, from Torula yeast (Grade V1): Sigma.

(d) Radioactive counting.

NCS solubiliser: Amersham/Searle corpn., Arlington Heights, Illinois.

POPOP (1,4-bis-(2,5-phenyloxazolyl)-Benzene): Sigma.

PPO (2,5-diphenyloxazole): Sigma.

(iv) Miscellaneous materials.

Diethylpyrocarbonate: Sigma.

Ethylene glycol-bis-(β -aminoethyl ether)-N,-N'-tetra acetic acid (EGTA) : Sigma.

Foetal calf serum: Commonwealth Serum Laboratories, Melbourne, Australia.

Gum Arabic: purified as described by Kuehl (1964).

Iodoacetic acid: Sigma, recrystallised from petroleum

ether (B.P. 60 - 80°), and stored in the dark.

Nonidet P-40 (NP40) : BDH.

Oligo(dT)-cellulose: Gift from Dr. J. Mercer.

Phenol: BDH, redistilled under N_2 and reduced pressure, stored at -15° under N_2 prior to use.

Sarkosyl (sodium dodecyl sarcosinate): Ciba-Geigy, Basle, Switzerland.

Sodium dodecyl sulphate: BDH, 99% pure.

Sucrose, Ultrapure, RNase free: Schwarz-Mann.

Waymouth's medium MB 742/1, dried powder: Difco.

All other chemicals used were of analytical reagent grade, or of the highest available purity.

(v) Preparation of solutions.

All solutions were prepared in glass-distilled water, and treated with diethylpyrocarbonate, followed by autoclaving, to eliminate any contamination with ribonuclease. Glassware was rendered RNase-free by either autoclaving, incubation at 110° overnight, washing with 1 M KOH followed by rinsing with sterile glass-distilled water, or by a combination of these procedures. Spatulas, etc., were washed with alkali and rinsed in sterile water, as described. Pipettes and micropipettes were washed in glass-distilled water containing diethylpyrocarbonate and dried for 16 hours at 110°.

B. TISSUE.

Fertilised eggs of White Leghorn fowls (*Gallus domesticus*), strain Para. 3 were obtained from the Parafield Poultry Research Station of the Department of Agriculture, Parafield, South Australia. The eggs were stored at 10° for no more than seven days, and incubated at 37°, 54% humidity in a forced draught incubator (Saunders Products Pty. Ltd., Adelaide). Feathers designated, for example, as "14-day feathers" were from embryos incubated for a total of 14 days.

Embryos were removed from the eggs, and washed either with Hanks balanced salt solution, or with NKM (150mM NaCl, 5mM KCl, 2mM $MgCl_2$). They were then immersed in the same solution, and the feathers removed by plucking with jewellers' forceps. The feathers were then washed (in either Hanks solution or NKM) by repeated low speed centrifugation. Where it was desirable that the feathers sustain minimal structural damage during preparation (e.g., when tissue was required for microscopy), fragments of skin, with feathers attached, were removed from the embryo, washed, and the feathers gently teased from the skin with forceps.

C. GENERAL METHODS.

(i) Estimation of nucleic acid concentration.

Nucleic acid concentrations were measured by

absorbance at 260nm, using a Zeiss PMQ II spectrophotometer. Extinction coefficients of 22 and 25 were used for 1 mg/ml solutions of native DNA and RNA, respectively. Where specific colorimetric methods were used, they have been described in the appropriate chapters.

(ii) Electron microscopy.

Samples were fixed for 1 hour in a solution of 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, then washed in 0.18 M sucrose, 0.1 M sodium cacodylate for 30 minutes. Post-fixation was in a solution of 2% (w/v) osmic acid in 0.1 M sodium cacodylate for 30 minutes, followed by a wash in 1% (w/v) uranyl acetate and dehydration with graded acetones. The samples were embedded in araldite, and sectioned with an LKB microtome, using glass knives. The sections were picked up on carbon-coated grids, and stained with lead citrate for 15 minutes. The sections were examined in a Siemens Elmiskop I microscope.

(iii) **Preparation of purified keratin mRNA**

Messenger RNA was routinely prepared in this laboratory by several alternative methods, as described below:

In some preparations, mRNA was isolated from the mRNP particles produced by EDTA dissociation of polysomes, as described by Kemp *et al.*, (1974b), and

purified by repeated sucrose gradient centrifugation.

Keratin messenger RNA was also isolated using chromatography on oligo(dT)-cellulose. Polysomes, prepared as described by Partington et al., (1973), were dissociated in 0.5 M NaCl, 0.5% SDS, and the RNA-protein solution applied directly to oligo(dT)-cellulose (Krystosek et al., 1975). The column was washed in the same buffer to remove unbound material, and the bound RNA eluted with 10mM tris-HCl(pH 7.4) 1mM EDTA. The "bound" RNA was concentrated by precipitation with 2.5 volumes of ethanol, after first adding 0.1 volume of 1 M Na acetate (pH5). The RNA was collected, after incubation at -20° for several hours, by centrifugation at 25,000xg for 30 minutes, and dried in vacuo. The RNA was then dissolved in 0.1 M tris-HCl(pH 9.0), 0.1% SDS, heated to 60° for 10 minutes to reduce aggregation, and aliquots loaded onto 11.2ml linear sucrose gradients (10 - 40% sucrose in 0.1 M NaCl, 1mM EDTA, 10mM tris-HCl,pH 7.4). Gradients were centrifuged for 16 hours at 178,000xg, 4° in a Beckman SW41 motor, and fractionated using an ISCO model 640 gradient fractionator. The peak of material sedimenting at 12S was collected, and precipitated with ethanol as described. The 12S RNA was purified by at least one further sucrose gradient centrifugation.

Alternatively, the polysomes were dissociated

in 0.1 M NaCl, 0.1 M tris-HCl(pH 9.0), 1% sarkosyl, and the RNA partially purified by twice extracting with an equal volume of phenol-$CHCl_3$ (1:1) followed by precipitation with 2.5 volumes of ethanol. The RNA was collected by centrifugation, and dissolved in 0.5 M NaCl, 10mM tris pH 7.4, 0.1% SDS for oligo(dT)-cellulose chromatography. The bound fraction was eluted, and mRNA purified, as described above.

(iv) Synthesis of keratin cDNA.

DNA complementary to keratin mRNA was synthesised using the DNA polymerase from avian myeloblastosis virus in the reaction mixture described by Kemp (1975), with deoxycytidine as the labelled nucleoside. The highest specific activity [^{3}H]-dCTP used (26.2 C/mmol) yielded cDNA with a specific activity of approximately 10^7 cpm per μg where cDNA of lower specific activity was required, the [^{3}H]-dCTP was diluted with unlabelled triphosphate. After incubation for one hour at 37°, the RNA template was removed by digestion with 0.3 M NaOH for 2 hours at 37°, and the cDNA separated from unincorporated triphosphate by chromatography on Sephadex G-50, in 0.2 M NaCl, 1mM EDTA 10mM tris-HCl, pH 7.4, followed by precipitation with ethanol (as described in (iii), above).

The cDNA produced by this procedure was generally of a molecular weight comparable to that of

12S keratin mRNA, as judged by polyacrylamide gel electrophoresis in 98% formamide (Pinder et al., 1974). Gel electrophoresis of a typical preparation is shown in Figure 2.1.

(v) Hybridisation reactions.

To minimise the effects of evaporation and of adhesion of nucleic acid species to glassware, all reactions were performed either in silicone-treated glass tubes, with the reaction mixture overlaid with paraffin oil, or in sealed, siliconised capillary tubes. Where DNA was present in the reaction, the nucleic acid was sonicated to shear the DNA. Hybridisations were carried out in 0.18 M NaCl, 1mM EDTA, 10mM tris-HCl (pH 7.4), 0.5% SDS - the mixtures were first denatured at 100° for 5 minutes, then incubated at 60° to the desired $C_o t$ (where the reaction was RNA-driven, this has been called $R_o t$). Unless specifically stated, hybridisations were performed under conditions where the unlabelled species was in excess over the cDNA probe.

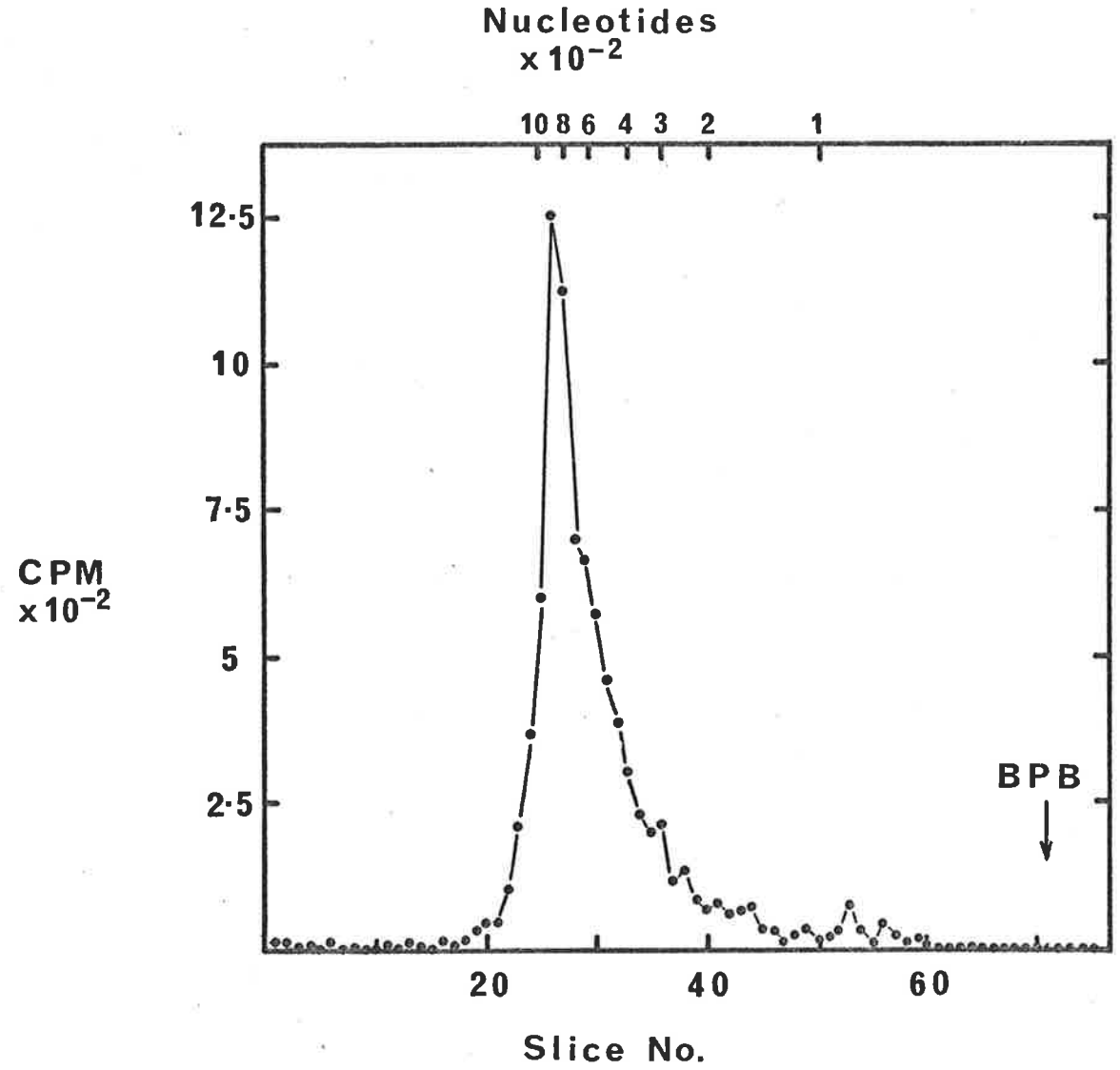
In experiments where the kinetics of hybridisation were investigated, different $C_o t$ values were achieved either by using a series of dilutions of the nucleic acid solution, or by taking aliquots from a single reaction mixture at appropriate time intervals. In some cases, particularly where a wide range of $C_o t$ values was required, a combination of both these methods

FIGURE 2.1.

Molecular Size of Keratin cDNA.

15,000 cpm of keratin cDNA, specific activity 10^6 cpm/μg, synthesised as described in the text, was dried *in vacuo*, dissolved in 25 μl of loading solution (Pinder *et al.*, 1974) and denatured at 60° for 1 minute. 20 μl was electrophoresed onto a single formamide-acrylamide gel (Pinder *et al* 1974), the gel then being soaked in sterile water for 2 hours, after which it was frozen, and sliced into 1 mm slices. The slices were dissolved in NCS-scintillation fluid (see text), and counted. The length, in nucleotides, was determined from the mobility of marker RNAs (28S, 18S, 5.8S and 5S rRNA, plus tRNA) in parallel gels.

The position of the bromophenol blue marker is indicated (BPB).


Nucleotides
x 10⁻²
10 8 6 4 3 2 1
12·5
10
7·5
5
2·5
CPM
x 10⁻²
BPB
20
40
60
Slice No.

was used.

The extent of hybridisation was assayed using the single-strand specific nuclease of Aspergillus oryzae (Ando, 1966). Hybridisation mixtures were diluted into (low salt) buffer (50mM NaCl, 30mM Na acetate, pH 4.6, 1mM $ZnSO_4$, 5% glycerol), divided into equal parts, and partially purified S_1 nuclease added to one half. (The enzyme had been purified to the end of step 4 as described by Vogt (1973)). Two units (Vogt, 1973) of S_1 nuclease were added per 6 µg DNA in the reaction - where cDNA comprised the only DNA in the reaction, 6µg of denatured calf thymus DNA was included as carrier. Digestion was carried out at 45° for 30 minutes. In some cases, the digestion was performed in high salt (300mM NaCl) buffer, at a temperature of 37°. Such cases are indicated at the appropriate places in the text. The extent of hybridisation was calculated from the acid-insoluble radioactivity in the nuclease S_1 treated and untreated samples.

(vi) Determination of radioactivity.

In general, samples were precipitated with 10% trichloroacetic acid, in the presence of bovine serum albumin as carrier, at 0°. After 30 - 60 minutes, the precipitates were collected on glass-fibre filters (Whatman, GF/A). The filters were then washed twice with 5 ml of 10% TCA, then extensively with ether,

placed into 2.5 ml glass vials and dried at 110°. Toluene-based scintillation fluid (3.5g PPO, 0.35g POPOP per litre of toluene) was then added, and the samples counted in a Packard liquid scintillation spectrometer.

In cases where small volumes (up to 10 µl) of aqueous samples were to be counted, these were dissolved in 2 ml of "toluene-triton" scintillation fluid (5 volumes of toluene scintillation fluid plus 3 volumes triton X-100).

Radioactivity in gel slices was determined after solubilising each slice with 0.2 ml NCS solubiliser plus 0.025 ml of 8M NH_4OH, to which was added 2 ml of scintillation fluid.

Samples were counted for as long as was feasible (at least 5 minutes each, but usually 10 or 20 minutes) to minimise counting errors.

Footnote:

Where the work concerned relative levels of a molecular species within the tissue for example at different ages, replicate experiments were performed and, in each experiment, at least duplicate estimations were carried out. The values between experiments did not differ by more than the range of difference between the duplicates within each experiment, i.e., by no more than ± 10%.

CHAPTER: 3

DNA IN THE DEVELOPMENT OF THE EMBRYONIC CHICK FEATHER

A. INTRODUCTION.

Morphological studies (reviewed in Chapter 1) have shown that feather growth is first evident at about 8 days, when it is characterised by a condensation of dermal cells over which epithelial cells have begun to proliferate. By 10 days the feather germs are clearly identifiable as raised lumps, in which the cells are rapidly dividing. From 11 - 13 days the tissue undergoes an extensive increase in length (Figure 1.1), after which elongation continues, but at a slower rate. Kischer and Furlong (1967) have observed maxima in the specific activity of DNA polymerase in whole skin extracts, at stages corresponding to the period of rapid cell division and tissue growth at 10 and ~12 days. The later elongation of the tissue has been attributed (Watterson, 1942; Matulionis, 1970) more to changes in cell volume and shape than to cell divisions.

Kemp et al (1974a) were unable to detect keratin in cells which were capable of DNA synthesis (as judged by the ability to incorporate [^{3}H]-thymidine), using electron microscopy of 12-day feathers. This suggests that DNA synthesis and keratin synthesis are mutually exclusive events in the feather, such that keratin synthesis is possible only after a final cell division, of the "quantal" type proposed by Holtzer et al., (1972, 1973).

The effect of such a quantal mitosis would be to make available for transcription portions of the genome

which were not previously exposed; in this case, the keratin genes. Kemp (1975) has shown that the haploid chick genome contains 100 - 240 keratin gene sequences, of which some 25 - 35 are expressed in the embryonic feather. Walker and Rogers (1976a, b) have calculated that there are at least 22 different keratin proteins synthesised in the tissue, and that these are likely to be the products of different genes. The presence of multiple genes in the tissue may be necessary to allow a high rate of production of messenger RNA species. However, it is possible that the high rate of keratin synthesis observed in the tissue (Kemp et al., 1974a) may require an overproduction of the genes, to give a larger number of sequences which are available for transcription. This type of amplification has been observed, for example, in the ribosomal RNA genes of Xenopus oocytes (Brown and Dawid 1968; Perkowska et al., 1968). In contrast, the structural genes for several proteins are present in one, or a few copies per haploid genome, and are not amplified in the tissue in which they are expressed; examples are the genes for globin, in a number of species (Bishop et al., 1972; Packman et al., 1972; Paul et al., 1973; Harrison et al., 1974; Gambino et al., 1974) for ovalbumin (Sullivan et al., 1973; Harris et al., 1973), and for chick δ-crystallin (Zelenka and Piatigorsky, 1976). Histone gene sequences, on the other hand, are reiterated (Kedes and Birnsteil,

1971; Wilson et al., 1974; Scott and Wells, 1976; Jacobs, 1976) in the genome, which may represent a crude form of gene amplification. An even cruder form of amplification is demonstrated by the gene for silk fibroin, which is present in only one copy per haploid genome (Suzuki et al., 1972; Gage and Manning, 1976), although the cells in which the gene is expressed are polyploid (Suzuki et al., 1972), thus effectively amplifying the entire genome.

While it is possible that amplification of the keraton genes may occur at about the stage of the onset of keratin synthesis, an equally possible alternative is that DNA may be lost from the tissue. Kischer and Furlong (1967) demonstrated an increase in DNase activity at about the time when keratin synthesis begins. This rise in DNase activity suggests that DNA may be being degraded during keratinisation.

In this chapter the gross content of DNA in the tissue has been quantitatively measured, in an effort to determine at which stage mitosis ceases during growth of the tissue, and, indirectly, how much of the feather growth may be attributed to changes in cell volume. The question of whether or not DNA is lost from the cells, the stage at which this might occur, and the possibility of specific gene amplification in the keratinising cells was also investigated.

B. METHODS.

(i) DNA content of feather tissue.

(a) Relatively large tissue volumes.

After plucking and washing the feathers, the packed volume was determined as previously described (Kemp et al., 1974a). The tissue was then homogenised in 0.1 M NaOH, and the macromolecular constituents precipated by the addition of an equal volume of 1M $HClO_4$. The precipitate was collected by low speed centrifugation, dissolved in 0.3M NaOH, and incubated for 3 hr. at 37°, after which the solution was again adjusted to 0.5 M in $HClO_4$, and the insoluble material collected by centrifugation. The pellet was redissolved in 0.1 M NaOH, and the DNA content of aliquots measured by the method of Burton (1956).

Other methods of solubilization of the tissue, e.g. the homogenates prepared in isolating nuclei (Chapter 5) were also employed. The values obtained by these procedures were indistinguishable from those obtained by the method above, and are included in the results presented.

(b) Single feathers.

Individual feathers were carefully plucked from well-washed embryos, and each separately incubated in 0.1 ml of 50 µg/ml proteinase K, as described by Gross-Bellard et al., (1973), at 37° until dissolved. NaCl and ethidium bromide were

then added to final concentrations of 0.5 M and 5 μg/ml, respectively, in a total volume of 0.5 ml. 0.1 ml of 1 M KCl was then added, the samples cooled to 4°, and the precipitate of potassium dodecyl sulphate removed by low speed centrifugation.

Total nucleic acid concentration in the supernatant was measured by ethidium bromide fluorescence by the method of LePecq and Paoletti (1966), using a Perkin-Elmer model 203 fluorescense spectrometer. DNA concentration was measured after panereatic RNase digestion (10 μg/ml, 7 hr. at 37°) of the nucleic acid. the standard solutions of calf thymus DNA were treated in the same manner as the feather digests.

(ii) DNA content of single cells.

Pieces of skin with feathers attached were removed from the embryo, washed in NKM, and fixed in methanol-acetic acid (3:1, v/v). Individual feathers were then removed by carefully teasing them off the skin, washed successively in 75%, 50%, 25% aqueous methanol, water, and finally incubated in 1 M HCl at 60° for 15 minutes. After incubation the feathers were washed in water, placed onto microscope slides with a drop of water and cut with a razor blade into three approximately equal segments, which were then separated on the slide. The tissue was then squashed under a coverslip (which was subsequently removed), and stained by the Feulgen procedure. The stained squashes were then dehydrated

and mounted. Appropriate nuclei were then scanned with an integrating microdensitometer (Barr and Stroud Ltd., Great Britain) at 565 nm. Cells clearly identifiable as erythrocytes in the squashes served as internal standards for quantitation of the DNA.

(iii) Deoxyribonuclease content of embryonic feathers.

Tissue extracts were prepared as described by Kischer and Furlong (1967), except that 1% NP-40 was included in the homogenising buffer.

The activity of DNase was measured by the amount of material absorbing at 260 nm which was released from native coalfish DNA as species soluble in 0.1 M HCl, 50% aqueous ethanol. Incubations were typically carried out for 3 hr. at 37°. A blank containing buffer instead of enzyme was always included, and this value was deducted from the observed values. Assays were routinely performed in duplicate.

Acid DNase was measured in 0.1 M Na acetate, pH 5, in the absence of $MgCl_2$ (see results below). Neutral DNase was assayed at pH 7.5, essentially as described by Kischer and Furlong (1967), except that phosphate and β-mercaptoethanol were omitted, and the system buffered with 25 mM tris acetate, pH 7.5.

(iv) Preparation of DNA for hybridisation.

DNA was isolated from preparations of total nucleic acid (Chapter 4, B (iii) (a)). The samples

were hydrolysed in 0.3M NaOH for 18 hr. at 37°, neutralised with HCl, and the DNA precipitated with 2.5 volumes of ethanol. The precipitate was collected by centrifugation, and dissolved in buffer for hybridisation (see chapter 2, C(v)).

Hybrids were assayed with nuclease S_1 (Vogt, 1973) in high salt buffer as described by Kemp (1975).

C. RESULTS.

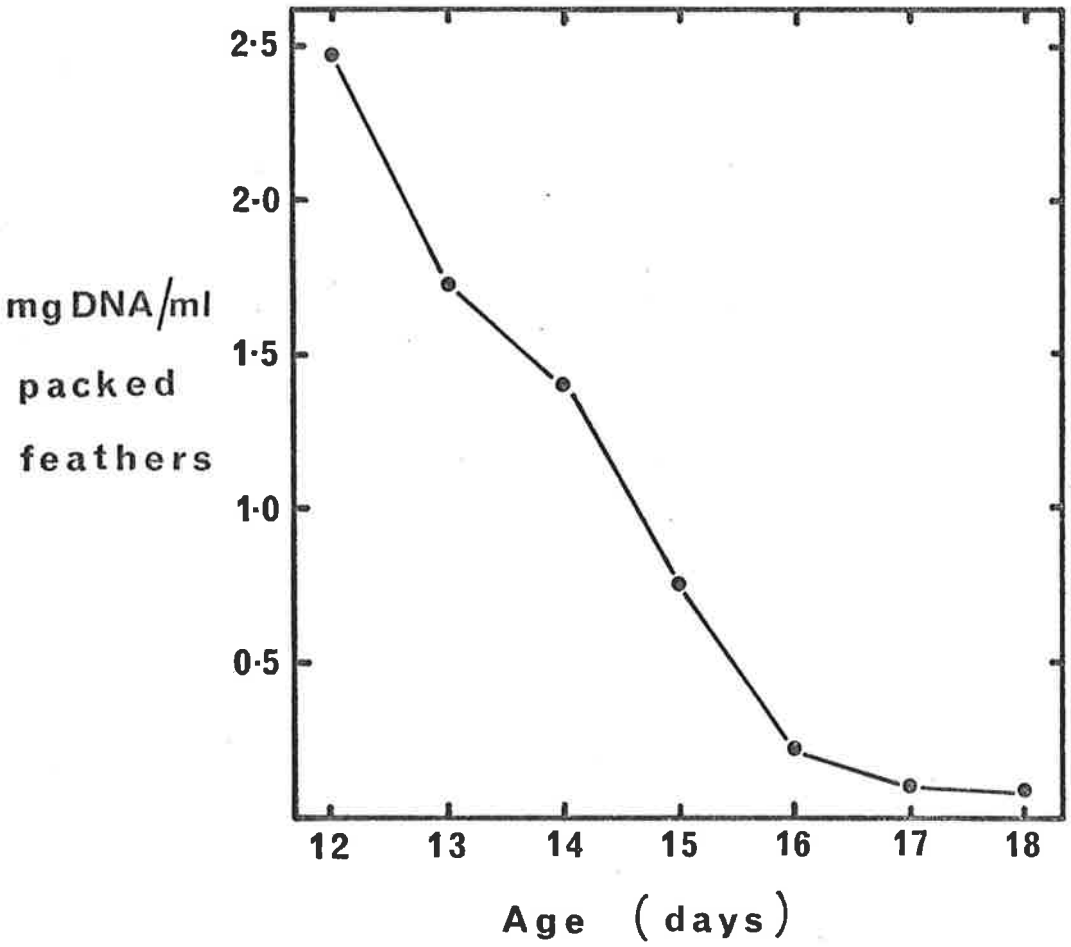
(i) DNA content of feathers during development.

In initial experiments employing large volumes (0.1-1 ml) of tissue, DNA content was expressed as "mg/ml tissue", the criterion used by Kemp et al., (1974a) to describe total protein and keratin content of the developing feather. In that case, the description of tissue content appeared to be quite acceptable, as the protein and keratin contents were seen to increase, in accordance with prediction. When the DNA content is expressed in this manner, there is a steady apparent decline in DNA content after 12 days (Figure 3.1). 12-day tissue was the youngest used in this study, and represents the stage at which keratin synthesis is initiated in the tissue (Matulionis, 1970; Kemp et al., 1974a). Thus if this method of presentation represented the true in vivo situation, it would be necessary to postulate that DNA degradation occurs at the time of the onset of keratin synthesis; which may be in accord with the rise in DNase activity noted by Kischer and Furlong (1967).

FIGURE 3.1.

DNA Content of Feather Tissues.

Feathers were plucked from embryos of different ages, washed in Hank's solution, and the packed volume determined by centrifugation of the tissue in graduated glass centrifuge tubes (Kemp et al., 1974). The tissue was solubilised with 0.1 M NaOH, the macromolecules precipitated with 0.5 M $HClO_4$, and the DNA content of the precipitate determined by the method of Burton (1956). The data for each age are the mean values of at least five estimations.


2·5
2·0
1·5
1·0
0·5
mg DNA/ml
packed
feathers
12
13
14
15
16
17
18
Age (days)

Considerable evidence exists, however, to suggest that this is an inadequate mode of presentation, and that it does not reflect accurately the developmental situation. It is possible to observe normal nuclei in cells at 15 days in which keratin synthesis is well advanced (Figure 3.2), which would suggest that little change in DNA content occurs prior to this stage of development. It is obvious, too, that any quantitation based on tissue volume does not take into account changes in cell volume. Both Watterson (1942) and Matulionis (1970) have observed that much of the elongation of the feathers may be due to an increase in cell volume, rather than to a change in cell number. However, for the change in DNA content observed in Figure 3.1 to be due entirely to an increase in cell volume this would require a change in cell volume of some 30-fold, in the absence of DNA synthesis. Such a change in cell volume would be largely represented by a change in length of the feather; the observed change in length (Watterson, 1942; P. Y. Dyer personal communication) is only about 3.5 fold, a figure considerably lower than that proposed above.

Two factors, therefore, would explain the apparent trend shown in Figure 3.1. Some, but not all, of the apparent decline in DNA may reflect changes in cell volume, whilst there must also be DNA degradation and nuclear degeneration, presumably late in development.

DNA undoubtedly is lost from the feather as the tissue ages. Thus, when 100mg of down feathers from

FIGURE 3.2.

Electron Microscopy of 15-day Feather Nuclei.

Electron microscopy of fixed, sectioned 15-day feathers was performed as described by Chapter 2 (C,(ii)). The cells are shown in cross-section. A - D are sections from near the tip of the feather, whilst E is from near the base of younger (14-day) tissue. The apparent absence of nuclei in some 15-day cells results from the observation that the cells are elongated at this age, so that a section may not include the nucleus.

K - keratin

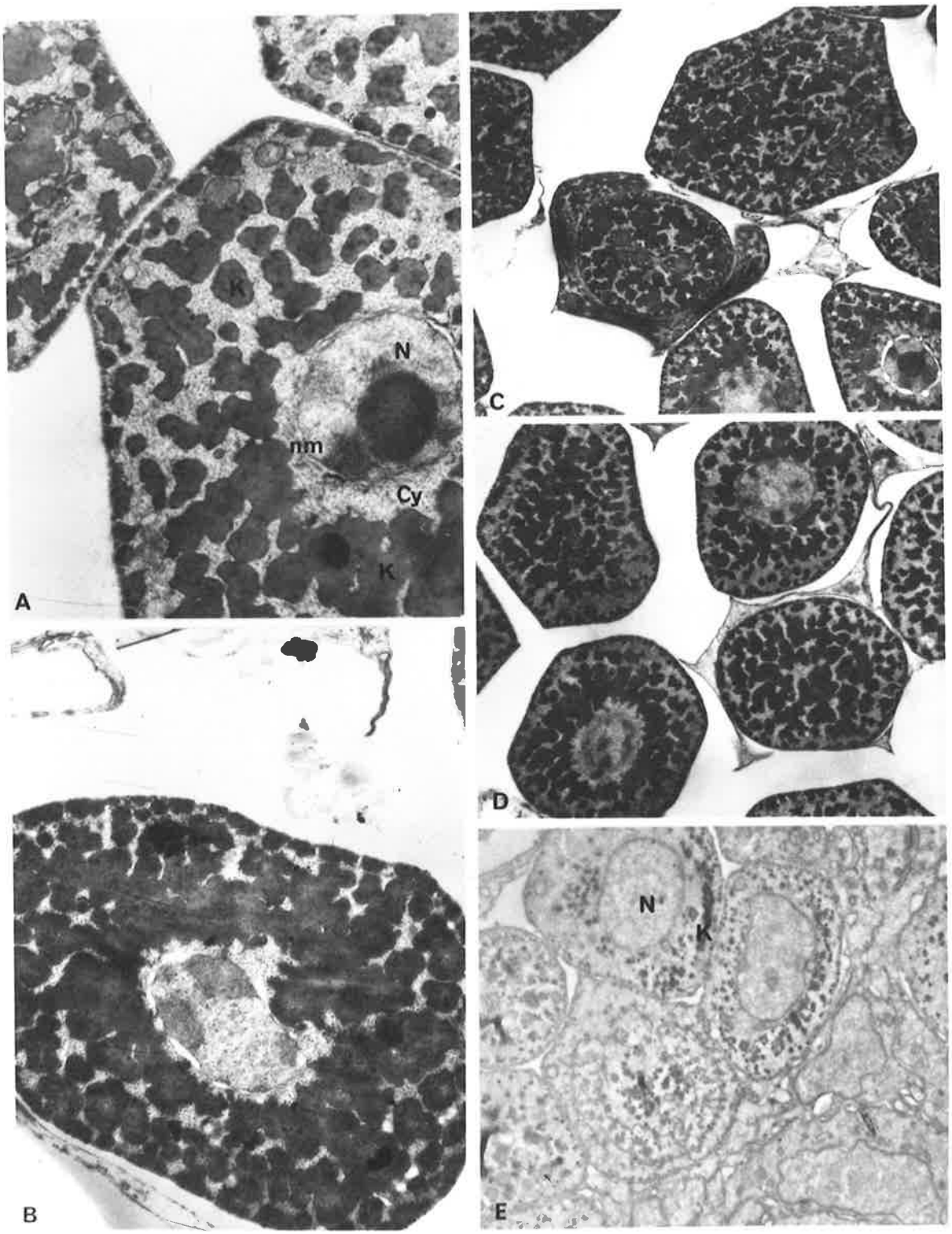
N - nuclei

nm - nuclear membrane

Cy - cytoplasm

Magnification A, B 9960 x

C - E 3320 x


K
N
nm
Cy
K
A
C
D
B
N
K
E

newly hatched (21-day) chicks was treated in the manner described in methods (i)(a), it was impossible to detect any DNA by the diphenylamine reaction (Burton, 1956). Under the conditions employed, 0.2 μg would have been detected.

Individual feathers were thus examined for DNA content to determine to what extent elongation of cells and degradation of DNA were involved in the apparent loss of DNA observed, and at what stage the DNA was lost. The fluorimetric method of LePecq and Paoletti (1966) was used for this quantitation, although problems were encountered in the preparation of samples. Because it is not feasible to use extremes of pH in solubilising the tissue, the feathers were digested with proteinase K (Gross-Bellard et al., 1973). While this readily dissolved the tissue, sodium dodecyl sulphate in the digestion buffer not only inhibited completely the pancreatic RNase digestion, but also led to enhanced fluorescence of the ethidium bromide in the absence of nucleic acid. Accordingly, dodecyl sulphate was removed as the insoluble potassium salt; at low salt concentrations it was found that this treatment effectively precipitated the nucleic acids (Table 3.1). High NaCl concentrations were used to overcome this effect.

The DNA contents of individual feathers from embryos aged 12-18 days were estimated by this procedure (Figure 3.3). The values are the averages of feathers

TABLE 3.1.

Recovery of nucleic acid after removal of dodecyl sulphate with KCl.

NaCl, mM.	Initial A_{260}	Final A_{260}*	% Recovery.
2	.139	0	0
80	.157	.019	12
400	.145	.136	94

Single feathers from a 14-day embryo were dissolved with proteinase K (Gross-Bellard et al., 1973) in a total volume of 0.1 ml. The NaCl concentration of this solution was 10 mM. 0.4 ml of either H_2O, .1 M NaCl or .5 M NaCl was then added, and the absorbance at 260 nm measured using a Zeiss PMQ II spectrophotometer. 0.1 ml of 1 M KCl was then added, the insoluble material removed by centrifugation, and the absorbance re-estimated.

* These values have been corrected for dilution after addition of KCl.

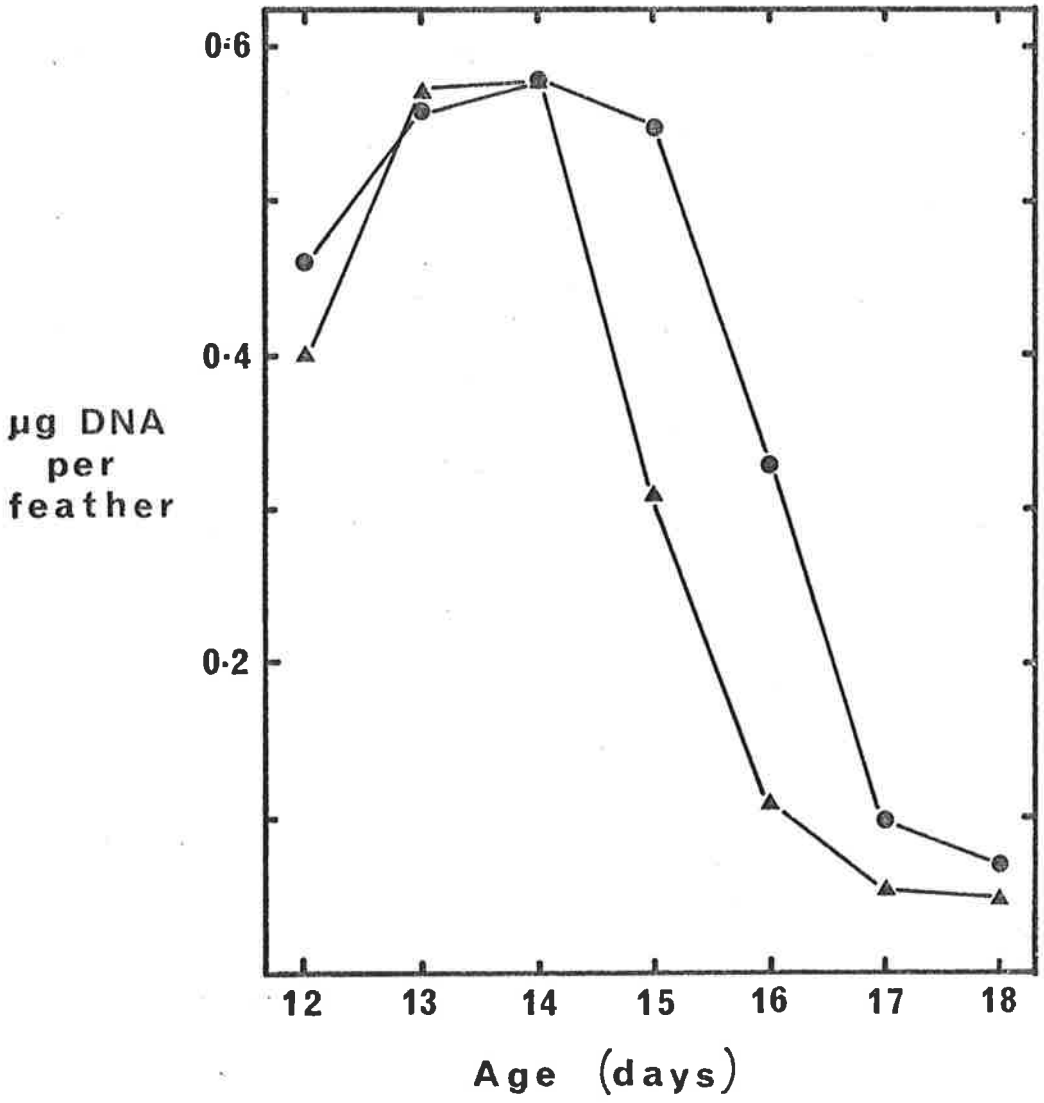
FIGURE 3.3.

DNA Content of Single Feathers.

Individual feathers were plucked, washed, and dissolved by digestion with proteinase K as described in the text. The DNA content of the extracts was determined by ethidium bromide fluorescence, after digestion with pancreatic RNase to eliminate fluorescence due to RNA.

●—● observed results, which are the averages of 10 feathers from 4 birds of each age.

▲—▲ predicted curve, based on the data in figure 3.1, with approximate feather volumes being calculated from the data of figure 1.1.


0·6
0·4
0·2
µg DNA per feather
12
13
14
15
16
17
18
Age (days)

from several embryos at each age, with estimations being performed on at least 10 feathers from each. There is an apparent increase in the DNA content between 12 and 13 days, possibly due to the final rounds of mitosis. It is also apparent that from 15-17 days a sharp decline in the DNA content of each feather occurs. Also shown in the figure is a curve calculated from the data of Figure 3.1., and estimated feather volumes based on feather length (assuming a constant average cross-sectional area during development). The calculated curve has been normalised such that the calculated and observed values at 14 days are identical. In this calculated data, the loss of DNA appears to occur approximately one day earlier: this may be due to differences in the development of the embryos used for each set of experiments, or to the assumptions made in performing the calculations. The conclusions that mitosis finishes at 13 days, and that degradation of DNA begins after about 15 days of development rest on the assumption that there has been no loss of DNA from 14 day feather cells (i.e. that synthesis of DNA at one end of the feather does not parallel degradation of DNA at the other).

The DNA content of individual 14-day feather cells was estimated using Feulgen staining of nuclei in feather squashes. No cells were observed which lacked a stained nucleus - thus, all of the cells have

retained at least some DNA. It was possible to distinguish three major, morphologically distinct types of nuclei in the specimens (Figure 3.4). The small, densely stained nuclei seemed to be associated with erythrocytes from the central capillaries of the feather, the larger, rounded, diffusely stained nuclei with non-keratinised cells, and the elongated nuclei with cells in which keratin synthesis was well advanced.

The content of DNA in the stained nuclei was determined by microdensitometry. Similar distributions in the stain bound per nucleus were observed (Figure 3.5) for all three nucleus types. Each cell at 14 days was therefore considered to have a DNA content equal to that of erythrocytes, i.e. 2.6 pg (Davidson and McIndoe, 1949; Davidson *et al.*, 1950).

This finding that all 14-day feather cells are diploid, taken together with the data of Figure 3.3, enables a calculation of the average cellular DNA content over the development period of the embryonic feather. This calculated data is shown in Figure 3.6, and serves to emphasise the apparent loss of DNA from the tissue on ageing. This loss of DNA might conceivably be due in part to a physical loss of cells from the tissue - erythrocytes, and probably some pulp cells are lost. Nonetheless, it is not feasible to propose that DNA loss reflects entirely a loss of cells, since this would require that some 90% of the cells would be lost by

FIGURE 3.4.

Feulgen Staining of 14-day Feathers.

Squashes of feathers from 14-day embryos were prepared as described in the text, and were stained with Feulgen reagent. After dehydration and mounting, the samples were examined and photographed using a Leitz microscope.

A. Nuclei from a region of the feather in which keratin synthesis is well advanced. The nuclei are diffusely stained, and are elongated (El.)

B. Nuclei from a basal region of the feather. The large, diffuse (L.D.) nuclei originate from feather cells, while the smaller, densely stained nuclei are those of erythrocytes (Er.)

C, D. Nuclei from the basal, and middle regions of the feather, showing further examples of the nucleus types.

Magnification 1950 x

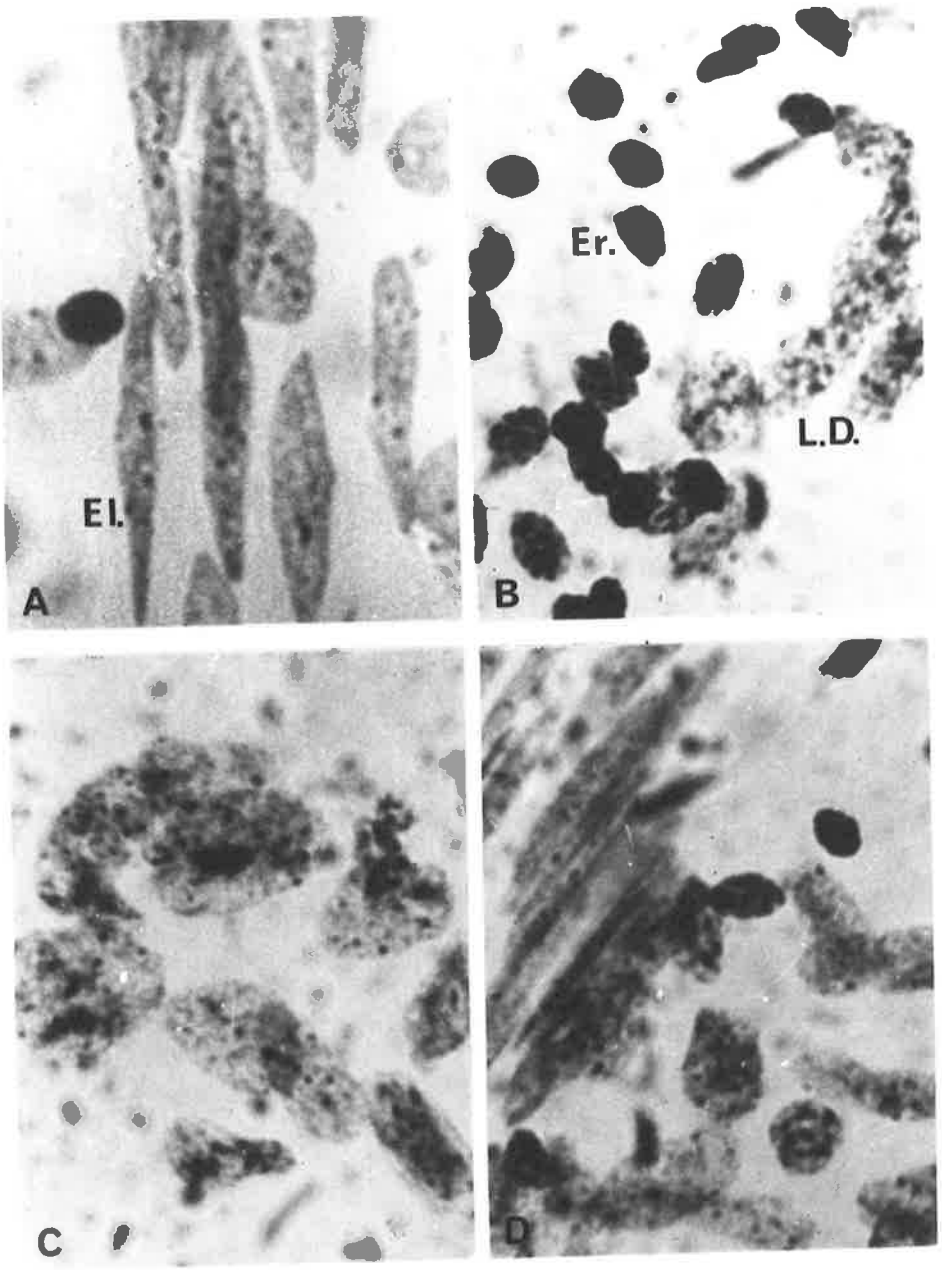

El.
A
Er.
L.D.
B
C
D

FIGURE 3.5.

DNA Content of Feulgen-Stained Nuclei.

Nuclei of the types shown in Figure 3.4, stained with Feulgen reagent were scanned in an integrating microdensitometer (Barr and Stroud, Ltd., Great Britain). Each nucleus was scanned three times, and corrected for background by scanning an adjacent area of cytoplasm. The data are pooled from two 14-day feather-squashes, prepared from different birds.

a. Erythrocyte nuclei. 50 nuclei were scanned; mean densitometer value 50.0 $\pm$ 4.5 (s.e.m.)

b. Large, diffuse feather nuclei. 50 nuclei were scanned; mean densitometer value 50.1 $\pm$ 4.5 (s.e.m.)

c. Elongated feather nuclei. 35 nuclei were scanned; mean densitometer value 49.7 $\pm$ 6.3 (s.e.m.)

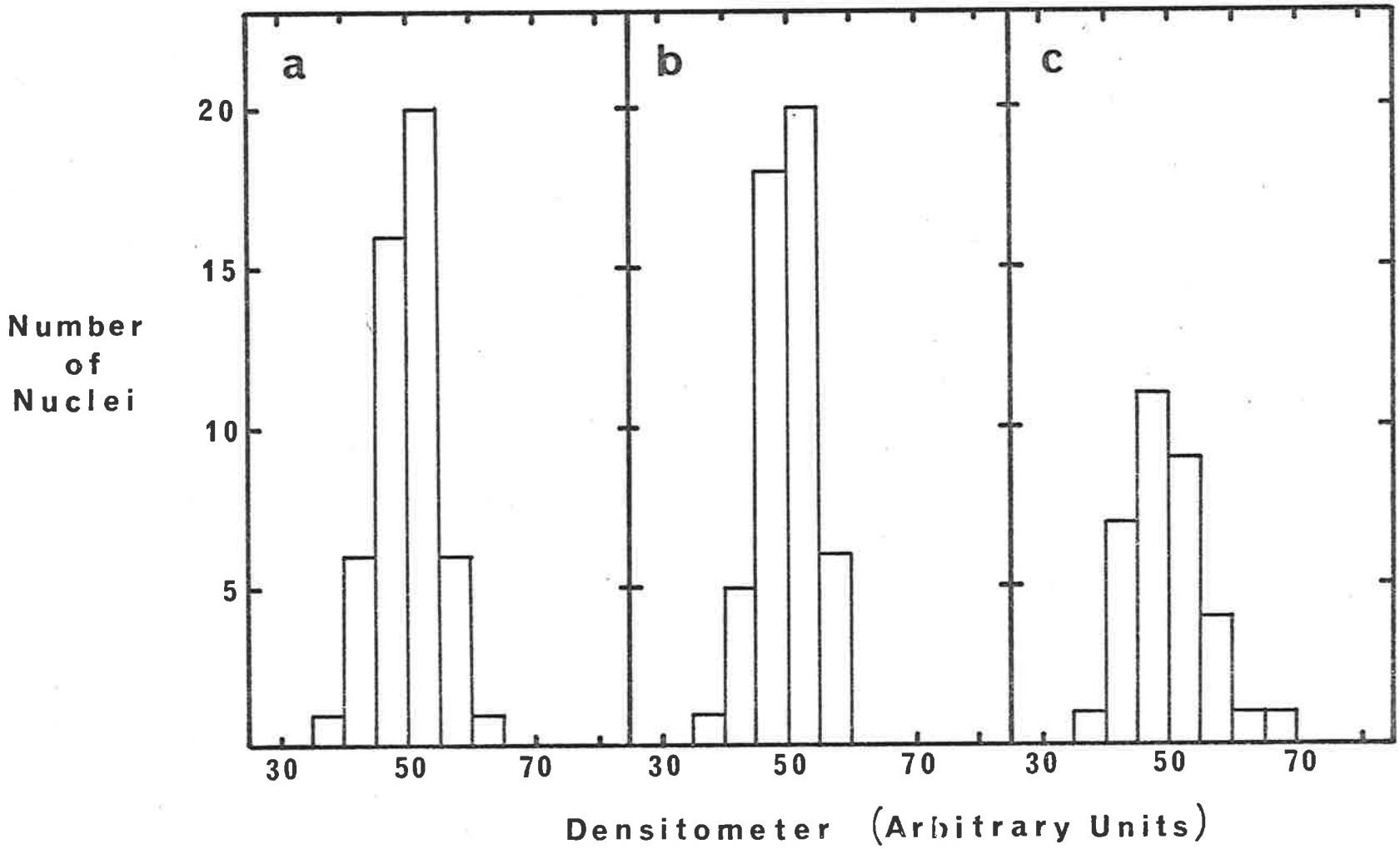
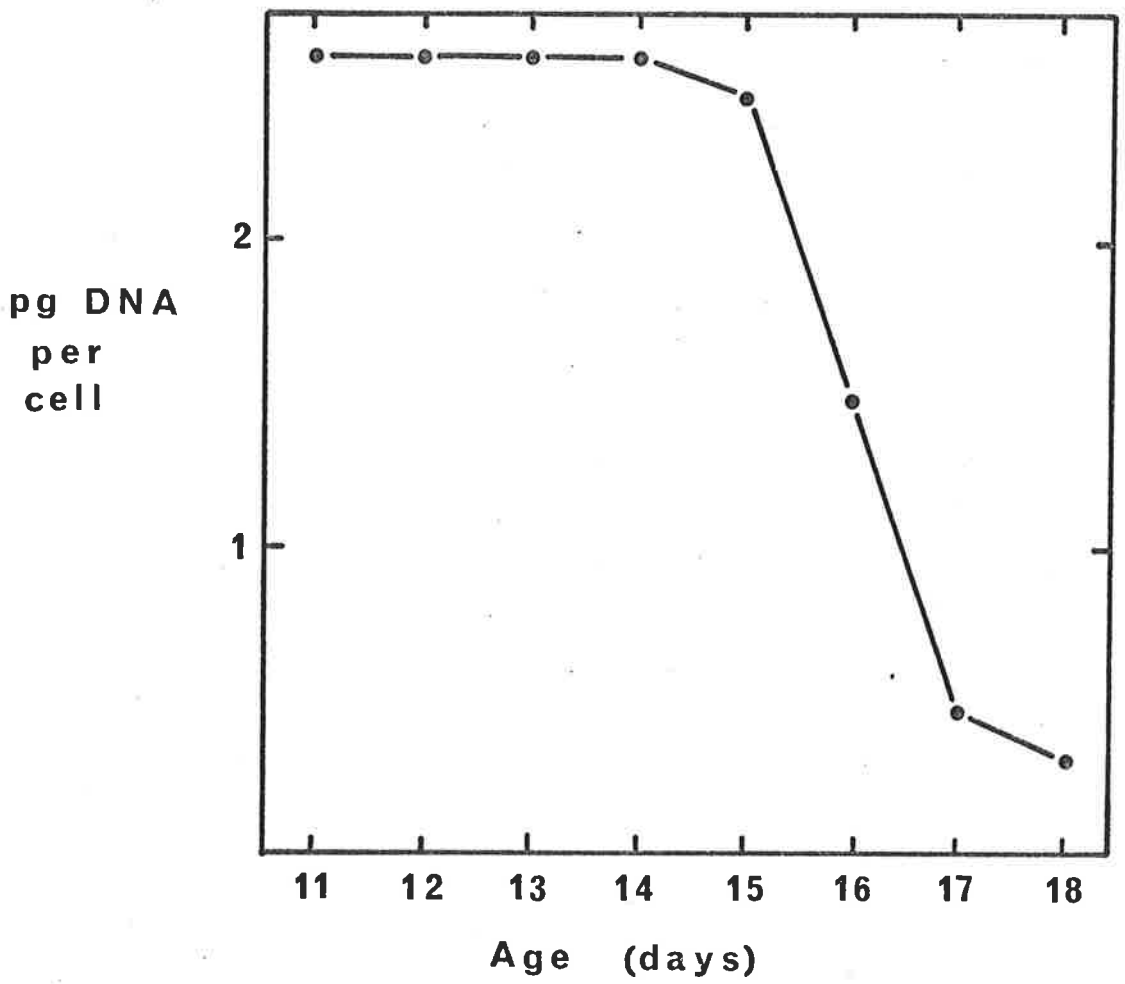

a
b
c
20
15
10
5
30
50
70
30
50
70
30
50
70
Number of Nuclei
Densitometer (Arbitrary Units)

FIGURE 3.6.

Cellular DNA Content in Development.

The cellular DNA content at each age was calculated from the data of figure 3.3, taking the DNA content of cells as being 2.6 pg up to 14-days (at which stage all the cells are still diploid).


pg DNA
per
cell
2
1
11
12
13
14
15
16
17
18
Age (days)

18 days (and an even higher proportion by 21 days).

(ii) Feather deoxyribonucleases.

Kischer and Furlong (1967) reported the presence of two deoxyribonuclease (DNase) activities in extracts from whole embryonic chick skins. This finding was reinvestigated using feathers alone, to determine whether any relationship existed between tissue DNA levels and DNase activity.

The preparation of cell extracts for DNase assay was modified from that of Kischer and Furlong (1967) by the inclusion of 1% NP-40 in the homogenising medium. This gave a higher level of protein in the extracts (~1.7 fold higher protein was obtained), and higher DNase activities. The specific activity of the DNase (units /mg of protein) was unaltered by the inclusion of NP-40 in the extraction, suggesting that the presence of detergent served to solubilise protein which may have ordinarily been trapped in the cell debris.

DNase activity was measured from the absorbance at 260nm of acid/ethanol soluble material released from native DNA, and the date expressed as units of enzyme activity. One unit was defined as the amount of enzyme rendering 1 μg of DNA acid/ethanol soluble in 10 minutes at 37°.

Two DNase activities were present in feather tissue. Under optimal conditions, each gave a linear release of acid-soluble material with time (up to 3 hours

incubation) and enzyme concentration. The conditions of assay were altered from those previously published (Kischer and Furlong, 1967); the effect of these modifications is shown in Table 3.2. As a consequence of these findings, acid DNase was assayed in 0.1 M Na acetate, pH 5.0, in the absence of $MgCl_2$. Neutral DNase was assayed in 25mM tris acetate (pH 7.5), 13.3 mM $MgCl_2$.

The pH optima of the enzyme activities (Table 3.2) suggest that the activities were those detected previously (Kischer and Furlong, 1967). The specific activity of both enzymes increased with age (Figure 3.7a); at 17 days the specific activity was considerably higher than at 16 days. This could be due, in part, to the lower levels of protein extracted at 17 days.

In each case, the extract was prepared from a known volume of tissue; the data for specific activity could then be expressed as units per ml of tissue. A value for the number of cells per ml of tissue was calculated for each age (Table 3.3), using the data in figures 3.1 and 3.6, and the figures so obtained were then used to calculate the DNase activity per cell (Figure 3.7b).

It can be seen (Figure 3.7) that the two DNase activities essentially parallel one another over the period from 12 to 16 days - the neutral DNase shows a slightly higher relative activity at 17 days. The most striking aspect of these results is the dramatic rise in the

TABLE 3.2.

Properties of feather deoxyribonucleases.

A. Acid DNase. 125 µl reaction mixtures contained 50 µg native DNA, 100 µg 16-day extract (total protein), .1 M Na acetate pH 5.0, plus additions as shown. The samples were incubated 3 hrs at 37°, and the digestion stopped by addition of 1 ml of 0.1 M HCl in 50% ethanol. Insoluble material was precipitated at 0° for 30 min. and removed by centrifugation. The absorbances of the supernatants were measured at 260 nm using a Gilford spectrophotometer. Blanks (no enzyme) were deducted. The absorbance values were converted to units - 1 unit converts 1 µg of DNA to acid soluble material in 10 minutes at 37°.
The pH optimum was determined as above, using Na acetate buffers ranging from pH 4 - 6.

B. Neutral DNase. 125 µl reaction mixtures contained 80 µg DNA 43 µg 16-day extract (protein), 25 mM tris acetate pH 7.5, 13.3 mM $MgCl_2$, plus additions as indicated. A different enzyme preparation to that in A was used. Incubation and estimation of activity are as in A.

TABLE 3.2.

Properties of feather deoxyribonucleases.

A. Acid DNase.

pH optimum ~5

Additions.	Activity(units).
6.7 mM $MgCl_2$	1.00
None	1.50
4 mM EDTA	1.48

B. Neutral DNase.

pH optimum ~ 7.5

Additions.	Activity(units).
None	0.62
25 mM phosphate	0.25
25 mM phosphate, 1mM β-mercaptoethanol	0.25
1 mM β-mercaptoethanol	0.53
$MgCl_2$ omitted	0.56
$MgCl_2$ omitted, 4 mM EDTA	0.27

FIGURE 3.7.

DNase in Feather Tissue in Development.

Extracts of feather tissue were prepared as described by Kischer and Furlong (1967), with the inclusion of 1% of Nonidet P-40 in the extraction buffer. DNase was assayed as described in the legend to Table 3,2. Three extracts, prepared from different embryos, were assayed at each age.

a. The specific activity of extracts at different ages. The protein content of extracts used for DNase estimation was estimated by the method of Lowry et al.(1951).

b. The DNase content of single feather cells. The soluble protein content of extracts, per ml of tissue, was determined, and the approximate values for cells per ml of tissue at each age (Table 3.3) used to convert the data in (a) to values for the enzyme activity of average cells at each age.

● acid DNase

o neutral DNase

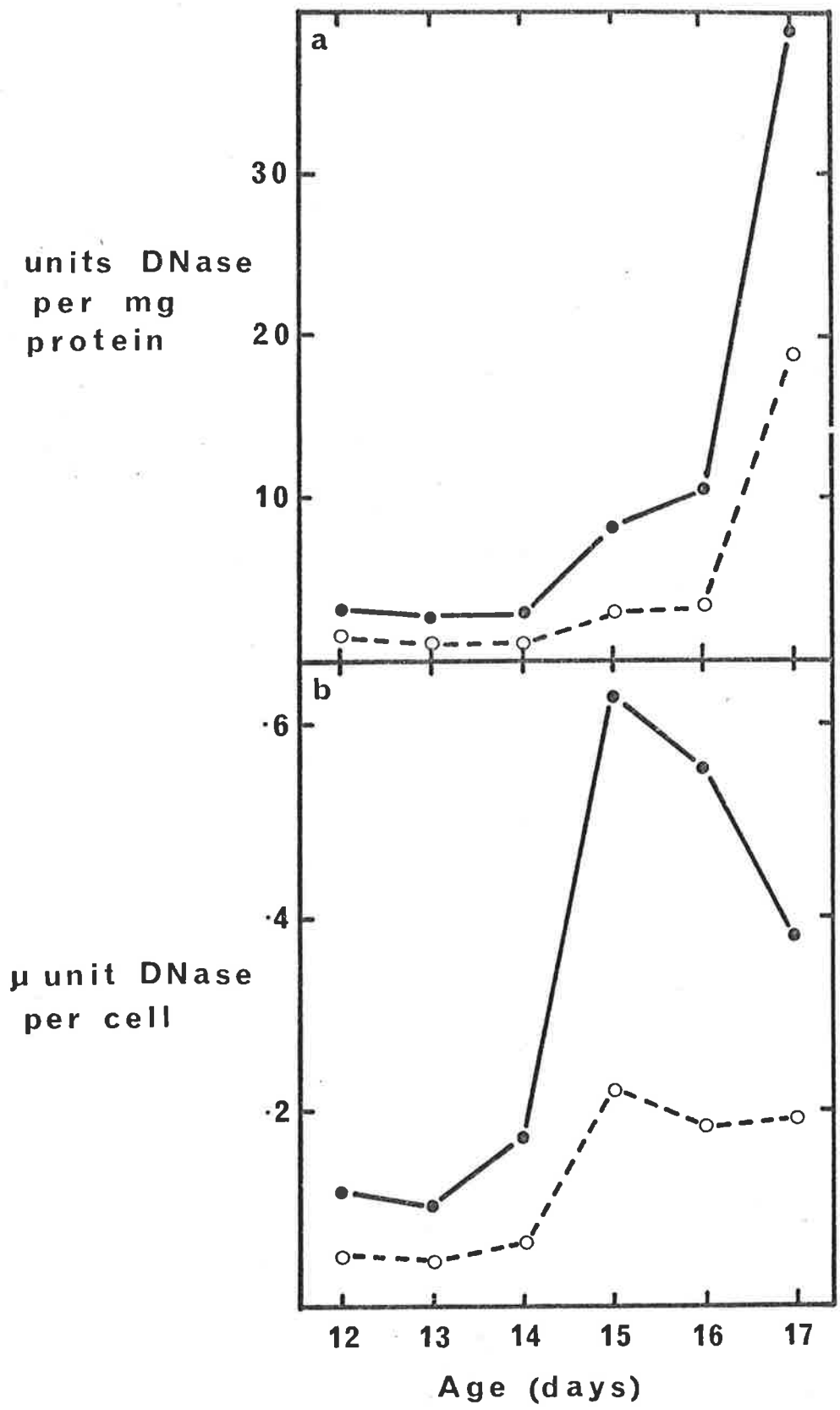

a
30
20
10
units DNase per mg protein
b
·6
·4
·2
µ unit DNase per cell
12
13
14
15
16
17
Age (days)

TABLE 3.3.

Calculated number of cells per ml tissue at different ages.

Age (Days).	Cells/ml ($\times 10^{-8}$).
12	9.50
13	6.64
14	5.42
15	3.08
16	2.45
17	2.23
18	1.74

The values were calculated from the figures for DNA/cell plotted in Figure 3.6, and for DNA per ml tissue in Figure 3.1.

activity of DNase in the cells at 15 days (increases of 6.2 fold and 4.7 fold relative to 13 days for the acid and neutral activities, respectively). DNase activity then remains at a high level over the period during which DNA was found to be lost from the cells.

(iii) Keratin gene content of feather cells.

The finding that feather cells are diploid precludes a crude amplification of feather keratin gene content by polyploidy, such as is observed for the fibroin gene in Bombyx mori silk gland (Suzuki et al., 1972). However, the method employed was by no means sensitive enough to observe the amplification of a small part of the genome.

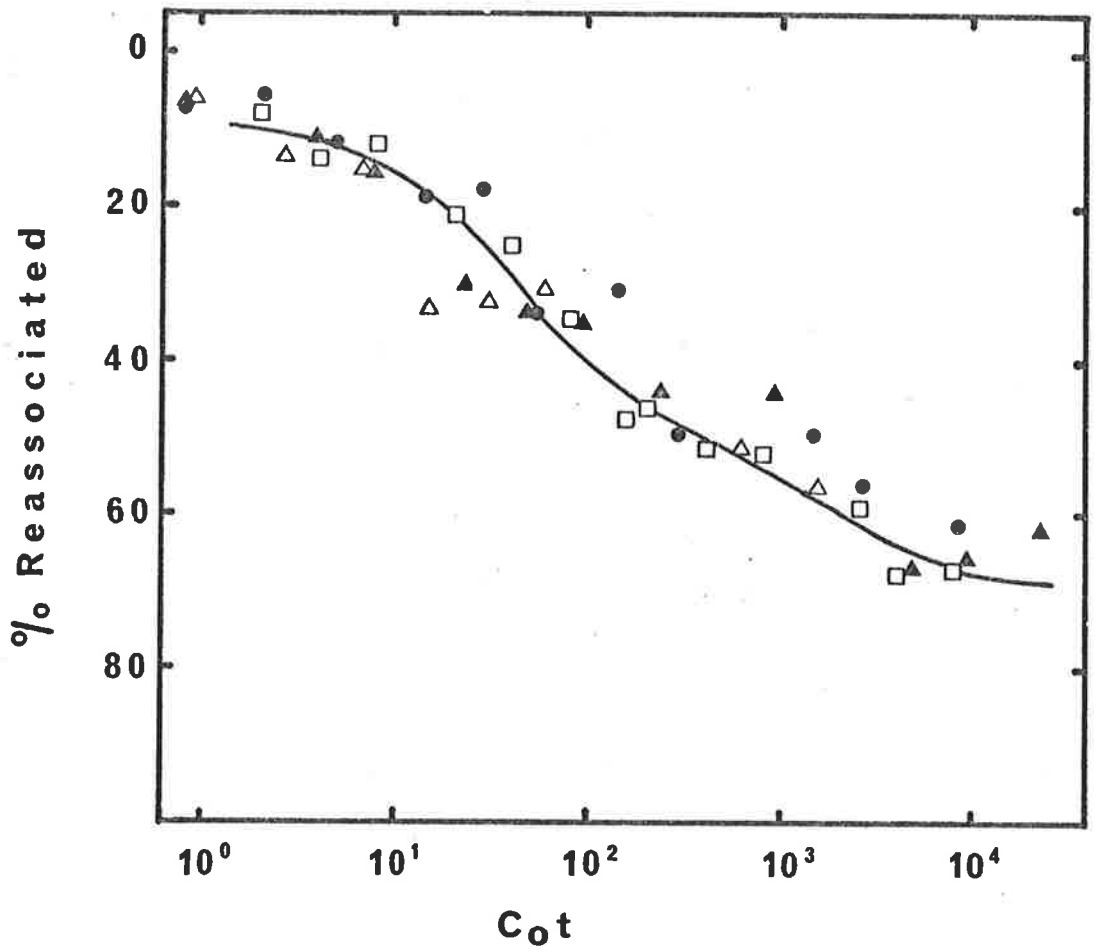
DNA isolated from feathers at 12, 13 and 14 days of development was hybridised with [^{3}H]-cDNA prepared from keratin mRNA (Chapter 2,(c)(iv)). The unlabelled feather DNA was in vast excess over the cDNA probe. The extent of hybridisation was examined using S_1 nuclease (Vogt, 1973) in high salt buffer, under the high salt digestion conditions described by Kemp (1975). The kinetics of the reannealing reaction were indistinguishable from those observed by Kemp (1975), who used adult hen erythrocyte DNA to assay the keratin gene content of the chick genome (Figure 3.8). Thus the content of keratin genes in the feather is 100 - 240 sequences per haploid genomes as in erythrocyte (Kemp, 1975), and hence no

FIGURE 3.8.

Keratin Gene Content of Feather DNA.

DNA isolated from 12-, 13- and 14-day feather tissue was mixed with keratin cDNA, denatured at 100°, and allowed to renature at 60°. Aliquots were removed at appropriate intervals, and the extent of renaturation estimated using S_1-nuclease (Vogt, 1973) in high salt buffer. The curve is that for chicken erythrocyte DNA hybridised with keratin cDNA, taken from Kemp (1975).

- □ erythrocyte DNA (Kemp, 1975)
- ● 12-day feather DNA
- ▲ 13-day
- △ 14-day


% Reassociated
0
20
40
60
80
10^0
10^1
10^2
10^3
10^4
$C_o t$

amplification of the genes for keratin occurs at the onset of keratin synthesis; presumably the number of active genes is adequate to yield the large quantity of mRNA required for keratin synthesis. Indeed probably only some 25 - 35 of these genes are expressed in the embryonic feather (Kemp, 1975).

The presence of 25 - 35 active keratin genes per haploid genome (i.e. 50 - 70 genes per cell) probably in itself reflects a form of gene amplification, although it is not tissue specific in the sense of ribosomal gene amplification in Xenopus oocyte (Brown and Dawid, 1968; Perkowska et al.,1968), in which the amplification is restricted to DNA of the one cell type.

D. DISCUSSION.

(i) Quantitative description of DNA in feather tissues.

It is apparent from data presented in figures 3.1 and 3.3 that the method chosen by Kemp et al., (1974a) to quantitate protein in developing tissues (mg/ml tissue) does not yield an accurate reflection of the in vivo situation. While from the data of figure 3.1 it was possible to infer that a loss of DNA occurred from the tissue, it was nonetheless difficult to accurately describe when this occurred. On the other hand, the use of fluormetric techniques to quantitate the DNA in individual feathers proved to be a more sensitive method, and also yielded values which were biologically more

meaningful. Knowing the DNA content per cell at 14 days (Figure 3.5), it was possible to then calculate DNA contents of cells at all the ages studied (Figure 3.6). This is certainly the best method of describing the content of any material in a tissue.

It is possible, using the values in figures 3.1 and 3.6, to calculate approximately the number of cells per ml of packed tissue at each age (Table 3.3). This is particularly useful, in that it enables conversion of values of the type obtained for figure 3.1 to a more meaningful value based on single cell contents. This approach was used, for example, in the determination of the DNase contents of the tissue (Figure 3.7).

(ii) Behaviour of DNA in feather development.

As discussed in chapter 1 and in the introduction to this chapter, the earlier stages of feather growth are characterised by rapid cell division. DNA synthesis in the feathers probably continues beyond 12 days - some cells at this stage are still capable of incorporating [^{3}H]-thymidine into DNA (Kemp et al.,1974a). This is confirmed by the increase in DNA content of single feather between 12 and 13 days (Figure 3.3). At 12 days, however, keratin synthesis has begun in the more advanced cells (Matulionis, 1970; Kemp et al, 1974a), although none of the cells making keratin incorporate [^{3}H]-thymidine (Kemp et al., 1974a). This strongly

suggests that the onset of keratin synthesis is immediately preceded by a cessation in DNA synthesis and mitosis. This could be indicative of a quantal mitosis (Holtzer et al., 1972, 1973), the function of which is to expose portions of the genome which were hitherto unavailable for transcription.

No amplification of the keratin genes in feather tissue was observed (Figure 3.8). In this respect it resembles other differentiating tissues, such as the reticulocyte or oviduct, in which amplification of the globin (Bishop et al., 1972; Packman et al., 1972; Paul et al., 1973; Harrison et al., 1974; Gambino et al., 1974) or ovalbumin (Sullivan et al., 1973; Harris et al., 1973) genes does not occur. There are, however, some 100 - 240 keratin genes in the haploid genome of the chicken (Kemp 1975); of these, 25 - 35 are expressed in the embryonic feather (Kemp, 1975). It is not known whether the expression of some of these genes is restricted to specific cell types (e.g. barbules), so that in a given cell there may be less than the maximum number of 50 - 70 genes being expressed. This reservation aside, it seems likely that the high gene content represents a form of amplification for specific genes in itself, and that further amplification is unnecessary.

Finally, as the process of keratinisation nears completion, the DNA is lost from the cells (Figures 3.5 and 3.6). This correlates closely with rises in DNase

activities in the cells (Figure 3.7). This rise in DNase activity had been observed earlier (Kischer and Furlong, 1967), although the previous findings ere different to those observed here. Firstly, the DNase activities in this study were found to parallel one another until very late in development, in contrast to the earlier work where acid DNase increased abruptly at about the onset of keratin synthesis, while neutral DNase remained essentially static. Secondly, the abrupt increases in activity took place apparently one to two days later in this study.

Why the DNA is lost at this stage is not clear. The loss of material may be necessary to ensure mechanical strength in the tissue - DNA loss may be delayed until late in the process to enable the activation of functions responsible for degradation of other cell constituents. Tnus the programming of differentiation of the feather may include a final burst of destruction of the nonkeratin materials in the cell, and would thus also include destruction of the programme.

The DNA of feather cells thus goes through three stages in development - an early proliferative phase during cell growth, a phase which is static with respect to growth, but active in transcription, followed by a final phase during which it is degraded.

CHAPTER 4

TEMPORAL RELATIONSHIPS BETWEEN THE SYNTHESES OF KERATIN mRNA, TOTAL RNA AND KERATIN.

(N.B. Part of the work discussed in this chapter has been published - Powell, B.C., Kemp, D.J., Partington, G.A., Gibbs, P.E.M. and Rogers, G.E., (1976) Biochem. Biophys. Res. Commun. 68 1263 - 1271)

A. INTRODUCTION.

The work described in this chapter represents an attempt to define the temporal relationship between RNA synthesis, and specifically mRNA synthesis, and the synthesis of keratin in embryonic chick feathers.

Total RNA levels have been described qualitatively in morphological studies. Koning and Hamilton (1954) used cytological staining procedures to investigate the distribution of RNA in the feather, and demonstrated high levels in the epidermal cells. Counting of ribosomal particles in electron micrographs of feathers at different stages of development (Matulionis, 1970) demonstrated an increase in ribosome content of the presumptive barb and barbule cells during the period of the onset of keratin synthesis. No increase was observed in the other cell types.

In other studies, the levels of messenger have been defined qualitatively, or semi-quantitatively. Between 12 and 14 days of development, there is a marked increase in the level of polysomes relative to monosomes (Humphreys et al., 1964; Yatvin, 1966a), with the polysomes containing predominantly 4 - 5 ribosomes. When compared with other systems (e.g. Heywood and Rich, 1968), this is the size expected for the translation of keratin (molecular weight 10,000; Harrap and Woods,1964b). The greater level of polysomes is accompanied by an

enhanced synthesis of keratin by cell-free protein synthesising systems prepared from the older tissue (Powell et al., 1976). These findings are indicative of an increase in the level of active mRNA as the tissue becomes committed to keratin synthesis. On the other hand it is arguable that an untranslated form of the messenger is stored and activated without increasing the real level of the mRNA.

The messenger RNA has been isolated and purified to apparent homogeneity (Partington et al., 1973; Kemp et al., 1974 b,c), and has been shown to sediment at 12S in sucrose gradients (Partington et al., 1973). The messenger RNA binds to cellulose (Partington et al., 1973; Kemp et al., 1974b), suggesting that it has a 3'-poly(A) sequence (Kitos et al., 1972; Schutz et al., 1972; DeLarco and Guroff, 1973). The quantity of 12S material retained by cellulose from 14-day polysomal RNA is considerably higher than that found in 12-day material (Powell et al., 1976).

The presence of a poly(A) sequence is further demonstrated by the ability of the purified mRNA to act as a template for oligo(dT)-dependent transcription by the RNA-dependent DNA polymerase from avian myeloblastosis virus (Kemp et al., 1975; Kemp, 1975). The complementary DNA produced by this reaction may be used as a specific probe for keratin mRNA sequences - the

level of 12S mRNA in 14-day polysomal RNA was greater than that in 12-day material when examined by sucrose gradient centrifugation and hybridisation of gradient fractions with the cDNA (Powell *et al.*, 1976).

The work described above suggests that the relative level of translatable mRNA in the tissue increases as the tissue becomes involved in keratin synthesis. The purpose of the following work is to describe this in more quantitative terms, thereby defining the total levels of mRNA in the tissue during development, including non-translated and stored forms of mRNA, if any.

B. METHODS.

(i) Quantitative estimation of feather RNA content.

(a) Relatively large tissue volumes.

The RNA content was estimated on the alkali-labile, macromolecular fraction obtained when preparing samples for DNA estimation (Chapter 3,B(i)(a)), using the orcinol reaction as described by Dische (1955). Yeast RNA was employed as a standard.

(b) Single feathers.

RNA concentration was measured in the extracts from single feathers (Chapter 3,B(i)(b)), using the difference in fluorescence intensity of ethidium bromide before and after RNase treatment (LePecq and Paoletti, 1966) and comparing with the values obtained with a

standard solution of yeast RNA.

(ii) Feather ribonuclease.

The same tissue extracts employed for DNase assay (Chapter 3,B(iii)) were assayed for RNase activity, by measuring the release of material soluble in .1M HCl, 50% ethanol from yeast RNA. The commercial yeast RNA was purified by phenol extraction at pH5, followed by ethanol precipitation. Assays, performed in duplicate, were routinely incubated at 37^{o} for 90 minutes. The value obtained with blanks, containing no enzyme extract, was deducted from the observed values.

Neutral RNase was assayed in 0.1 M tris-acetate buffer (pH 7.4). Acid RNase was assayed in 0.1 M Na acetate (pH 5.5), 25 mM K phosphate, 4mM β -mercaptoethanol, 10mM $MgCl_2$.

(iii) Isolation of nucleic acid for hybridisation.

(a) Total feather nucleic acid.

Washed feathers (0.5ml or less) were suspended in a total volume of 5ml in 10mM tris-HCl, pH 9.0, 5mM EDTA, 1% SDS, and homogenised at maximum speed in the microhomogeniser of a Sorvall Omnimix. The homogenate was extracted with an equal volume of water-saturated phenol, the phases separated, and the phenol phase and interphase back extracted with 2 ml of buffer lacking SDS. The aqueous phases were then combined, extracted twice with 3 ml of water-saturated phenol,

adjusted to 0.1 M in NaCl, and precipitated with 2.5 volumes of ethanol at -20°, overnight. The precipitated material was collected by centrifugation at 25,000 xg for 30 minutes, drained and dried *in vacuo*. The RNA content was determined by the orcinol reaction (Dische, 1955).

(b) Cytoplasmic and polysomal RNA.

Cytoplasmic RNA was prepared by phenol extraction, as described above, of post-mitochondrial supernatants prepared as described by Partington *et al.*, (1973). For polysomal RNA, a similar extraction was performed on polysomes isolated from identical post-mitochondrial supernatants (Partington *et al.*, 1973).

(iv) Keratin.

The total protein and keratin contents of older tissue (16 - 18 days) was determined as described by Kemp *et al.*, (1974a).

C. RESULTS.

(i) Total RNA content of feathers during development.

Initial experiments to quantitate the RNA content of feather tissue revealed marked changes over the period during which keratinisation occurs. As in earlier experiments with DNA (Chapter 3) and protein (Kemp *et al.*, 1974a), the RNA content was expressed relative to the tissue volume. Using this as a means

of comparing the RNA contents for tissue of different ages revealed a steady apparent decline in RNA (Fig.4.1) after 13 days, which continues until about 17 days.

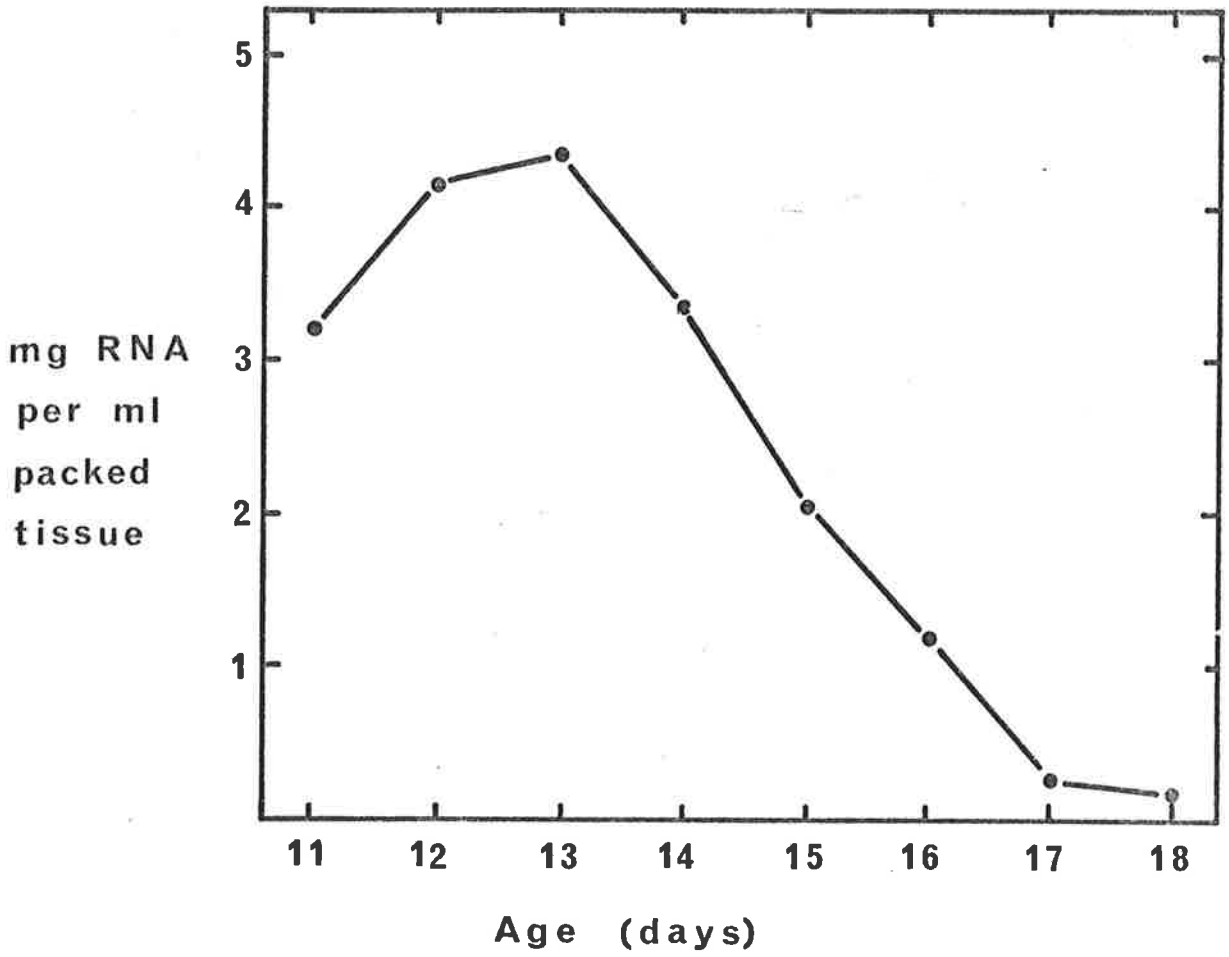
This apparent decline of RNA in the feather may be, in part, an artifactual result produced by changes in tissue volume, as was observed previously with DNA. This possibility was investigated further by estimating the RNA content of individual feathers, which should yield a more realistic assessment of RNA behaviour during development.

Both DNA and RNA cause enhanced fluorescence of ethidium bromide, the intensity of fluorescence being proportional to the nucleic acid concentration, and, where there is both DNA and RNA in the solution, the increase in intensity is equal to the sum of the increases due to equivalent amounts of DNA and RNA separately (LePecq and Paoletti, 1966). The RNA content of single feathers was therefore estimated from the difference in the fluoresence intensities observed before and after pancreatic RNase digestion of the total nucleic acid extracts prepared from single feathers. Pancreatic RNase, under the conditions employed here, completely eliminated the stimulation of fluorescence by yeast RNA. The increase of fluorescence caused by a 1μg/ml RNA solution was found to be 0.38 of the value observed for 1μg/ml DNA - this is slightly less than the value of 0.46 observed by LePecq and Paoletti (1966).

FIGURE 4.1.

RNA Content of Tissue in Development.

The packed volume of the tissue was determined, and the tissue solubilised as described in the legend to figure 3.1. The alkali-soluble component of the macromolecular fraction was isolated, and the RNA content estimated by the orcinol method (Dische, 1955). The values are the averages of at least 4 independent estimations at each age.


mg RNA per ml packed tissue
5
4
3
2
1
11
12
13
14
15
16
17
18
Age (days)

The result of these estimations, shown in figure 4.2, indicated that from 12 - 15 days a nett increase in RNA content occurs. Although these values were obtained as the differences between two sets of data (and hence could be expected to be subject to greater error), the ratio of RNA to DNA obtained at each age was comparable to that observed when the figures based on packed volume were compared. The increase in total RNA presumably represents mainly ribosomal and transfer RNA synthesis, since incubation of feathers with radioactive nucleosides results in most of the incorporated label being found in these species (Partington, 1974). After 15 days there is a rapid decrease in the level of RNA in the feathers, resembling that observed for DNA content.

The data obtained for RNA content in the tissue have been recalculated to yield figures for RNA per cell (Figure 4.3). These values were calculated from either the data of figure 4.1, using calculated figures for the number of cells per ml of tissue (Table 3.3), or from the ratios of RNA to DNA in single feathers, combined with figures for DNA per cell (Figure 3.6). The RNA per cell figures essentially reflect the trends observed for RNA per feather, with the maximum cellular level of RNA being observed at 14 - 15 days.

The total cellular RNA content rises over the early stages of keratin synthesis in the tissue. As this

FIGURE 4.2.

RNA Content of Single Feathers.

The RNA content of proteinase K digests of single feathers was estimated from the difference in the fluorescence intensity of ethidium bromide fluorescence produced on ribonuclease digestion of total nucleic acid, as described in the text. At each age, the value is the mean of that obtained for 10 feathers from each of 4 embryos.

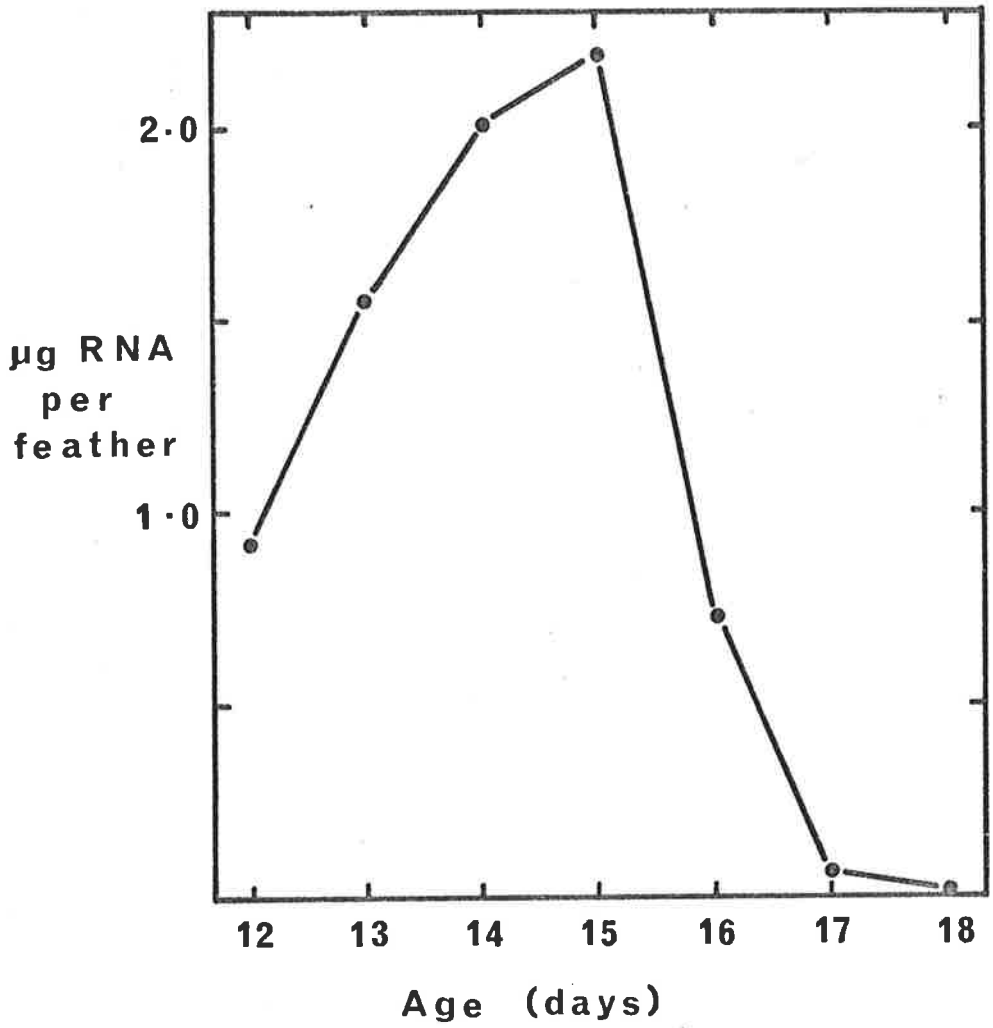
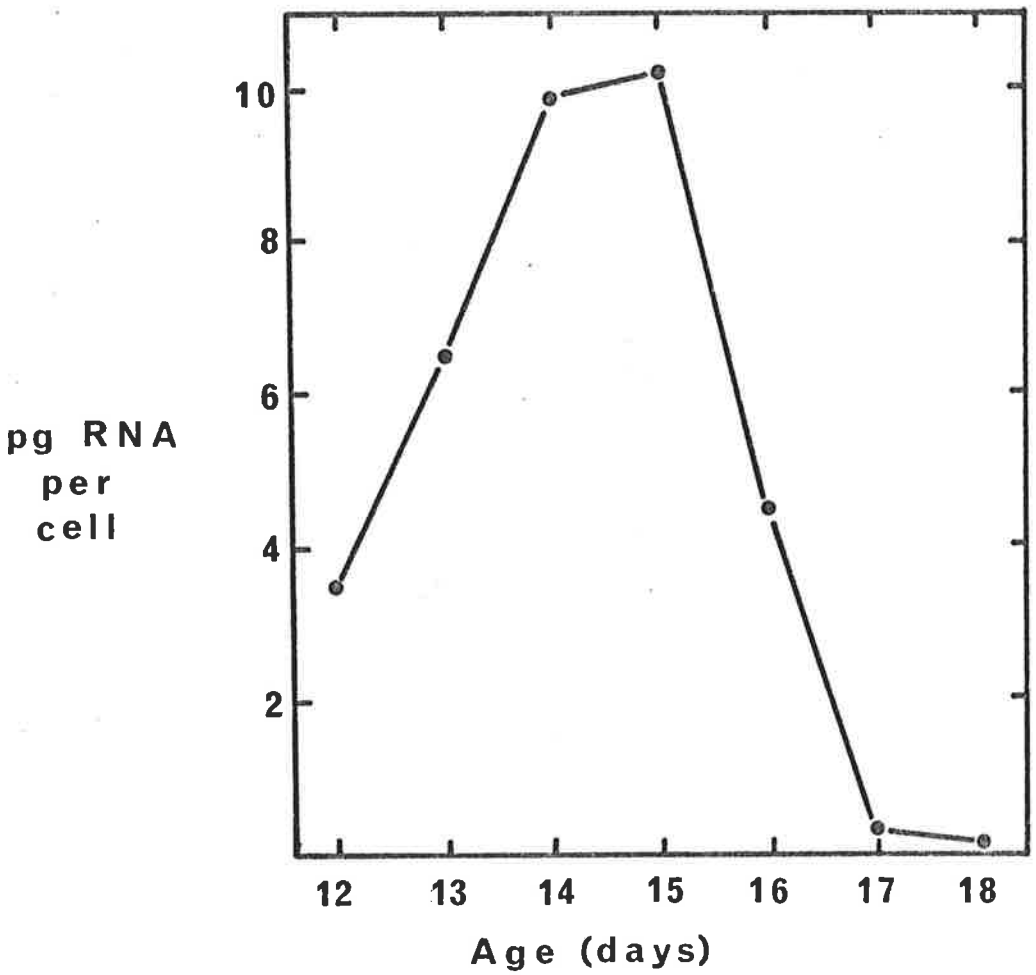

2·0
1·0
µg RNA per feather
12
13
14
15
16
17
18
Age (days)

FIGURE 4.3.

RNA Content of Single Feather Cells.

The values were calculated from those in figure 3.6, and the values obtained for RNA:DNA ratio obtained in a number of experiments, including data shown in figures 3.1, 3.3, 4.1 and 4.2


pg RNA per cell
10
8
6
4
2
12
13
14
15
16
17
18
Age (days)

is largely rRNA and tRNA synthesis, it represents a build-up of translational machinery in the cell, allowing keratin synthesis to proceed at a high rate. After 15 days, the level of RNA drops dramatically, at which stage keratin synthesis is also slowing down (see D, below).

(ii) Feather Ribonucleases.

The extracts which were used to examine feather DNase (Chapter 3) were also found to contain RNase activity. Two enzyme activities were found in the extracts; their properties are discussed below. Each gave a linear release of acid soluble material with time, up to at least 90 minutes, and with the quantity of enzyme added. The data are expressed in units similar to those used for DNase; one unit was defined as the quantity of enzyme required to render 1 μg of RNA acid-soluble in 10 minutes at 37°.

It was found that higher levels of RNase were present in extracts prepared using 1% Nonidet P-40 in the homogenising solution (Table 4.1). This appeared to be due to a larger proportion of the tissue protein being solubilised in the presence of detergent, rather than to a specific release of RNase, since the specific activity of the RNase was unaltered by the inclusion of NP-40 (Table 4.1). The detergent did not affect the RNase activity per se (Table 4.1).

TABLE 4.1.

Effect of Nonidet P-40 on extraction and activity of Neutral RNase.

Experiment.	NP-40 in extract.	Units.	µg protein.	Units /mg protein.
1	-	9.2	20	459
	+	16.5	34.8	474
2	-	12.0	28	429
	-*	12.2	28	436
	+	12.8	28	457

* NP-40 added to assay mixture.

Equal volumes of 16-day feathers (from the same embryos) were homogenised in buffer either lacking or containing 1% NP-40, as described (Kischer and Furlong, 1967). The soluble protein content of the extracts was determined by the method of Lowry et.al., (1951).

In experiment 1, equal volumes (20 µl) were assayed for RNase activity - the total volume of the assay was 100 µl.

In experiment 2, the volumes were adjusted so that an equal weight of protein was present in each assay.

The two RNase activities differ markedly in their properties. One of these (acid RNase) had a pH optimum of approximately 5.5. It was activated slightly by the addition of $MgCl_2$, and of phosphate (Table 4.2), and was slightly inhibited by the presence of 4mM EDTA.

The second activity was a neutral RNase, having a pH optimum of approximately 7.4. It was inhibited by both $MgCl_2$ and by phosphate (Table 4.2), although high levels of each were required to give significant inhibition. It was also strongly inhibited by EDTA, suggesting that the enzyme may require a divalent metal ion for activity. Furthermore, EGTA, which binds Ca^{++} specifically in the presence of Mg^{++} (Schmid andReilly, 1957) also strongly inhibited the enzyme, suggesting that calcium may be the metal involved.

This metal ion requirement was investigated in the following experiment. The enzyme and substrate were preincubated at 0° in the presence of 4mM EDTA, after which a solution containing the metal ion of interest was added, to give a final concentration of uncomplexed metal ion of 4mM. The mixtures were then assayed for ribonuclease activity at 37° as usual. A control lacking EDTA was included, and the recovery of RNase in each assay was expressed relative to this (Table 4.3). Several metal ions were effective in

TABLE 4.2.

Properties of feather RNases.

	Acid RNase.	Neutral RNase.
pH optimum	~5.5	~7.4
Control	1.00	1.00
4mM $MgCl_2$	1.19	.92
20mM $MgCl_2$	1.32	.73
20mM phosphate	1.26	.82
100mM phosphate	1.46	.48
4mM EDTA	.65	.07
4mM EGTA		.03

Reaction mixtures (100 µl) contained yeast RNA (1.5 mg), buffer (.1 M Na acetate, pH 5.5 for acid RNase, .1 M tris acetate, pH 7.4 for neutral RNase) and 16-day extract (50 µg total protein), plus additions as indicated. Samples were incubated at 37°, for 90 minutes and stopped by the addition of 2 ml of 0.1 M HCl, 50% ethanol. Insoluble material was removed by centrifugation, the supernatant diluted 5-fold in water, and the A_{260} of the diluted sample read in a Gilford spectrophotometer. Typically, the acid RNase control contained 5 - 10 units, the neutral control 20 units. For ease of comparison, the results are expressed relative to the control as 1.00.

pH optima were determined using a series of Na acetate or tris acetate buffers of different pH (ranges 3 - 6, 6.5 - 9, respectively).

TABLE 4.3.

Neutral RNase - effect of metal ions on EDTA treated enzyme.

Metal ion added.	Relative activity.
None	1
Mg^{++}	0
Zn^{++}	10
Cu^{++}	42
Ni^{++}	43
Fe^{+++}	55
Co^{++}	102
Mn^{++}	110
Fe^{++}	114
Ca^{++}	176

The reaction mixtures were pretreated with 4 mM EDTA at 0^{o} for 15 minutes, after which a solution of metal ion was added to give a total concentration of 8 mM, and the enzyme assayed as described in the legend to Table 4.2. The data are expressed relative to a control which was not treated with EDTA, and which contained 21.5 units; this value was taken as 100.

restoring all or part of the activity of the enzyme, although calcium ion was the most effective. The reason for the high recovery is unclear, although it was reproducible between experiments. These results, although preliminary, suggest that the enzyme may have a requirement for calcium ion, possibly at the level of the substrate, since treating the enzyme extract with chelex-100 resin prior to assay did not affect the enzyme activity.

Extracts of tissue of different ages were assayed for both ribonucleases, and in each case the specific activity of the enzyme increased with age (Figure 4.4a). The activity was also calculated per cell, as described for DNase (Chapter 3 C(ii)), and it is noticable that the activity is maximal at 16 days (Figure 4.4b) for both enzymes. Since this is the stage at which the rate of loss of RNA from the tissue is greatest, it seems likely that these activities are directly involved in degrading the RNA.

(iii) Keratin mRNA content of tissue.

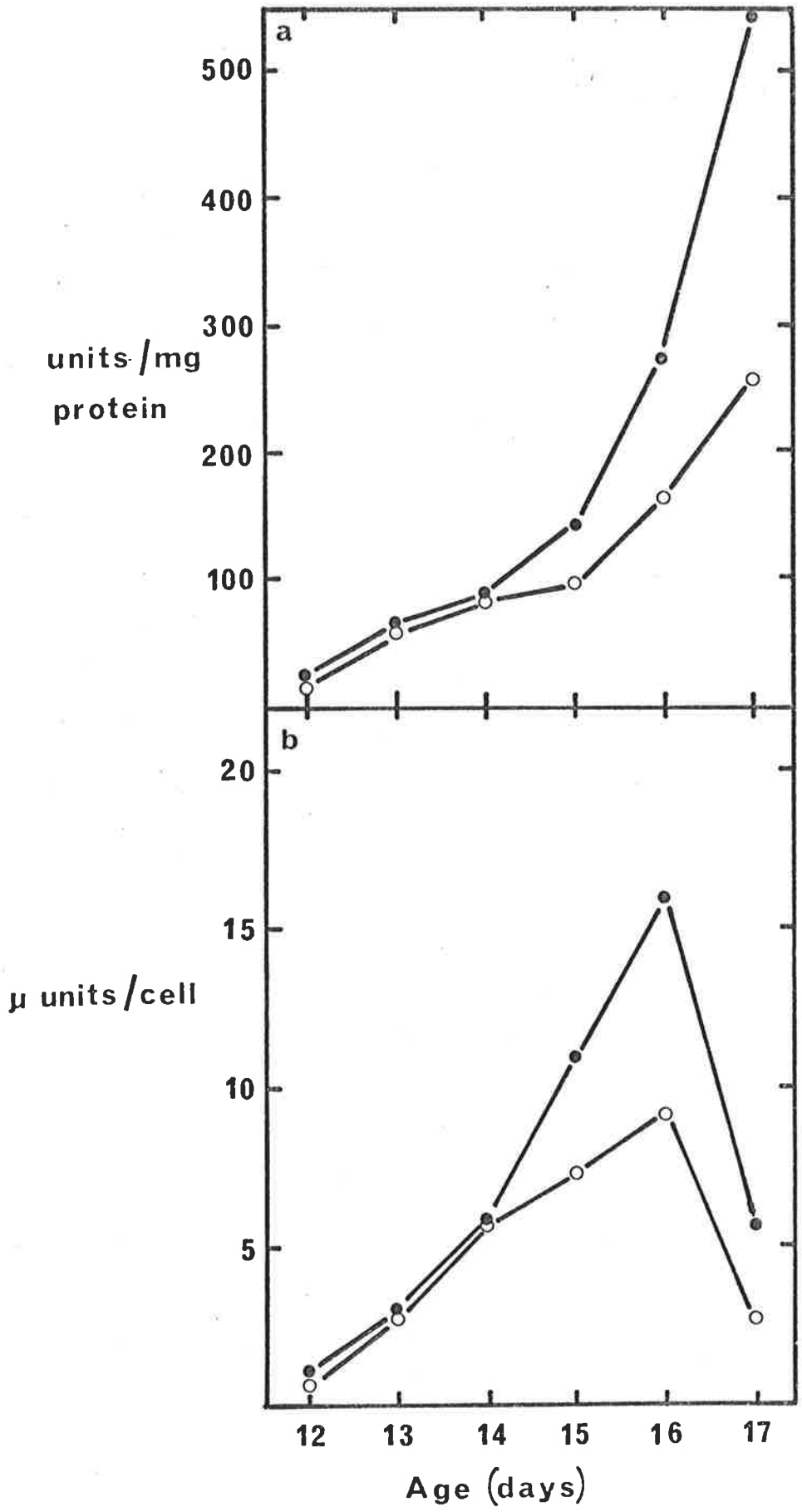
In the average feather cell, the total RNA content increases dramatically over the period during which the synthesis of keratin begins, and remains at a high level until late in development (Figure 4.3). Most of the RNA made is rRNA and tRNA, although there is evidence that the relative activity of mRNA also

FIGURE 4.4.

RNase Levels in Feathers During Development.

The RNase levels of extracts of tissue prepared in the presence of 1% NP.40, as described by Kischer and Furlong (1967) were assayed as described in the text and in the caption to Table 4.3. Neutral RNase was assayed in tris-acetate buffer (pH 7.4), and acid RNase in sodium acetate buffer (pH 5.5), containing 10 mM $MgCl_2$, and 50 mM Na phosphate. Specific activities (a) were calculated from the protein content of the extract. The cellular levels, shown in (b), were calculated as described in the legend to figure 3.7.

- ● neutral RNase
- o acid RNase


a
500
400
300
200
100
units /mg
protein
b
20
15
10
5
μ units /cell
12
13
14
15
16
17
Age (days)

increases during this period (Humphreys *et al.*, 1964: Yatvin, 1966a: Powell *et al.*, 1976). DNA complementary to purified keratin mRNA was prepared (Kemp, 1975), and hybridisation of this cDNA with total feather nucleic acid was used as a means of quantitating the total mRNA content of feather tissue.

Hybridisation was performed with RNA in excess over cDNA; the extent of hybridisation was plotted as a function of $R_o t$ (the product of ribonucleotide concentration (mol/litre) and time (sec): Bishop *et al.*, 1974a), essentially according to Britten and Kohne (1968). It was observed that the nucleic acid from feathers at all ages tested (11 - 16 days) contained messenger sequences, but that the rate of hybridisation varied with age (Figure 4.5), being greatest for 14 day nucleic acid and least for 11 day material. The $R_o t$ values are based on the RNA content of the nucleic acid, and the contribution of DNA sequences to the reaction has not been considered. The rate of hybridisation is dependent upon the number of sequences present which are capable of reacting, i.e. the greater the number of sequences present, the faster the reaction proceeds. It is convenient to describe the rate of hybridisation by the mid-point ($R_o t_{\frac{1}{2}}$), and estimates of the fractional mRNA sequence content of a sample may then be derived (c.f. Cox *et al.*, 1974) from the relationship

$$\frac{\%\ \text{sequence in sample}}{\%\ \text{sequence in mRNA}} = \frac{R_o t_{\frac{1}{2}}\ (\text{mRNA})}{R_o t_{\frac{1}{2}}\ (\text{sample})} \quad \text{---(1)}$$

FIGURE 4.5.

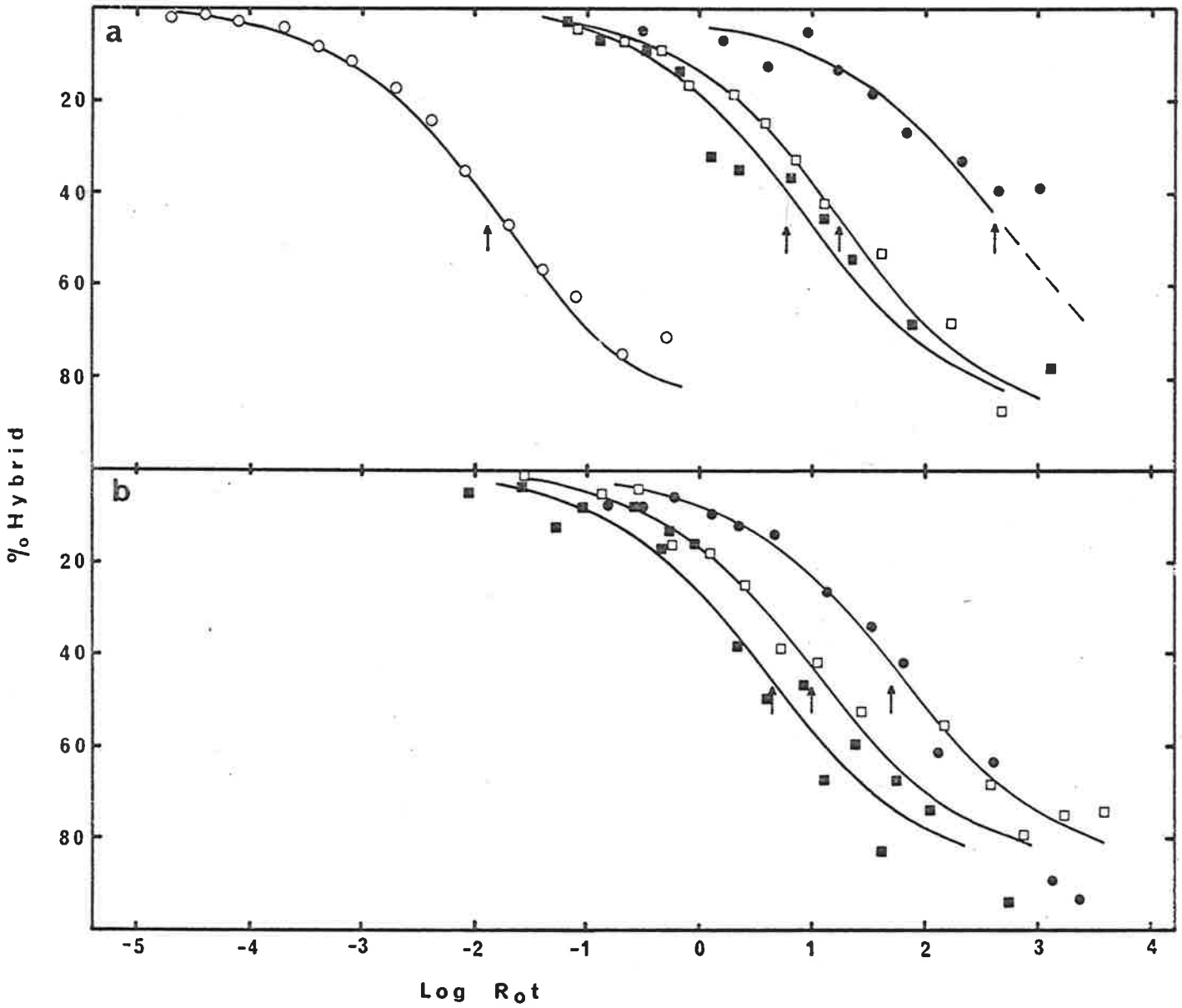
Hybridisation of Total Feather Nucleic Acid with Keratin cDNA.

Total feather nucleic acid was isolated by phenol extraction at pH9.0 (see text), then dissolved in buffer for hybridisation. The reaction was carried out with RNA in vast excess over cDNA. The samples were denatured at 100°, and incubated at 60°. Hybridisation was assayed with nuclease S_1. The $R_o t$ values were calculated from the RNA content of the nucleic acid preparations, which was estimated by the orcinol method. The contribution of DNA sequences to the reaction has not been considered.

a. Hybridisation of purified mRNA (o), 11-day (●), 13-day (□) and 15-day (■) total nucleic acid.

b. Hybridisation of 12-day (●), 14-day (■) and 16-day (□) nucleic acid.

Arrows indicate the mid-points ($R_o t_{\frac{1}{2}}$) of the reactions.


a
b
20
40
60
80
% Hybrid
-5
-4
-3
-2
-1
0
1
2
3
4
Log Rot

where the per cent sequence in mRNA is 100.

Using this relationship, and $R_o t_{\frac{1}{2}}$ values of 1.3×10^{-2} and 4.5×10^{0} for pure mRNA and 14-day total RNA respectively, the mRNA sequence content of 14-day RNA is calculated to be 0.29%. If, however, all 14-day polysomes are synthesising keratin, approximately 1.5-2% of polysomal RNA would be mRNA, so that it appears that the value obtained for total RNA is low, particularly as a large proportion of feather polysomes synthesise keratin (Powell et al., 1976).

One possible reason for this low observed value is a selective loss of messenger RNA at some stage during the preparation. This was investigated using a 12-day post-mitochondrial supernatant as an RNA source. This "cytoplasmic" fraction was divided into equal portions, and purified messenger RNA added to one half, the final concentration of mRNA being 1.0% of the total RNA. The RNA prepared from these two samples was then hybridised with cDNA. 12-day RNA showed a $R_o t_{\frac{1}{2}}$ of 6.2×10^{1} (Figure 4.6), while the sample to which mRNA was added showed a $R_o t_{\frac{1}{2}}$ of 6.5×10^{0}. The two samples, respectively, contained 0.021% and 0.20% messenger sequence. Thus it is apparent that 80% of the added messenger RNA was lost during purification.* The low value was not due to impurity of the messenger used, which hybridised with a $R_o t_{\frac{1}{2}}$ of 1.1×10^{-2} (c.f. figure 4.5). This selective loss of messenger

---

*Losses may be reduced or eliminated by using phenol-chloroform in conjunction with Sarkosyl as detergent.

FIGURE 4.6.

Recovery of Keratin mRNA after Phenol Extraction.

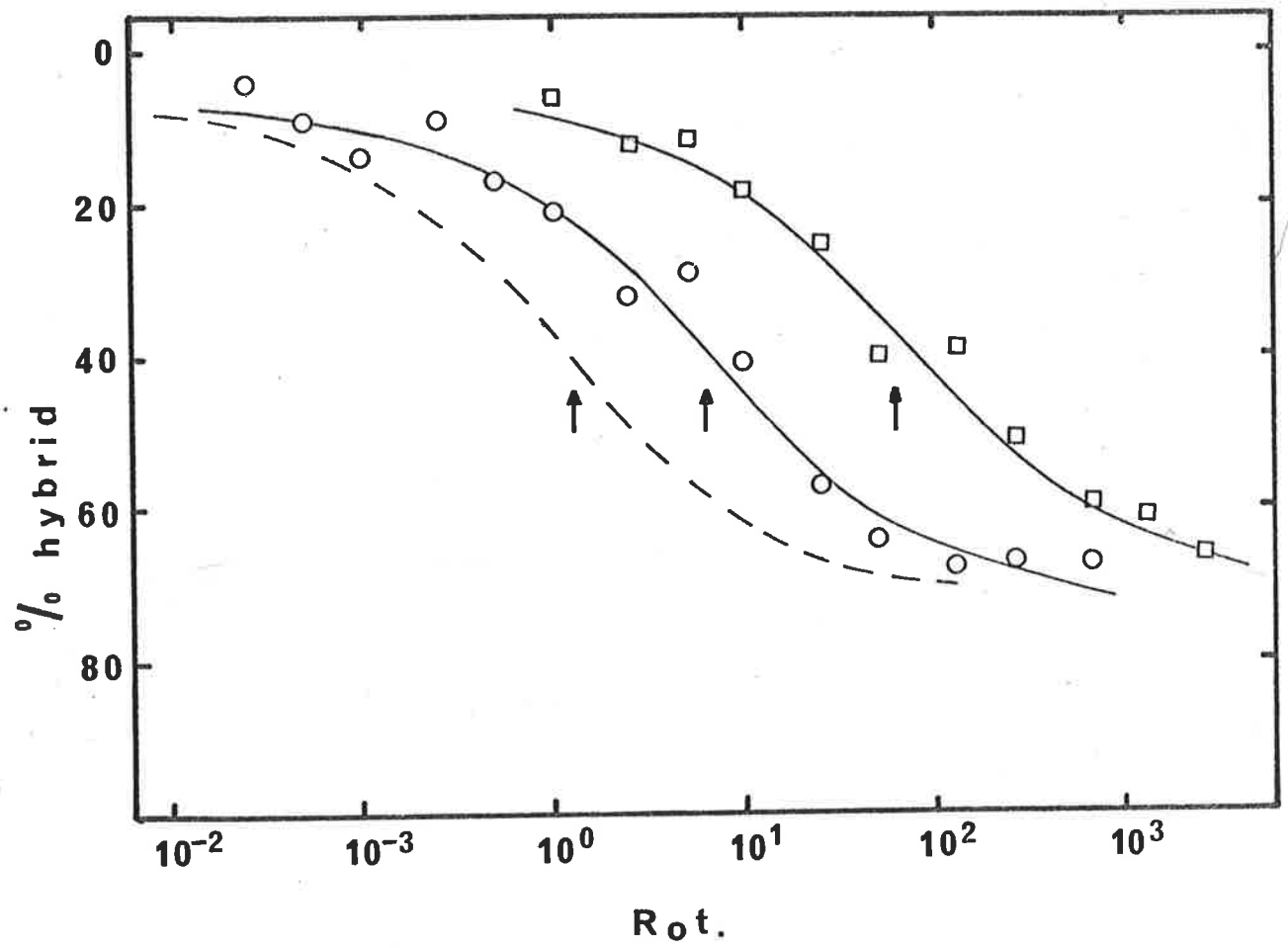
Keratin mRNA was added to one half of a preparation of 12-day post-mitochondrial supernatant, to a concentration of 1.0% of the total RNA. RNA was then extracted from each half of the preparation, and was hybridised with keratin cDNA. The extent of reaction was assayed with nuclease S_1.

□ 12-day cytoplasmic RNA

o 12-day cytoplasmic RNA to which mRNA had been added.

----- predicted curve for 12-day cytoplasmic RNA to which mRNA had been added, based on the concentration of the added mRNA.

Arrows indicate the $R_o t_{\frac{1}{2}}$ points.


0
20
40
60
80
% hybrid
10^{-2}
10^{-3}
10^{0}
10^{1}
10^{2}
10^{3}
Rot.

RNA was assumed to be constant for different preparations (for technical reasons tissue older than 12-days could not be effectively tested), and values for mRNA content calculated from equation (1) were corrected to take this low recovery into account.

The fraction of mRNA in total RNA preparations was calculated from the $R_o t_{\frac{1}{2}}$ values, and corrected for loss of mRNA during isolation (Table 4.4). From this data, the number of messenger RNA molecules per cell could be calculated, using the total RNA content of the cell (Figure 4.3) and the molecular weight of 2.5×10^5 determined for keratin mRNA (Kemp et al., 1974b). The messenger RNA content is very low (~460 molecules per cell) at 11-days, when keratin is detectable in only the most advanced cells near the tip of the feather (Matulionis, 1970), but increases some 750 fold over the next three days of development (Figure 4.7). This increase in absolute messenger RNA content is accompanied by a relative increase in the fraction of mRNA (Table 4.4) which confirms the trends toward higher relative activity of mRNA at 14-days (Humphreys et al., 1964; Yatvin 1966a; Powell et al., 1976). The other noteworthy feature of the messenger levels in the cell is the decline in messenger RNA by 16 days of development, by which stage keratin synthesis is nearly completed (see D, below).

TABLE 4.4.

Relative mRNA content of total nucleic acid.

Age (days).	$R_o t_{\frac{1}{2}}$.	% mRNA.
Pure mRNA	1.3×10^{-2}	100
11	4.0×10^{2}	0.016
12	5.0×10^{1}	0.13
13	1.7×10^{1}	0.38
14	4.5×10^{0}	1.44
15	5.5×10^{0}	1.18
16	1.0×10^{1}	0.65

The $R_o t_{\frac{1}{2}}$ values were taken from Figure 4.5 % mRNA was calculated from the relation

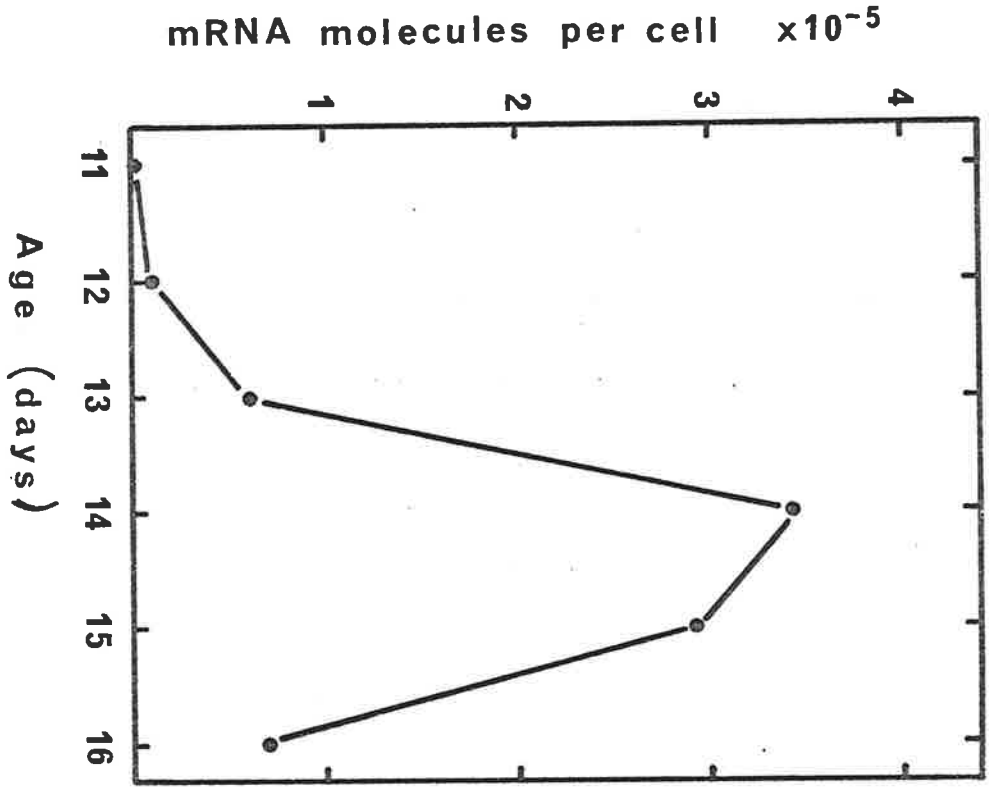
$$\frac{\% \text{ mRNA in sample}}{\% \text{ mRNA in mRNA}} = \frac{R_o t_{\frac{1}{2}}(\text{mRNA})}{R_o t_{\frac{1}{2}}(\text{sample})}$$

The values have been corrected for loss of mRNA during isolation (see text), by multiplying the % mRNA obtained from the equation above by 5.

FIGURE 4.7.

Keratin Messenger RNA Content of Cells with Age.

The content of messenger RNA in the average cell at each age was calculated from the figures of Table 4.4, and the data for total RNA per cell shown in figure 4.3.


mRNA molecules per cell x10⁻⁵
1
2
3
4
Age (days)
11
12
13
14
15
16

(iv) Subcellular distribution of mRNA.

The data in the previous section suggests that the total messenger content of the tissue increases over the period 11 - 14 days, in accordance with observations made on keratin synthesis (Kemp et al., 1974a). In view of this dramatic change in total messenger, it is of interest to determine the proportion of this which is involved directly in protein synthesis in polysomes.

The relative levels of messenger RNA in cytoplasmic RNA at different ages are given in Table 4.5. (The values given in this table for the cytoplasmic fraction were obtained in this laboratory by Mr. B.C.Powell, and are quoted here with his permission). Also shown are the levels of cytoplasmic mRNA per ml of tissue, and, for comparison, the levels of total mRNA calculated from the data in Table 4.4 and figure 4.1. The values for total mRNA and cytoplasmic mRNA are quite similar, suggesting that much of the mRNA is cytoplasmic. From the ratios of cytoplasmic to total mRNA, an average value of 96% of the total mRNA appeared to be cytoplasmic in origin. Because of uncertainties in the data, it is not certain how accurate this figure is, but it appears very likely that more than 90% of the mRNA is cytoplasmic.

The presence of mRNA in the cytoplasm may not necessarily mean that it is actively being

TABLE 4.5.

Comparison of mRNA levels in cytoplasmic and total RNA.

Age (days)	mg cytoplasmic RNA/ml tissue.	$R_o t_{\frac{1}{2}}$	% mRNA	µg mRNA per ml tissue	µg mRNA per ml tissue (from total RNA)	mRNA in cytoplasm (% of total mRNA)
11	3.20	4.8×10^2	0.014	0.45	0.51	88
12	3.95	4.0×10^1	0.16	6.3	5.4	117
13	4.03	1.7×10^1	0.38	15.3	16.5	93
14	4.01	6.0×10^0	1.08	43.3	48.2	90

The data cited in this table for cytoplasmic RNA were determined by B.C. Powell of this laboratory and are cited with his permission. The RNA content of post-mitochondrial supernatants prepared as described (Partington, et al, 1973) was determined by the orcinol reaction (Dische, 1955). The values for % mRNA were determined as described in the legend to Table 4.4, and have been corrected for loss during isolation. The values for total mRNA were calculated using the data of Figure 4.1 and Table 4.4.

translated. The proportion of mRNA in the polysomal fraction was investigated as follows. A post-mitochondrial supernatant was isolated from 14-day feathers, and divided into approximately equal portions. RNA was extracted directly from one portion, while the other was subjected to high speed centrifugation to yield a polysomal pellet, and the post-ribosomal supernatant. RNA was then prepared from each of these, and all three RNA preparations hybridised with keratin cDNA. The results, shown in figure 4.8, suggested an enrichment of mRNA in the polysomal RNA fraction relative to total cytoplasm, whilst the post-ribosomal supernatant contained a much lower level of messenger than either of these. From the data obtained from these three curves, the quantity of cytoplasmic mRNA in polysomes could be determined (Table 4.6). Nearly all of the mRNA (99%) was present in the polysomal fraction.

In another, similar experiment, > 85% of the mRNA was present in polysomes - in this case, however, the time taken in preparing the post-mitochondrial supernatant could well have allowed mRNA release from the polysomes. In the experiment described above, a high degree of run-off may have been overcome by using a relatively small tissue sample, and hence shortening the preparation time.

Assuming that these two sets of data represent

FIGURE 4.8.

Distribution of Cytoplasmic mRNA in 14-day Feathers.

Post-mitochondrial supernatant was prepared from 14-day feather tissue, and was either directly extracted with phenol, or was fractionated into polysomes and post-ribosomal supernatant prior to phenol extraction. The RNA from each of the fractions was then concentrated by ethanol precipitation, dissolved in buffer for hybridisation, mixed with a small quantity of keratin cDNA, denatured at 100° for 5 minutes and allowed to hybridise at 60°. Hybridisation was assayed with nuclease S_1.

a. post-mitochondrial supernatant (cytoplasmic) RNA
b. polysomal RNA
c. post-ribosomal supernatant RNA

Arrows indicate the $R_o t_{\frac{1}{2}}$ points.

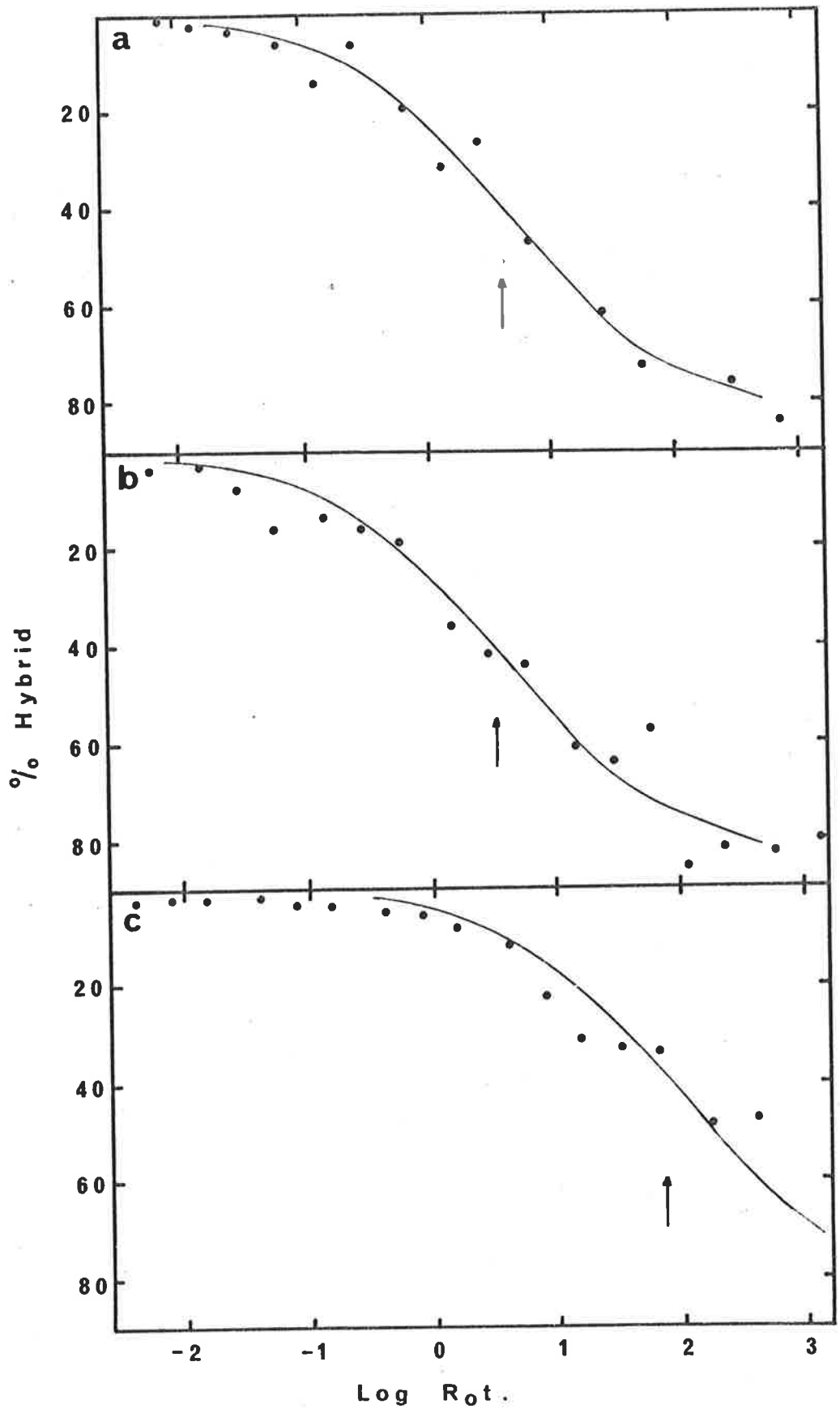

a
b
c
20
40
60
80
% Hybrid
-2
-1
0
1
2
3
Log Rot.

TABLE 4.6.

Distribution of cytoplasmic keratin mRNA.

Source of RNA	% of total cytoplasmic RNA	$R_o t_{\frac{1}{2}}$	% mRNA in sample.	µg mRNA per mg total cytoplasmic RNA	% of total cytoplasmic mRNA
Post-mitochondria supernatant.	100	4.2	0.310	3.10	100
Polysomes.	78	3.3	0.394	3.07	98.7
Post-ribosomal supernatant.	22	72	0.018	0.04	1.3

Post-mitochondrial supernatant was prepared from the feathers of one 14-day embryo, and polysomes isolated from this by centrifugation at 140,000 g for 90 minutes in a Beckman Ti50 rotor (Partington et al., 1973). RNA was isolated from the post-mitochondrial and post-ribosomal supernatants and from the polysomes by phenol extraction (see text for details), and hybridised with keratin cDNA. The $R_o t_{\frac{1}{2}}$ values are taken from Figure 4.8, and the percent mRNA in each sample calculated from equation (1) (see text). The values were not corrected for mRNA loss during isolation.

a fairly close approximation to the situation in the cell, it is apparent that a high proportion (99% of 96%, i.e. 95%) of the total mRNA in the cells is actively being translated in polysomes. The figure to which the greatest uncertainty may attach is that for the relative abundance of mRNA in the cytoplasm, compared with total tissue. It is unlikely that untranslated forms of the mRNA exist, at least in the cytoplasmic fraction.

D. CALCULATION.

The in vivo rate of keratin synthesis.

This calculation was performed primarily to check whether the calculated values of mRNA per cell (Figure 4.7) were adequate to account for the level of keratin synthesised in feather development.

Data existed for the total protein, and keratin, content of the tissue (Kemp *et al.*, 1974a) up to 15 days of development. No data was available in the literature for older tissue; accordingly, total protein and keratin content for 12 - 18 day feather tissue was determined by the method of Kemp *et al.*, (1974a), and incorporated with their data. From these figures of protein per ml of tissue, and the values in Table 3.3, the protein and keratin contents of the average cell at each age were determined

(Figure 4.9). The number of molecules of keratin per cell was calculated using a molecular weight of 1.05×10^4 for the molecular weight.

Between 12 and 18 days, the content of keratin in the average cell increases from 4.8×10^8 to 3.1×10^{10} molecules (Figure 4.9). The latter figure was taken as the number of molecules in a completely filled cell.

Two assumptions are made in the following calculation, the validity of which are discussed below. Firstly, it was assumed that it takes three days to fill the cell with keratin. Secondly, the level of mRNA in the cell was taken at be 3×10^5 molecules (which is approximately the mean of the 14 and 15 day values, figure 4.7).

If it takes 3 days to fill the cell with keratin, i.e. with 3.10×10^{10} molecules, then keratin must be synthesised at a rate of 1.03×10^{10} molecules per day, or 7.17×10^6 molecules per minute.

Each mRNA, therefore, yields 24 molecules of keratin per minute. This value is the translational efficiency (Palmiter, 1975) of the mRNA, and is equal to the rate of initiation of protein synthesis on the messenger. Translational efficiency (T_{eff}) is also defined by the relationship (Palmiter, 1975).

$$T_{eff} = \frac{P}{t}$$

FIGURE 4.9.

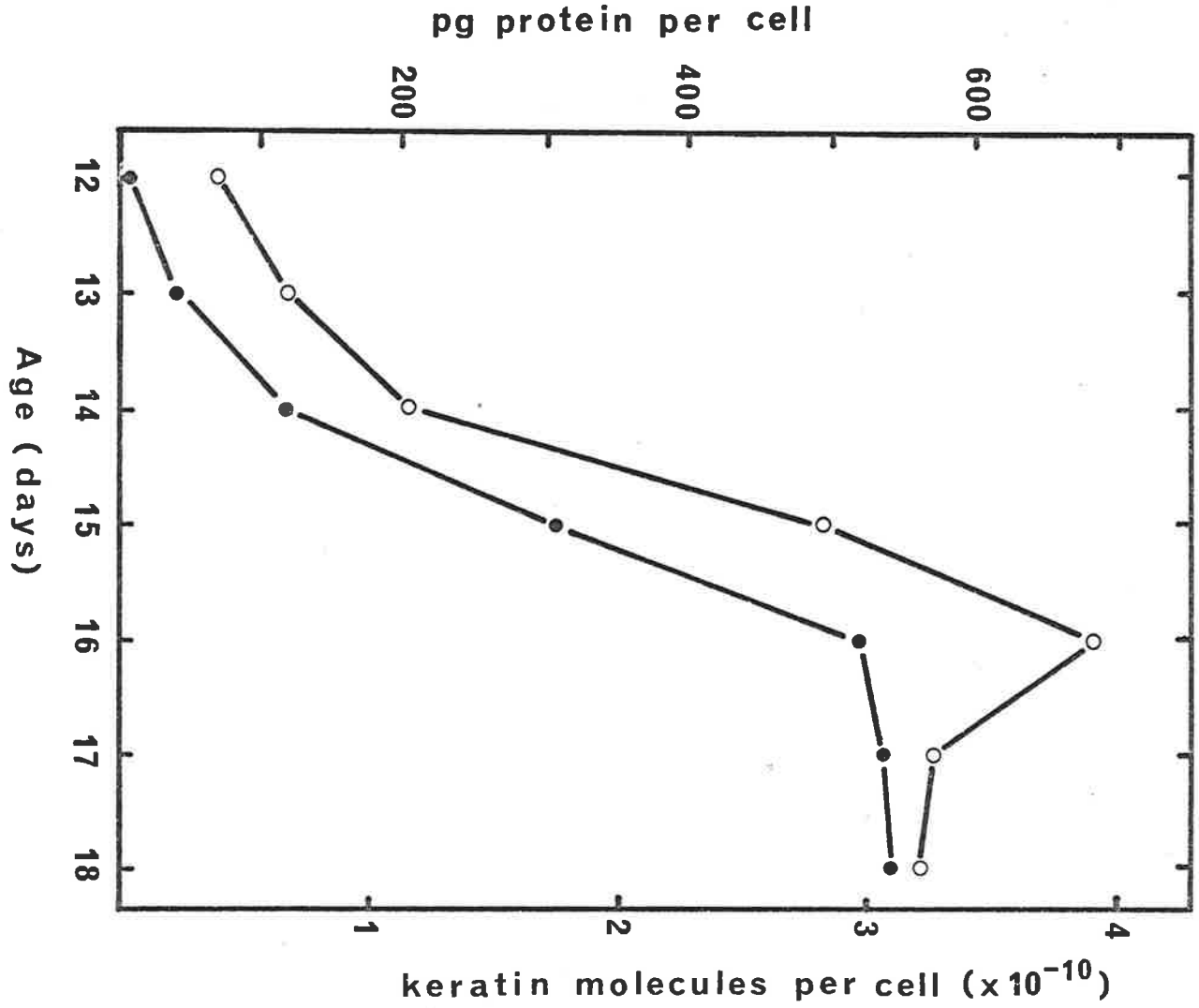
Cellular Content of Keratin in Development.

The total protein content per ml of packed tissue was determined as described by Kemp et al., (1974a), and the level of keratin in the protein estimated by quantitative polyacrylamide gel electrophoresis. (The data of Kemp et al., (1974a) for 12 - 15-day tissue has been included in this figure). From the keratin and total protein contents per ml of tissue, and the approximations for the number of cells per ml of tissue (Table 3.3), the weights of protein and of keratin per cell were calculated at each age.

o——o total protein

●——● keratin

The right-hand scale estimates the number of molecules of keratin per cell, based on a molecular weight of 1.05×10^4 for keratin (Harrap and Woods, 1964b).


pg protein per cell
200
400
600
12
13
14
15
16
17
18
Age (days)
1
2
3
4
keratin molecules per cell (x10^{-10})

where P is the polysome size, t is the ribosome transit time (i.e. the time taken for the ribosome to completely traverse the mRNA in synthesising one polypeptide chain). Taking the polysome size as 4.5 ribosomes (Humphreys et al., 1964; Yatvin, 1966a; Partington, 1974), the transit time, t, is 11.3 seconds. The keratin protein contains 100 amino acid residues, so that this yields an elongation rate of 8.8 amino acids per second. This value is not significantly greater than that observed in other systems, which is generally 2-8 residues per second (Dintzis, 1961; Staehelin et al., 1964; Hunt et al., 1969; Fan and Penman, 1970; Bordin et al., 1972; Waldron et al., 1974; Palmiter, 1975).

It would seem from the above calculation that the values obtained for mRNA content per cell are at least of the correct order of magnitude. Nonetheless, these calculations rest on two assumptions. The first is the time taken to fill the cell with keratin, which necessitated a rate of synthesis of 1.03×10^{10} molecules per day. The maximum rate observed in vivo (between 14 and 16 days, figure 4.9) was 1.15×10^{10} molecules per cell per day. This suggests that three days is likely to be a valid estimate.

The second assumption is rather less justifiable, as it utilises a messenger level which is the mean of the maximum values. The minimum level

of mRNA which seems acceptable is the half-maximum value (1.7×10^5 molecules per cell). This would require an elongation rate which is somewhat higher (15 amino acids per second). It is probable, however, that at the stage where the cell is synthesising keratin near the maximum rate, the level of mRNA is intermediate between these values.

Is the rate of elongation calculated here sufficient to allow the increases in keratin content over each period of one day? For the purpose of these calculations, an elongation rate of 10 amino acids per second was used, giving a translational efficiency of 27 molecules/min/mRNA molecule. The calculations are shown in Table 4.7. The levels of messenger RNA present in the tissue at 14, 15 and 16 days are adequate to yield all the keratin molecules produced in the ensuing 24 hours. For the production of keratin in the interval 13 - 14 days, a level of mRNA intermediate between that of 13 and 14 day cells would suffice. For the period 11 - 13 days, each daily increment of protein would require a level of messenger of the same order as that observed at the end of the interval.

In general then, it seems that the quantitative levels of keratin mRNA in feather cells are adequate to support keratin synthesis at a rate comparable to that in other systems.

TABLE 4.7.

Messenger RNA requirements for observed rates of keratin synthesis.

Age (n days)	Keratin synthesised[a]	Rate of synthesis[b]	Molecules mRNA required[c]	Molecules mRNA day n-1	present[d] day n.
12	4.8×10^8	3.33×10^5	1.23×10^4	4.60×10^2	1.09×10^4
13	1.94×10^9	1.35×10^6	4.99×10^4	1.09×10^4	5.99×10^4
14	4.20×10^9	2.92×10^6	1.08×10^5	5.99×10^4	3.44×10^5
15	1.09×10^{10}	7.57×10^6	2.80×10^5	3.44×10^5	2.92×10^5
16	1.22×10^{10}	8.47×10^6	3.13×10^5	2.92×10^5	7.01×10^4
17	1.00×10^9	6.94×10^5	2.57×10^4	7.01×10^4	?

a. Molecules of keratin synthesised per cell in previous 24 hours (from Figure 4.9).

b. Molecules synthesis per cell per minute (over previous 24 hours).

c. Assuming a translational efficiency of 27 molecules of protein/min/mRNA molecule.

d. From Figure 4.7.

E. DISCUSSION.

The results described in this chapter represent the average state of all cells in the tissue. A gradient of differentiation exists such that, at any one age, cells at the tip of the feather are more advanced than those at the base (Matulionis, 1970). Despite this complication the trends demonstrated in this study of the total tissue are likely to be a fairly close reflection of the temporal relationships in each cell as it becomes committed to keratin synthesis.

Keratin mRNA was estimated by hybridisation with cDNA, and it was demonstrated that between 11 and 14 days the relative level of mRNA in the tissue increases some 90-fold (Table 4.4). However, it was also noted that the total RNA content of the cells increased greatly over the period (Figure 4.3), so that in real terms the increase in mRNA is some 750-fold. Labelling studies with radioactive nucleosides indicate that most of the RNA synthesised in the feather is rRNA and tRNA (Partington, 1974). Presumably this increase in total RNA represents a build-up in the translational machinery of the cells, so that neither tRNA nor ribosomes will be rate limiting in translation. Feather polysomes generally have a large peak of single ribosomes (Humphreys *et al.*, 1964; Yatvin 1966a; Partington, 1974), suggesting that there is an excess of ribosomes in the

tissue. Total RNA increases may be localised in specific cells at any one time, as has been observed at both the light microscope (Koning and Hamilton, 1954) and electron microscope levels (Matulionis, 1970).

Feather tissue contains very little keratin mRNA which is not associated with polysomes, suggesting further that the mRNA is a limiting factor in keratin synthesis. A comparison of the levels of mRNA in the cytoplasmic fraction and in total RNA (Table 4.5), revealed that a large proportion of the mRNA (96%) was localised in the cytoplasm. This figure may be subject to some error, as the data used to calculate it was not obtained using the same embryos as a source of RNA, and it is possible that the comparison is not wholly valid.

Of the cytoplasmic mRNA, very little was found to be unassociated with the polysomal fraction - 99% of the mRNA sedimented with the polysomes, while only 1% remained in the post-ribosomal supernatant. This is in contrast to the situation observed in other systems. In both rabbit (Bonanou-Tzedaki et al., 1972; Gianni et al., 1972; Jacobs-Lorena and Baglioni, 1972, 1973; Olsen et al., 1972) and duck reticulocytes (Spohr et al, 1972a, b) a significant quantity of α-globin mRNA exists apart from polysomes. Similarly, large amounts of actin mRNA appear to be untranslated in chick embryo muscle (Bag and Sarkar, 1975).

The low level of keratin mRNA observed in the

post-ribosomal supernatant could arise in several ways. The most likely is run-off of polysomes during isolation, since no precautions were taken against this other than using a small volume of tissue to keep the isolation time as short as possible. Using similar conditions, Partington (1974) was unable to detect substantial differences in the polysome profiles when polysomes were isolated in the presence or absence of cycloheximide. It was found in a second experiment where the polysomes had ample opportunity to run down, as the time involved in preparation was significantly greater, that a considerably higher level of the mRNA was present in the post-ribosomal fraction. Thus it is conceivable that, *in vivo*, only a very small quantity of the mRNA at any one time is not associated with polysomes, while the level observed in isolated material arises as a consequence of the isolation procedure. The figure of 1% of mRNA not associated with polysomes could therefore be a maximum estimate of untranslated mRNA.

The presence of non-polysomal α-globin messenger in reticulocytes has been attributed to several mechanisms - the mRNA could be a precursor to the active polysome-associated form (Spohr *et al.*, 1972b), or it could be a consequence of an imbalance in protein synthesis (Bonanou-Tzedaki *et al.*, 1972). Recent evidence suggests that the α-globin mRNA may in fact be released from polysomes as a consequence of cell

aging (Marbaix et al., 1976) and would thus represent the earlier stages of cessation of protein synthesis. It is possible that any of these proposed mechanisms may occur in feather cells, but in only a small population of either relatively immature cells (if the Spohr et al., (1972b) hypothesis applies) or the most highly developed cells, if the last suggestion is correct.

It is also unlikely, from the developmental studies on the levels of keratin mRNA, that the messenger sequences are stored in the tissue prior to the onset of keratin synthesis. The levels of keratin mRNA in the tissue are just adequate to account for the quantities of keratin synthesised by the tissue (section D). Further, as discussed above, most of the messenger in the tissue appears to be actively translated. In this respect, embryonic feathers resemble erythroid cells where mRNA does not accumulate before the onset of globin synthesis, either in vivo (Chan et al., 1974; Groudine et al., 1974; Stewart et al., 1976), in cultured cells stimulated to differentiate with erythropoietin (Ramirez et al., 1975) or in leukaemia cells stimulated with dimethylsulphoxide (Ross et al., 1972). Similarly, in the hen oviduct, ovalbumin mRNA is synthesised after induction with estrogen (Cox et al., 1974; Harris et al., 1975; McKnight et al., 1975) but is not present prior to

induction. Whilst storage of maternal mRNA in an untranslated form is observed in sea-urchin eggs (Gross et al., 1973, Skoultchi and Gross, 1973), the evidence presented here makes this an unlikely possibility in feather differentiation.

Once synthesised in the tissue, the messenger RNA appears to be stable. Bell (1964) showed that actinomycin D, in doses high enough to inhibit 95% of RNA synthesis, inhibited protein synthesis by only 40% in 13-day tissue, and showed by autoradiography (Bell and Merrill, 1967) that protein synthesis in keratinising sheath cells was unaffected by this treatment. Polysome profiles from actinomycin-treated tissue revealed the presence of polysomes containing 4 ribosomes, presumably those synthesising keratin (Bell, 1964). Electrophoresis of protein synthesised in the presence of actinomycin D revealed that keratin synthesis was not affected by the treatment (my own unpublished observations).

Late in development, the messenger RNA content of the tissue decreases rapidly as the process of keratinisation nears completion (Figure 4.7). A similar observation was made in the case of hen oviduct during acute estrogen withdrawal; the messenger for ovalbumin is ordinarily quite stable, with a half-life of about 24 hours (Palmiter, 1973), but the rate of degradation is accelerated during the withdrawal process

(Palmiter and Carey, 1974). A slight increase in tissue RNase accompanied this degradation (Palmiter and Carey, 1974).

Late in development of the embryonic feathers, a rapid loss of cellular RNA is seen, including mRNA. This is accompanied by increases in the levels of two RNase activities identified in the feather, the maximum levels of RNase occurring at the stage where the rate of RNA loss is greatest. It is probable that these activities are involved in the degradation of RNA during the terminal stages of development.

Both RNase activities are present in the tissue throughout the period of keratinisation, and perhaps are involved in normal RNA turnover in the cells. It was noted in the previous chapter that cellular DNase activity increases dramatically at about 15 days. Thus it is possible that the late stages of feather differentiation follow the pattern proposed by Modak (1972) for lens differentiation. The rise in DNase may initiate DNA degradation, thereby terminating transcription. This would lead to an imbalance between transcription and normal RNA degradation, which would be accentuated by the elevated RNase levels; these levels reach a maximum value slightly later than DNase (the same extracts were used for estimating both RNase and DNase activities). Such an imbalance would promote cessation of protein synthesis in the cells.

CHAPTER 5

ISOLATED NUCLEI AS A SOURCE OF KERATIN mRNA PRECURSORS

A. INTRODUCTION.

A large body of evidence (reviewed in Chapter 1) suggests that a number of distinct events may occur between the synthesis of the primary RNA transcription product of a gene, and the final appearance of active mRNA in polysomes. The primary transcription product is a precursor to the final mRNA, in that it undergoes modifications prior to finally appearing as an active messenger species. In a number of cases, the final mRNA sequence appears to be included within a species of higher molecular weight than the polysomal RNA, although in other instances this appears not to be the case (see Chapter 1). Sequence analysis of precursors to specific mRNAs should yield valuable information concerning DNA sequences adjacent to the structural gene, provided that such sequences are transcribed.

With the large number of keratin genes estimated to be present in the chicken genome (Kemp, (1975) and the possibility that these are physically linked (Lockett and Kemp, 1975), it was of interest to attempt to isolate precursors to keratin mRNA. It is not unlikely, for example, that several of the keratin genes which are to be expressed in one cell type in the feather are present on one segment of the genome, and are transcribed as a single large molecule containing several messenger sequences. Other

possibilities are that each gene, and adjacent non-messenger sequences, are transcribed into individual precursors of high molecular weight, or that the messenger sequence seen in the cytoplasm is the entire transcript.

Because very little of the keratin mRNA present in the cytoplasm is untranslated (Table 4.6), the species which is finally transported to the cytoplasm is likely to be the final keratin mRNA. Any intermediates involved in its synthesis are therefore likely to be confined to the nucleus of the cell. Thus if nuclei, demonstrably free of cytoplasmic contamination, could be isolated from feather tissue, the search for precursors to keratin mRNA would be greatly facilitated. Such nuclei necessarily need to be intact, to minimise loss of their contents during isolation. While a wide variety of procedures exist for the isolation of nuclei from cells (see, for example, Busch et al., 1972) embryonic feather tissue is rather refractory because insoluble aggregates of keratin form when the tissue is disrupted. Such aggregates may also physically entrap other cytoplasmic contaminants, and need to be selectively removed from nuclear preparations.

Accordingly, methods were developed for isolating nuclei free from cytoplasm, which were used as a source of nuclear RNA and hence of keratin mRNA precurors.

B. METHODS.

(i) Isolation of feather nuclei.

Initially, a wide range of procedures for the isolation of feather nuclei were examined; of these, two yielded nuclei in reasonable quantities, and with low levels of keratin contamination when examined by phase contrast microscopy.

All operations were performed at 0-4°. Thirteen-day feathers were used in all preparations.

(a) "Sucrose - $CaCl_2$" nuclei.

The tissue was homogenised using a hand-held Potter-Elvehjem homogeniser in 10 volumes of 0.25M sucrose, 3mM $CaCl_2$, 0.1% Triton X-100, filtered through two layers of muslin cloth, and nuclei collected from the filtrate by centrifugation at 2,000 xg for 5 minutes. The pellet obtained was resuspended in fresh homogenising solution, the suspension layered over 0.88M sucrose, 3mM $CaCl_2$ and nuclei collected by centrifugation (2,000 xg, 10 minutes). The nuclei obtained were washed several times in 0.147M NaCl.

(b) Rotating knives.

This procedure is essentially a modification of those employed by Kuehl (1964) and by Zentgraf et al., (1969). Tissue (0.5ml or less) was suspended in a total volume of 5ml of a solution containing 0.4 M

sucrose, 3% w/v gum arabic, 3mM $CaCl_2$, 4mM n-octanol, 10mM tris HCl, the final pH being ~6.5. This suspension was homogenised in the microhomogeniser of a Sorvall Omnimix operated at maximum speed for 45 seconds, diluted with an equal volume of buffer and centrifuged at 2,000 xg for 5 minutes. The pellet was suspended in a dense sucrose solution (~ 2.35 M, refractive index 1.450), and the nuclei collected by centrifugation at 168,000 xg in a Beckman SW41 rotor for 20 minutes. The supernatant and proteinaceous pellicle were discarded, and the pellet of nuclei washed several times in 0.147 M NaCl.

(ii) Characterisation of nuclei.

Preparations were routinely examined by phase-contrast microscopy. In some preparations, nuclei were concentrated by centrifugation, and the pellet fixed, stained and sectioned for electron microscopy as described (Chapter 2 C.(ii)).

The protein content of nuclei was estimated on aliquots dissolved in 0.1 M NaOH by the method of Lowry et al., (1951). The DNA and RNA content of similar samples was estimated as described (Chapter 3,B(i)(a) and 4,B(i)(a) respectively). Aliquots of nuclear preparations were reduced and S-carboxymethylated for estimation of keratin content as described by Kemp et al., (1974a).

(iii) Histones.

Histones were isolated from washed nuclei as described by Appels and Wells (1972), dialysed against water, and freeze-dried. The histone proteins were examined on 15% polyacrylamide gels at pH 2.7 (Panyim Chalkley, 1969). In some experiments, histones were prepared as the sulphate salt, and concentrated by ethanol precipitation (Murray et al., 1968).

(iv) Isotopic labelling of feather RNA.

Feathers were plucked and washed in a modified Hanks solution lacking both calcium and magnesium and containing 1mM EGTA. They were then partially disaggregated in the same solution by digestion with 100 μg/ml trypsin (Difco 1:250) at 37° for 15 minutes, followed by slow stirring with a magnetic stirrer at room temperature for 10 minutes. The tissue was then washed several times with the modified Hanks solution, followed by several washes in Waymouth's medium (Waymouth, 1959) containing 10% foetal calf serum. Incubation was performed in the same medium containing 40 μC/ml [5,6-^{3}H]-uridine (specific activity 40C/mmole) at 37°, with gentle shaking, under an atmosphere of 5% CO_2 in air. After incubation for 1 hour, the tissue was washed several times with medium lacking uridine.

(v) Isolation of nuclear RNA

Nuclei were digested with proteinase K as described by Gross-Bellard et al., (1973) for 7 hours, the digest then being extracted with an equal volume of water-saturated phenol, adjusted to 0.1 M NaCl, and nucleic acids precipitated by the addition of 2.5 volumes of ethanol, and stored overnight at -20°. Nucleic acid was collected by centrifugation (25,000 xg, 2°), and dried in vacuo. DNA was removed by digestion with DNase I, followed by phenol extraction and ethanol precipitation, essentially as described by Soeiro and Darnell (1969). DNase I was treated with iodoacetic acid to eliminate RNase activity (Zimmerman and Sandeen, 1966).

(vi) Sucrose gradient centrifugation, and detection of keratin mRNA sequences.

Sucrose gradient certrifugation was performed under denaturing conditions using gradients of 4.6-22% sucrose in 70% formamide (Suzuki et al., 1972). The gradients were fractionated using an ISCO model 640 gradient fractionator. Aliquots of each gradient fraction were diluted with $^1/_5$ volume 7x hybridisation buffer, plus $^1/_5$ volume of [^{3}H]-cDNA in water (~1000 cpm were used per sample). Samples were then denatured at 60°and incubated at 37° for the times required. Each sample was then diluted 40-fold with 0.18M Na phosphate

(i.e. Na^+ molarity = 0.18, pH 7.0) containing 50 µg each of native and denatured calf thymus DNA, and assayed on hydroxyapatite at 60° (Britten and Kohn, 1968).

C. RESULTS.

(i) Comparison of methods for nucleus isolation.

The efficiencies of the two methods described were assessed by a number of criteria. Firstly, it was desirable that yields be good, and in this respect both procedures were of equal merit, with yields (based on recovery of DNA) ranging from 25 - 40%. The yields obtained using high speed rotating knives to homogenise the tissue were dependent on two factors. If the duration of homogenisation was increased beyond 45 seconds, the yield declined (Figure 5.1(a)), presumably because of damage to the nuclei. The method was also sensitive to changes in pH - some preparations of purified gum arabic (Kuehl, 1964) were extremely acidic and lowered the pH to ~3.5, at which pH a large amount of protein copurified with the nuclei. An optimal pH range appeared to be from pH6-7 (Figure 5, 1(b)) - the homogenising buffer was thus adjusted to pH 6.5 before use. Presumably the poor yields of clean nuclei below pH 6 were due to alterations in the ionic state of the gum arabic, the function of which may be to protect the nuclei against damage (Kuehl, 1964).

FIGURE 5.1.

Factors Influencing Isolation of Nuclei with Rotating Knives.

(a) Effect of varying homogenising time on yield of nuclei.

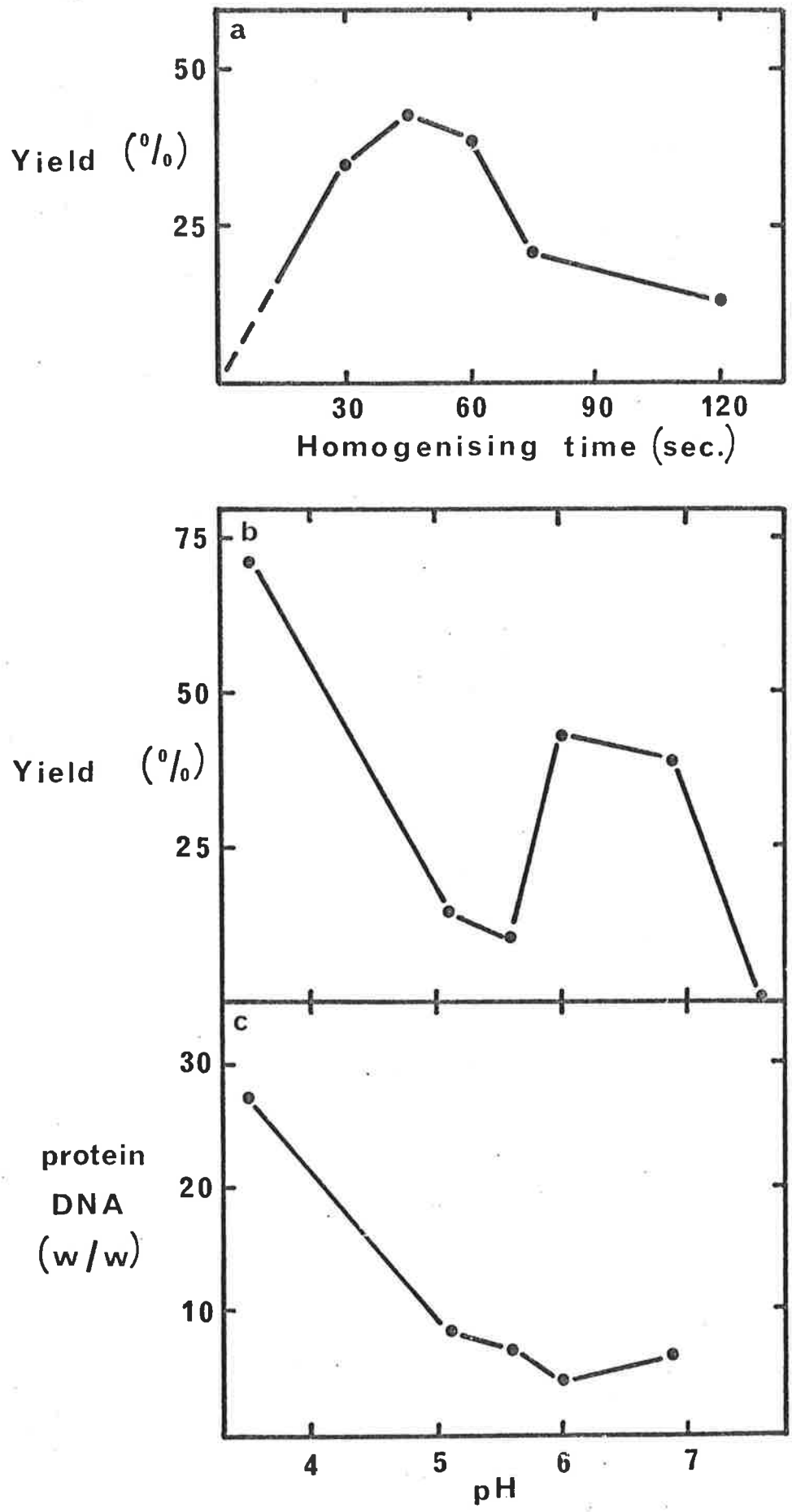
Feathers were suspended in a total of 5 ml of 0.4 M sucrose, 3mM $CaCl_2$, 3% gum arabic, 10 mM tris pH~6.5 and 4 mM n-octanol, and homogenised for the times indicated at maximum speed in the Sorvall Omnimix. The nuclei were purified by centrifugation through 2.35 M sucrose, and the yield estimated by recovery of DNA.

(b) Effect of pH on yield of nuclei.

Feathers were homogenised as described in (a), except that the pH of the medium was varied over the range shown. Nuclei were purified through 2.35 M sucrose. The yield was estimated by the recovery of DNA.

(c) Effect of pH on protein : DNA ratio of nuclei.

The protein content of the nuclei prepared in (b) above was estimated by the method of Lowry, et al., (1951), from which the protein : DNA ratio was calculated.


a
50
25
Yield (%)
30
60
90
120
Homogenising time (sec.)
b
75
50
25
Yield (%)
c
30
20
10
protein
DNA
(w/w)
4
5
6
7
pH

The nuclei were also examined to assess the degree of contamination with cytoplasmic materials. The ratio of protein to DNA in the preparations was used as a semiquantitative estimate of purity of the nuclei - the more highly purified the nuclei, the lower this ratio should be. In each case, the level of protein in the nuclear fractions was considerably lower than in the starting tissue (Table 5.1); the value was significantly lower for nuclei isolated using knives. It was found (Figure 5.1(c)) that the protein : DNA ratio in the latter case was highly pH-dependent.

A major contaminant in these preparations was expected to be keratin. The presence of this material was estimated qualitatively using light microscopy - aggregates of keratin were readily visible - and quantitatively using gel electrophoresis (Kemp et al., 1874a). Some preparations of nuclei using knives contained no detectable keratin, in others it was present as a minor component (Table 5.1). All preparations using the more gentle technique contained at least some keratin, albeit much less than in the starting tissue - most of the keratin aggregates forming on homogenisation in this case were readily removed by filtration through muslin cloth.

The higher level of keratin observed in preparations of "sucrose-$CaCl_2$" nuclei cannot entirely account for the higher protein content of these nuclei.

TABLE 5.1.

Composition of Feather Nuclei.

	13-day Tissue	Nuclei isolated by "Sucrose-calcium" method.	Nuclei isolated using rotating knives.
DNA	1.00	1.00	1.00
RNA	2.49	0.29	0.48
Protein	65.8	8.60	4.11
Histone	n.d.	1.44	1.23
Keratin	11.7	1.10	0.20

Nuclei were isolated by the methods described in the text, and the DNA, RNA and protein contents estimated on aliquots dissolved in 0.1 M NaOH (the details are given in the text). Histone was determined as protein soluble in 2 M NaCl, 0.25 M HCl (method of Appels and Wells, 1972). Keratin was determined by the method of Kemp et al., (1974a) after reduction and carboxymethylation of an aliquot of the preparation. Data have been expressed as ratios relative to DNA (weight : weight in each case) for comparative purposes. Each value represents the mean of several experiments. The 13-day tissue values were determined on the initial homogenates.

n.d. = not determined.

Examination of the nuclei with the electron microscope reveals that they are contaminated by adhering cytoplasm and by remnants of the plasma membrane. (Figure 5.2a). Such contaminants were not observed when rotating knives were used to homogenise the tissue (Figure 5.2b), suggesting that the lower protein content of these nuclei is due to the complete removal of cytoplasm and membrane. Inclusion of detergents such as triton X-100 at 0.1%(v/v) (Figure 5.2c) or 0.5%(v/v) nonidet P-40 (Figure 5.2d) in the homogenising medium did not alter the protein : DNA ratio of nuclei isolated with rotating knives, but did lead to considerable damage of the nuclear membrane. In the absence of detergent the nuclei obtained appeared to be free of cytoplasmic contaminants, and were intact, as judged by electron microscopy.

(ii) Histones.

Acid soluble proteins, described as histones in Table 5.1, could be isolated from nuclei prepared by either method. This designation was confirmed by polyacrylamide gel electrophoresis at low pH (Panyim and Chalkley, 1969) - the protein patterns on these gels resembled those of erythrocyte histone (Figure 5.3).

Preparations of histone isolated from "sucrose-$CaCl_2$" nuclei often showed higher levels of the erythroid cell - specific histone H5 (nomenclature of Bradbury, 1974)

FIGURE 5.2.

Electron Microscopy of Isolated Nuclei.

Nuclei were isolated as described in the appropriate places in the text, washed in 0.147 M NaCl and collected by centrifugation. The pellets were fixed, stained and sectioned, as described in the text.

A. Nuclei isolated by the "sucrose-$CaCl_2$" method
N - nucleus, Cy - cytoplasm, pm - plasma membrane.

B. Nuclei isolated by homogenising with rotating knives.

C. Nuclei isolated by homogenising with rotating knives, but with 0.1% (v/v) Triton X-100 included in the homogenising medium.

D. Nuclei isolated by homogenising with rotating knives, but with 0.5% (v/v) Nonidet P-40 included in the homogenising medium.

Magnification 9710x.

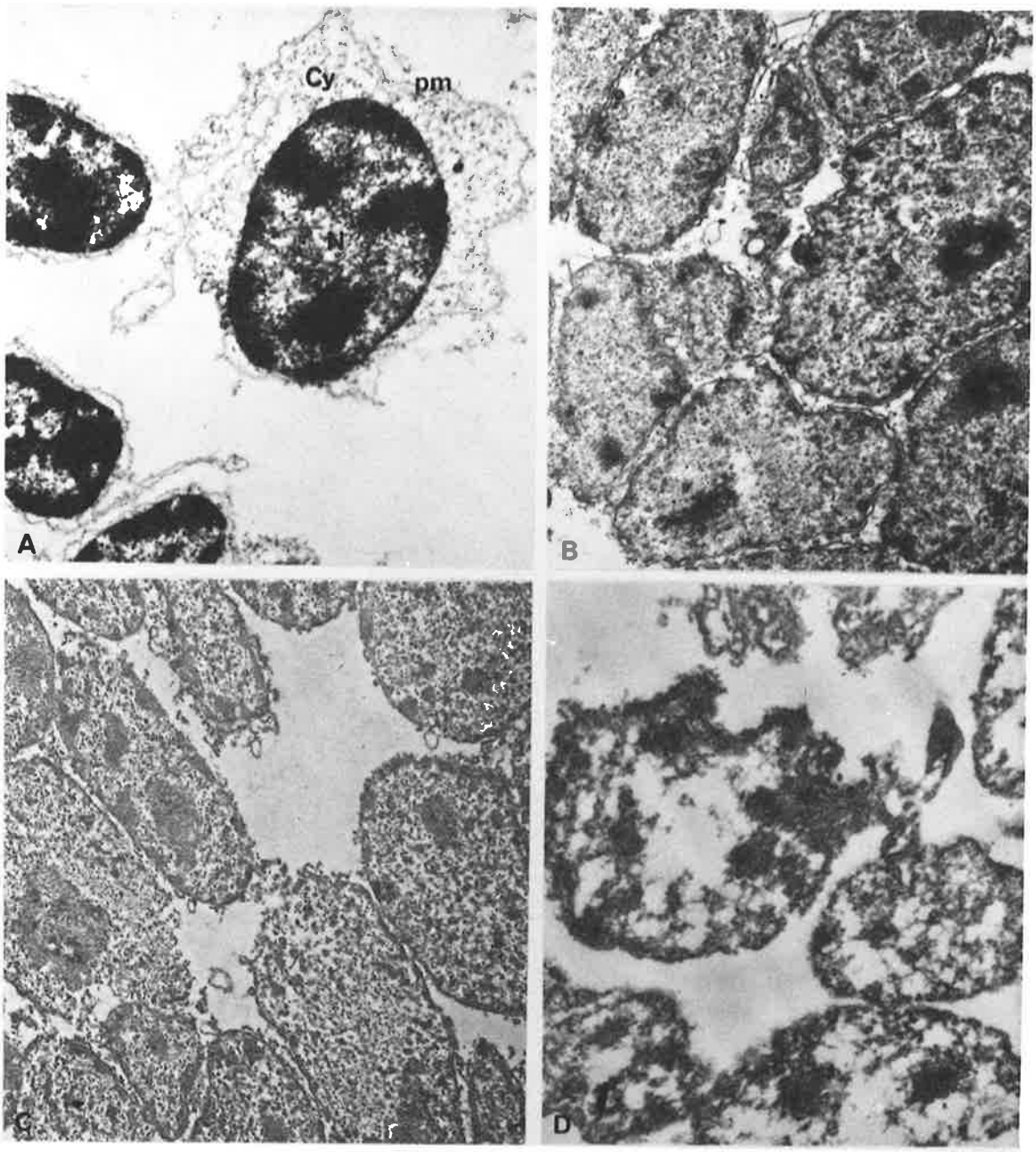
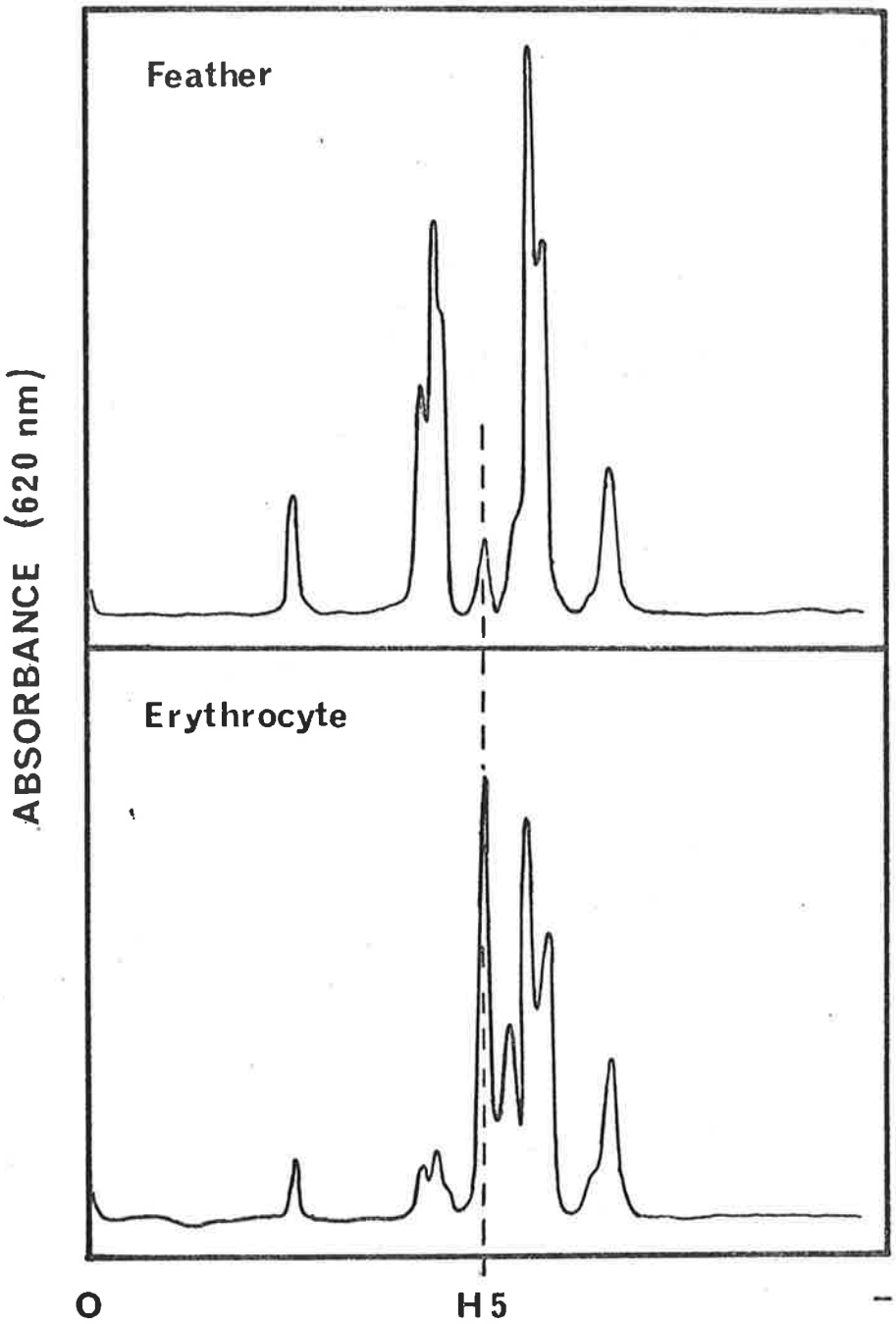

Cy
pm
N
A
B
C
D

FIGURE 5.3.

Polyacrylamide Gel Electrophoresis of Histones prepared from "Sucrose-$CaCl_2$" Nuclei, and from Erythrocytes.

Histones were isolated from "sucrose-$CaCl_2$" nuclei prepared from feather tissue, as described by Appels and Wells (1972). The proteins were dialysed against distilled water, freeze-dried, and subjected to gel electrophoresis at pH 2.7 as described by Panyim and Chalkley (1969). For comparison, a sample of chicken erythrocyte histones (gift of Dr. R. Harlow) was subjected to electrophoresis on a parallel gel. 14 µg of protein was loaded on each of the gels. The gels were stained with Amido Black, and scanned at 620 nm in a Gilford spectrophotometer fitted with a gel scanner, coupled to a W + W recorder.

O	-	origin
-	-	cathode
H5	-	position of the erythroid cell-specific histone H5.


Feather
Erythrocyte
ABSORBANCE (620 nm)
O
H5
–

than could simply be explained by the levels of erythrocytes in the tissue. If, however, the filtration step was omitted, and histones extracted from a crude cell debris fraction, the relative level of H5 was much lower (Figure 5.4). Similarly, the level of H5 in nuclei isolated using knives was lower than in those isolated by sucrose/$CaCl_2$ (Figure 5.4). These observations suggest that the more gentle homogenisation employed with the sucrose - $CaCl_2$ procedure leads to a selection for nuclei from the softer, less keratinised cells in the tissue, whereas vigorous homogenisation gives a more representative sample of nuclei from the tissue.

(iii) Isolation of nuclear RNA.

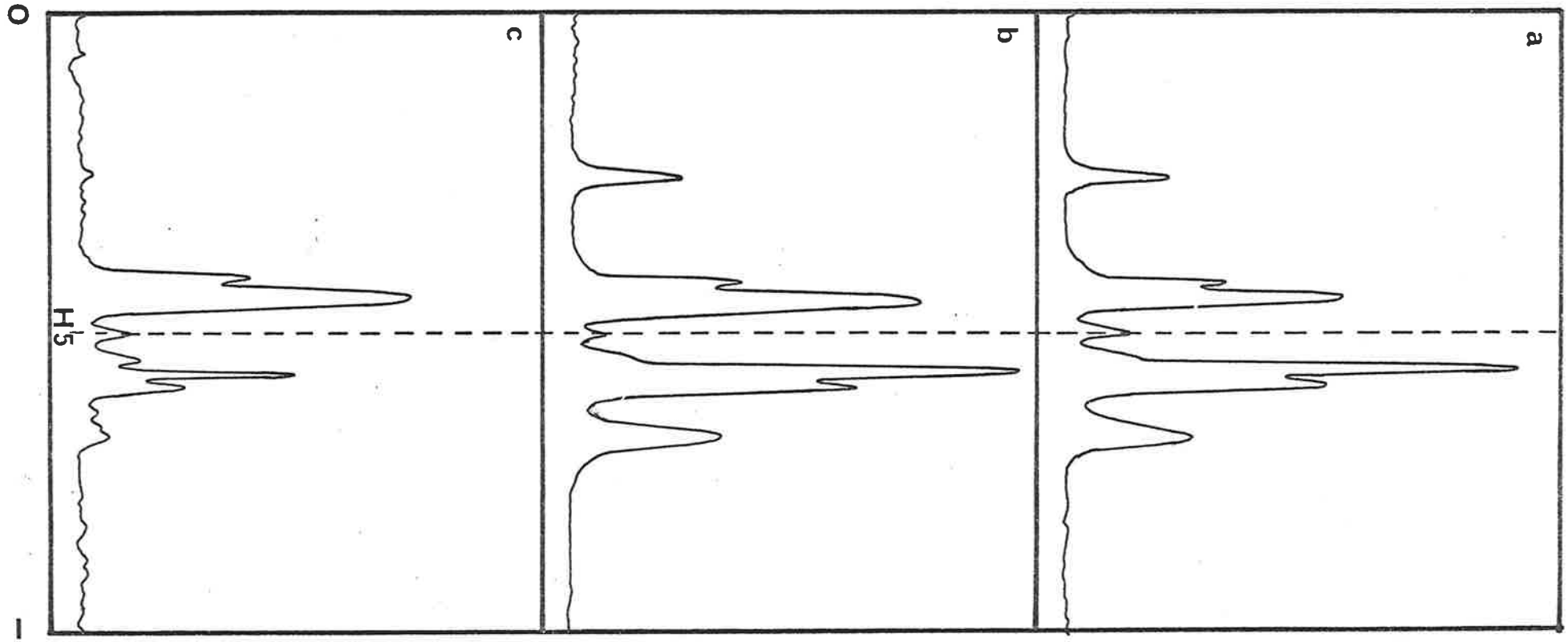
In preliminary investigations, the RNA was first labelled in cultured feathers with[^{3}H]-uridine. It was found that higher levels of incorporation could be achieved if the tissue was first partially disaggregated - this treatment resulted in a 2.5 fold increase in specific activity, although the distribution of the label was unaltered. Two procedures in addition to that described (B(v)) were examined for relative efficiency of preparation of RNA. The recovery of RNA by either hot phenol extraction (Soeiro and Darnell, 1969) or by phenol extraction of nuclei solubilised in urea (Holmes and Bonner, 1973) was found reproducibly to be lower than that obtained by the sequential enzymic

FIGURE 5.4.

Gel Electrophoresis of Feather Histones.

Histones were isolated from nuclei purified by the "Sucrose-$CaCl_2$" procedure (a), from a cell debris fraction from feathers homogenised as for the "Sucrose-$CaCl_2$" procedure, but from which the filtration step was omitted (b) and from nuclei isolated using rotating knives (c). The proteins were subjected to gel electrophoresis at pH 2.7, with 28 μg being loaded onto each gel. The gels were stained with Amido Black, and scanned at 620 nm as described in the caption to Figure 5.3.

O	origin
-	cathode
H5	marks the position of H5 histone in a parallel gel of erythrocyte histones (not shown).


ABSORBANCE (620nm)
0
1
H5
c
b
a

digestion procedure described (Table 5.2). In the latter procedure, high yields were obtained at each stage in the procedure (Table 5.2).

The integrity of the RNA isolated by each procedure was examined using sucrose gradient centrifugation under denaturing conditions in 70% formamide (Suzuki et al., 1972). Both the Holmes and Bonner (1973) procedure and that using enzymic digestions yielded RNA which sedimented heterogeneously, with some material greater than 28S being present (Figure 5.5). Although the 28S rRNA does not sediment far into the gradient, it was also apparent the RNA extracted with hot phenol was smaller and probably degraded during isolation.

From these findings, the method chosen for the purification of nuclear RNA for hybridisation studies was that involving proteinase K and DNase digestions.

(iv) Hybridisation of nuclear RNA fractions to keratin cDNA.

The kinetics of hybridisation of keratin mRNA to its cDNA were examined under the conditions used (50% formamide, 0.18 M Na^+, 37°), and, as judged by sensitivity to S1 nuclease, did not show any significant difference to the kinetics observed under standard conditions in the absence of formamide

TABLE 5.2.

Isolation procedures for nuclear RNA.

	cpm	% recovery	nett yield, %
Nuclei	13800	100	100
Hot phenol extraction. (Soeiro and Darnell, 1969).	1120	8.1	8.1
Holmes and Bonner(1973)			
Crude nucleic acid extract.	10400	75.4	
DNase digested.	3600	34.6	26.1
Sequential enzyme digestion.			
Protease K digest, extracted with phenol.	11840	85.8	
DNase digested.	8800	74.4	63.8

Nuclear RNA was labelled with [^{3}H]-uridine as described in the text, and nuclei prepared from the tissue using rotating knives. The nuclei obtained were divided into three equal portions, and nuclear RNA isolated from each. An aliquot was precipitated with 10% TCA at various stages during the procedure, to estimate recoveries.

FIGURE 5.5.

Sedimentation Analysis of Nuclear RNA.

RNA was labelled with [5,6-^{3}H]-uridine,* and the nuclei prepared from the tissue as described in the text. The nuclei were divided into three equal parts, and the RNA isolated from the individual fractions by the different procedures described in the text. The RNA was loaded onto 4.6 - 22% sucrose gradients in 70% formamide, and centrifuged for 12 hours at 37° in an SW41 rotor. The gradients were fractionated using an ISCO model 640 gradient fractionator, and 0.5 ml fractions were collected. The gradient fractions were precipitated with 2 ml of 10% TCA, plus 50 µg of BSA as carrier, and collected on GF/A filters. The filters were washed with TCA, ethanol and ether, dried, and counted.

Sedimentation is from left to right, and the position of 28S rRNA is indicated by the dashed line.

(a) RNA isolated by hot phenol extraction (Soeiro and Darnell, 1969).

(b) RNA isolated by the method of Holmes and Bonner (1973)

(c) RNA isolated by sequential digestion of the nuclei with proteinase K and with DNase I.

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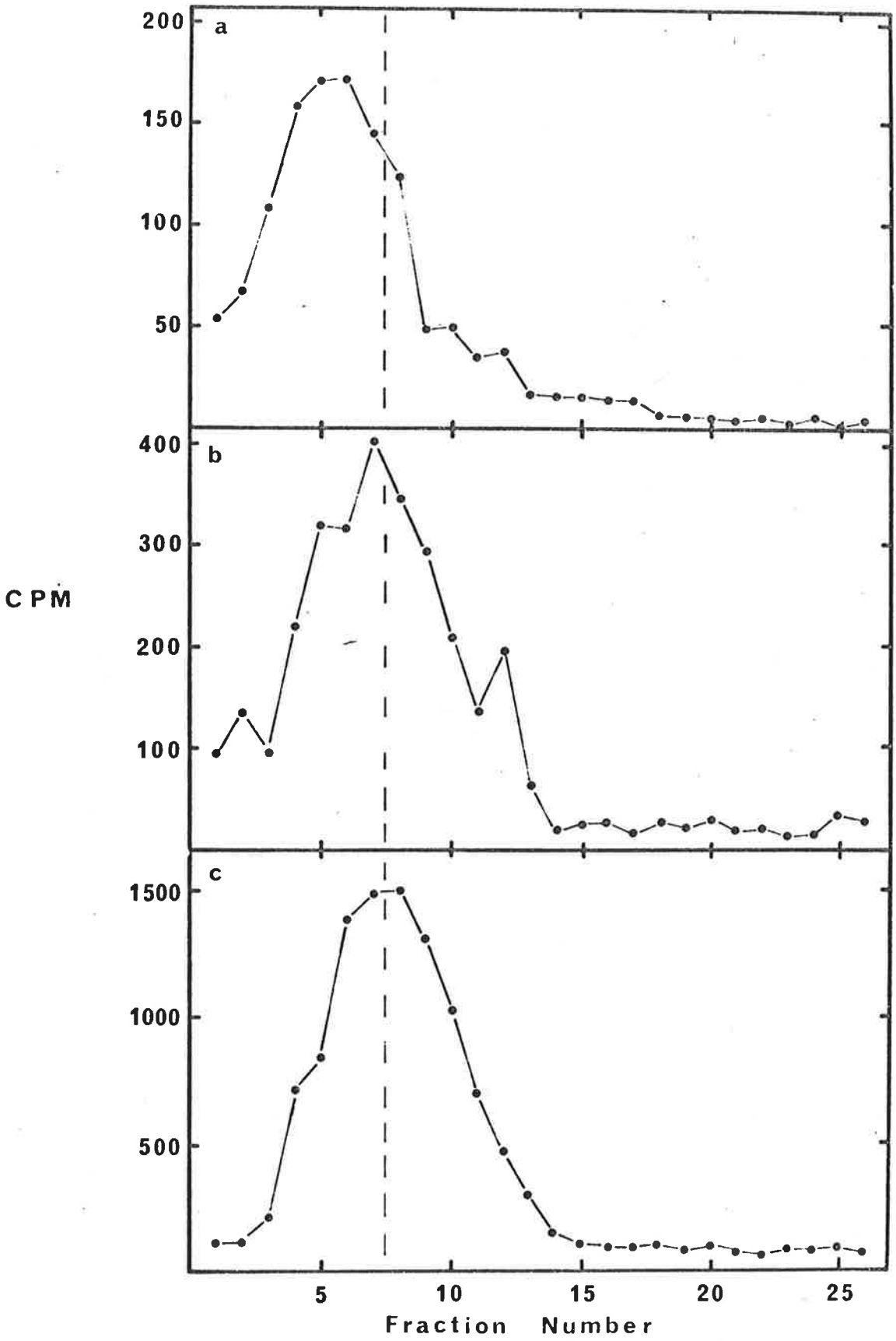
*For 1 hour.


a
b
c
200
150
100
50
400
300
200
100
1500
1000
500
CPM
5
10
15
20
25
Fraction Number

(Figure 5.6). For reasons unknown, however, the behaviour of these hybridisation reactions to nuclease S1 was not reproducible from experiment to experiment - occasionally all the hybridisation reactions in an experiment were completely insensitive to the enzyme.

Unlabelled nuclear RNA was separated on a sucrose gradient in formamide, and aliquots of the gradient fractions hybridised with keratin cDNA. The extent of hybridisation in each was estimated using hydroxyapatite where low levels of formamide did not affect binding. Furthermore, the apparent rate of reaction between mRNA and cDNA is approximately 10-fold greater when assayed by hydroxyapatite compared to S1 (Kemp, 1975), so that shorter hybridisation times could be employed. The concentration of keratin mRNA sequence was calculated from the observed extent of hybridisation, using a standard mRNA : cDNA hybridisation curve. It was observed that a fraction of the mRNA sequence present in the preparation sedimented faster than the main peak at 12S (Figure 5.7). This heterogeneous material may be due to aggregation of the messenger sequence with other RNAs in the preparation - a parallel gradient containing 13-day cytoplasmic RNA exhibited a similar profile. In many of the high molecular weight points the extent of hybridisation did not differ greatly from that observed with controls lacking RNA; it is possible that the differences may

FIGURE 5.6.

Hybridisation of Keratin mRNA with cDNA.

(a) Reaction mixtures (25 µl) contained keratin mRNA (0.004 - 1,60 µg/ml) and 1000 cpm of [^{3}H]-cDNA, in aqueous buffer (Chapter 2,C(v)). The mixtures were denatured at 100° for 5 minutes and incubated at 60° for 4 hours. The samples were diluted with 500 µl of high salt S_1 buffer containing 24 µg/ml single stranded DNA, and one half of the sample was digested with 2 units of nuclease S_1 (Vogt, 1973) at 37° for 30 minutes. The acid-insoluble radioactivity in the enzyme-treated and untreated portions of each sample was used to determine the extent of hybridisation. The arrow indicates the $R_o t_{\frac{1}{2}}$ of the reaction (6.0×10^{-3} mol.sec.l^{-1}).

(b) Reaction mixtures (35 µl) contained keratin mRNA and cDNA as in (a), in buffer similar to that used above except that it contained 50% formamide. Denaturation was at 60°, and hybridisation was at 37° for 5.5 hours. The extent of hybridisation was assayed as described in (a).

The arrow indicates the $R_o t_{\frac{1}{2}}$ (5.8×10^{-3} mol.sec.l^{-1}) .

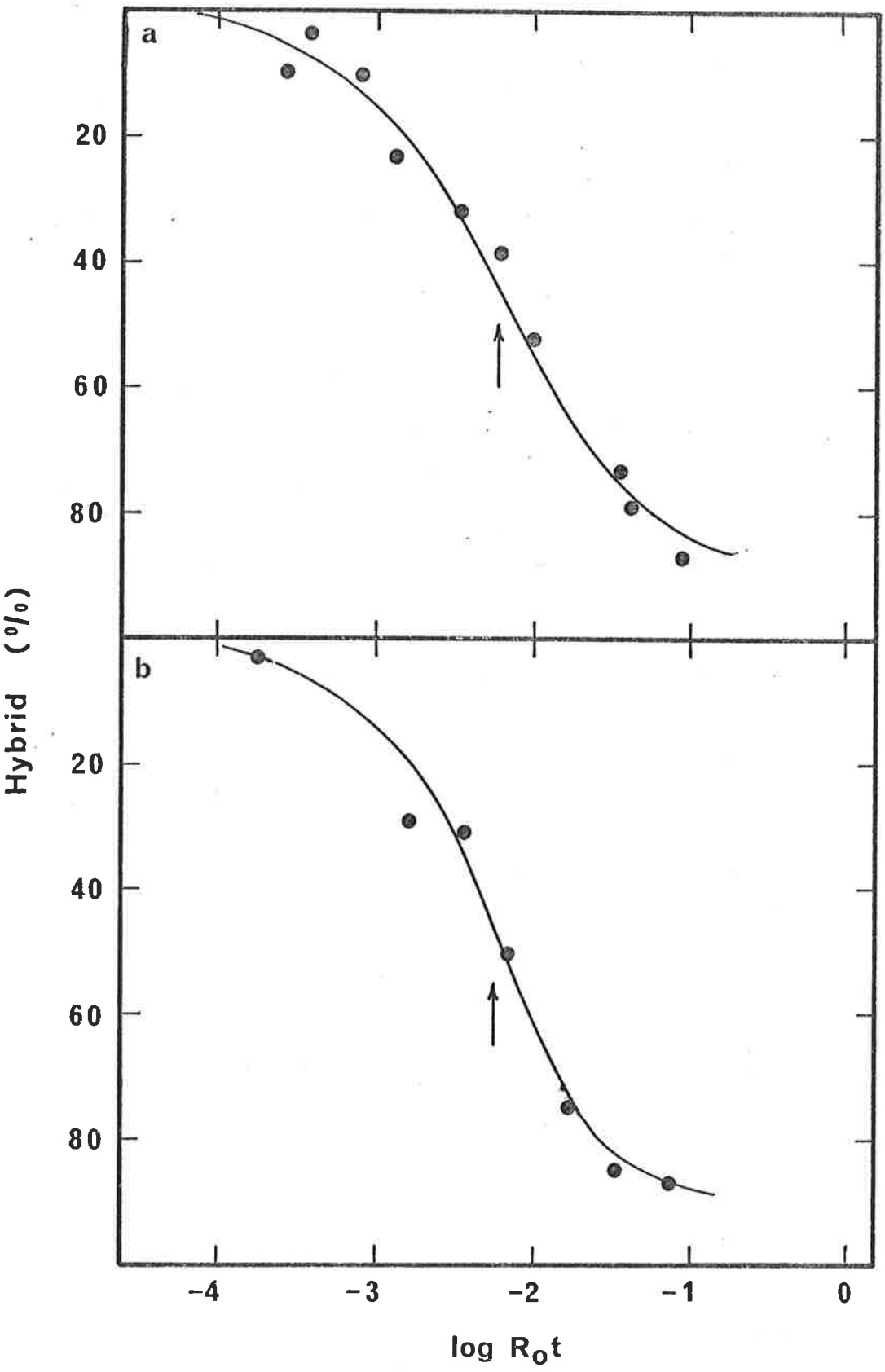
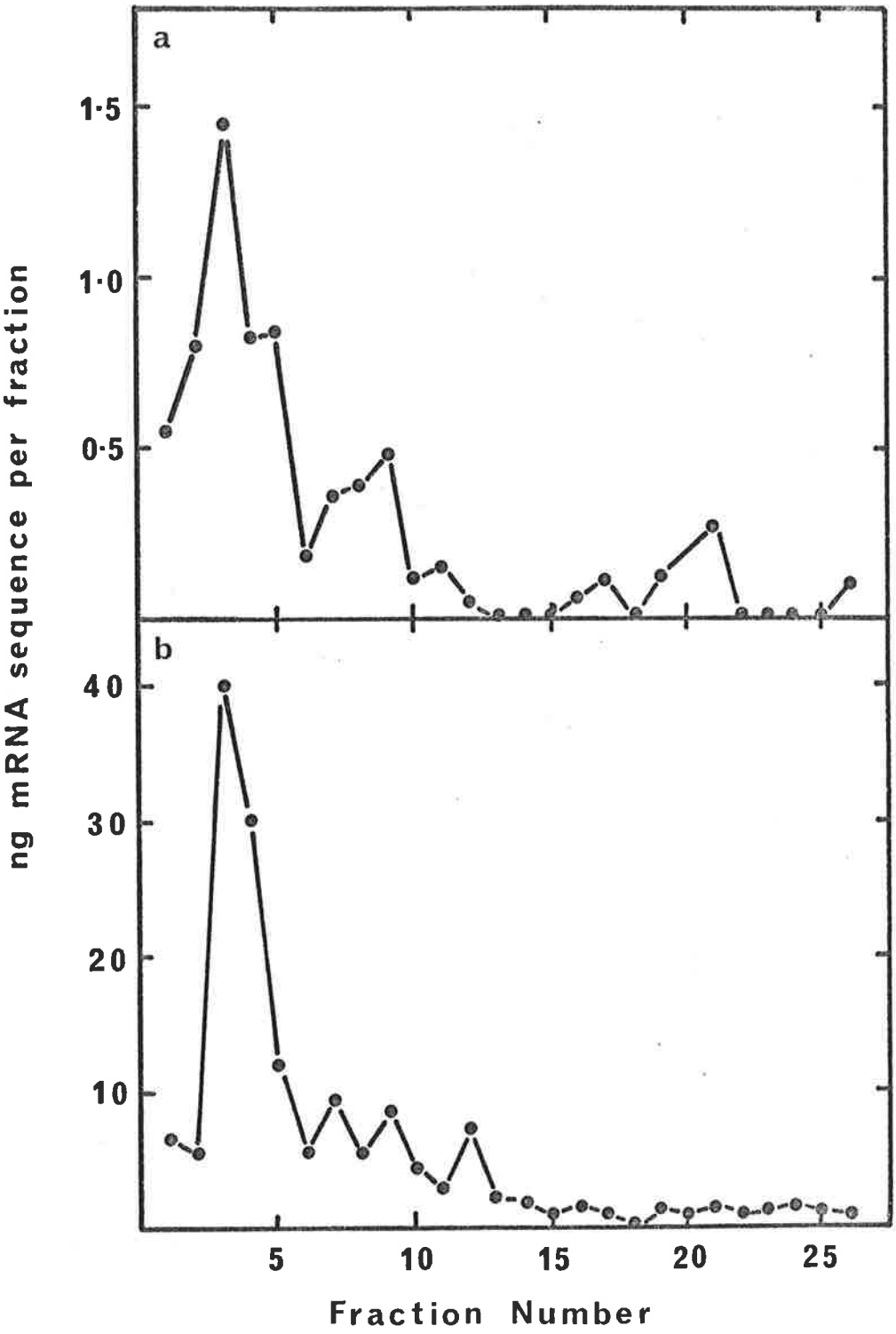

a
b
20
40
60
80
20
40
60
80
Hybrid (%)
-4
-3
-2
-1
0
log $R_o t$

FIGURE 5.7.

Sedimentation analysis of Nuclear mRNA Sequences.

(a) Nuclei were isolated from 1.8 ml of packed 13-day feathers, and RNA isolated from the nuclei by sequential digestion with proteinase K and DNase I as described in the text. The total RNA was subjected to sucrose gradient centrifugation as described in the legend for figure 5.5. 25 µl of each gradient fraction were diluted with 5 µl of 7x hybridisation buffer and 5 µl of cDNA in H_2O, and the samples denatured at 60°, and incubated at 37° for 17 hours. Hybridisation was assayed with hydroxyapatite as described in the text, and the concentration of mRNA determined by comparison with a standard mRNA : cDNA hybridisation curve.

(b) 192 µg of 13-day cytoplasmic RNA, isolated by phenol extraction at pH 9.0, was centrifuged on a parallel gradient. 25 µl of each fraction was diluted for hybridisation as above, denatured, and reacted at 37° for 6 hours. The samples were assayed on hydroxyapatite, as described.


a
b
1·5
1·0
0·5
40
30
20
10
5
10
15
20
25
ng mRNA sequence per fraction
Fraction Number

not be significant.

D. DISCUSSION.

All the tissue used in the experiments described in this chapter was taken from embryos at 13-days of incubation. At this stage keratin synthesis has become established in the tissue, although the levels of keratin are still relatively low (Kemp et al., 1974a: see also figure 4.9). Nonetheless, the data of figure 4.7 would strongly suggest that the rate of keratin mRNA synthesis is nearly maximal in the feathers at this stage. Nuclei could be prepared from such feathers by the techniques described, although it was observed by a variety of criteria that nuclei isolated by high speed homogenisation of the tissue in combination with a density selection step contained less non-nuclear contaminants than did those isolated by the more gentle procedure. Furthermore, it is probable, from the electrophoretic patterns of histones isolated from the nuclei, that the more vigorous technique yielded a more representative sample of feather nuclei, encompassing all cell types. This conclusion must be tentative, however, as it is apparent, from Figure 5.4c, that some limited proteolysis of the histones occurred during isolation.

It is unlikely that the H5 histone in these

preparations arises from anything other than erythrocytes present in the central capillary of the feathers. The levels of H5 in histone preparations from cell-debris fractions could readily be accounted for by the erythrocyte content of the feathers (calculated from the absorbance due to haemoglobin in a soluble fraction prepared from the feathers). The chick embryo lens has also been shown to lack H5 (Teng et al., 1974), indicating that H5 is specific to erythroid cells, and is not a characteristic of terminally differentiating, non-dividing, avian cells.

Nuclei lacking cytoplasmic contaminants were used as a source of nuclear RNA, which should be enriched in keratin mRNA precursors. The RNA was isolated by sequential digestion of the nuclei with proteinase K and DNase I. The possibility of RNA degradation occurring during this isolation procedure is low, as proteinase K is a potent RNase inhibitor (Wiegers and Hilz, 1971), and although residual RNase activity was observed in occasional batches of DNase I, this could be eliminated by pretreatment with iodoacetate (Zimmerman and Sandeen, 1966). Examination of the molecular weight distribution of mRNA sequences in the nuclear RNA indicated that no species of molecular weight greater than that of 12S keratin mRNA could be detected which could not be explained by the presence of aggregated forms of the mRNA. This indicates that either the conditions of denaturation described by

Suzuki et al., (1972) were inadequate to ensure denaturation of keratin mRNA-containing aggregates, or that these were capable of reforming rapidly after denaturation. On the basis of this experiment, it was unlikely that a precursor to keratin mRNA could be detected in the nucleus.

The levels of detection employed in this experiment may not have been adequate to locate a precursor of high molecular weight. The high level of aggregation of mRNA sequences (Particularly those sedimenting between 12 and 40S) may easily mask a precursor of that order of size which could conceivably be present in a low amount. The low levels of mRNA sequence present in the extracts further complicated this analysis. The reason for such a low level is not clear - losses may have occurred during the isolation procedure. It is not clear, for example, to what extent the normal processing mechanisms may have continued to operate in the nuclei during isolation, either in cleaving larger precursors to a final 12S product, or in transporting the completed mRNA into the extra-nuclear medium. It is also possible that non-specific RNase degradation may occur - the vigorous homogenising may disrupt lysosomes, releasing RNase and proteases in addition to other enzymes. That this may have occurred is suggested by the degradation of histones in some preparations, particularly where vigorous

homogenising or high levels of detergents were used in nuclear preparation. Losses of keratin mRNA sequences also occur during phenol extraction (see Chapter 4).

While the obvious tentative conclusion from this work is that no mRNA precursor occurs in isolated nuclei, it is necessary to stress that the time taken in isolating the nuclei may have allowed the various processing enzymes, or non-specific cellular RNase, to degrade the precursor sequences. Since the precursor (if any) may be short-lived *in vivo*, it was decided not to persevere further with this approach, and to use other methods of seeking the precursor (see Chapter 6).

CHAPTER 6

DETECTION OF THE STEADY-STATE LEVELS OF KERATIN mRNA PRECURSORS

A. INTRODUCTION.

The procedure used to purify nuclei which were free from cytoplasm was quite time-consuming, and, as a consequence could have allowed degradation of keratin mRNA precursors during isolation, either by normal processing or by nonspecific nuclease digestion. It was therefore felt that alternative approaches to the problem of detecting precursors might be more fruitful.

The work described in this chapter was performed with the total cellular RNA from feather cells. Because the nuclear species are likely to comprise only a relatively small proportion of the total messenger population of the cell, the tissue was taken from young (12-day) feathers, where the level of mRNA in the cell is quite low (Figure 4.7). On the other hand, in such tissue, nuclear precursors may be relatively more abundant than in somewhat older material. It was subsequently found (see below) that younger tissue had other practical advantages which proved useful in this study.

B. METHODS.

(i) Isolation of total RNA from 12-day feathers.

Feathers were plucked from 12-day embryos which had been washed extensively with NKM, and were placed directly into an emulsified mixture consisting

of 1 volume 0.1 M tris-HCl (pH 9.0), 1% sarkosyl, and 0.5 volume water-saturated phenol, at 0°. The emulsion was shaken vigorously at reasonably frequent intervals to aid dissolution of the tissue. The emulsion was finally briefly homogenised with 6 - 8 strokes of a hand-held Potter-Elvehjem homogeniser, after which 0.5 volumes (i.e. equal to that of phenol) of chloroform was added. The RNA was extracted at either room temperature (25°) or 37°, with frequent and vigorous mixing. The phases were separated by low speed centrifugation (2,000 xg, 5 minutes). The organic phase and interphase were extracted with 0.5 vol. H_2O, after which the aqueous phases were pooled, and twice extracted with 1 volume of phenol-chloroform (1:1), as above. Finally, the aqueous phase was adjusted to 0.1 M in Na acetate, pH 5, and nucleic acid precipitated by the addition of 2.5 volumes of ethanol. The precipitate was allowed to form overnight at -20°, and was then collected by centrifugation (25,000 xg, 30 minutes), drained and dried <u>in vacuo</u>.

The DNA was removed by digestion with DNase I, as described by Soeiro and Darnell (1969), and the RNA remaining precipitated with ethanol as described above, and collected by centrifugation.

(ii) <u>Polyacrylamide gel electrophoresis of RNA</u>.

RNA was subjected to polyacrylamide gel electrophoresis in 98% formamide, as described by

Pinder et al., (1974). Samples of RNA, prepared as described above, were dried, and then dissolved in H_2O. The concentration was estimated (by absorbance at 260nm, see Chapter 2) and an aliquot lyophilised. This dried sample (generally 25 - 30 μg of total RNA) was dissolved in the loading buffer described by Pinder et al., (1974), heated to 60° for 1 minute, and loaded onto the gel - the gel size was 8.5 x 0.6 cm, and was 4% in acrylamide.

After electrophoresis, the mobility of RNA samples was estimated in stained or unstained gels. Unstained gels were scanned at 280 nm in a Gilford spectrophotometer. Gels containing RNA only were stained with 0.05% toluidine blue (dissolved in 55 mM Na acetate, pH 5.5, 0.1 mM EDTA), and were scanned at 600 nm. In some cases, notably where total nucleic acid was loaded on the gels, staining was performed with "stains-all" (Dahlberg et al., 1969) - these gels were also scanned at 600 nm. For comparison of different gels in the same experiment, the relative mobility of the RNA species was determined, by comparison with the bromophenol blue dye marker.

(iii) Recovery of RNA from polyacrylamide gels.

Gels were soaked in sterile water for 2 hours after electrophoresis, then frozen in dry ice and cut into 1 mm slices using a single-blade gel slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey, England).

Individual slices were placed into 2.5 ml glass vials with 200 - 250 µl of buffer (10mM tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS) containing 15 µg yeast RNA, and incubated for 12 - 15 hours at 4°.

The RNA was collected from the eluates by either pooling the material from appropriate fractions, adding 0.1 volume of 1M Na acetate, pH 5 and 2.5 volumes of ethanol and collecting by centrifugation, or by drying aliquots of the eluate *in vacuo*.

(iv) Chromatography on oligo(dT)-cellulose.

RNA was fractionated on a column of oligo(dT)-cellulose (1.2 x 0.7 cm) essentially as described by Aviv and Leder (1972), except that NaCl replaced KCl in all buffers, and the intermediate (0.1 M salt) wash was omitted. The RNA in bound and unbound fractions was precipitated with ethanol as described above.

C. RESULTS.

(i) Isolation of total 12-day feather RNA.

To minimise the possibility that processing of nuclear RNA could continue during the isolation of total cellular RNA, conditions were employed which were designed to rapidly halt normal cellular processes. It was found that the method employed, lysis in strong detergent at pH 9.0 in the presence of phenol, at low temperature, was particularly appropriate for 12-day

tissue. Disruption of the tissue was rapid - the tissue was essentially destroyed after no more than 1 - 2 minutes, as judged by the presence of tissue fragments in the emulsion. Older tissue was found to be rather less readily lysed under these conditions. Feathers from 12-day embryos also offer the advantages of containing relatively low levels of cytoplasmic mRNA (thus perhaps containing a greater relative abundance of nuclear species), and of RNase (Figure 4.4).

The total nucleic acid extracted by this procedure appeared to contain intact RNA. Chick ribosomes contain equimolar amounts of 28S and 18S rRNA, of molecular weights 1.58 and 0.70 x 10^6, respectively (Loening, 1968), and should therefore be present in a ratio of 2.25 : 1, by weight. Large molecules would be expected to be more susceptible to nicking by RNase than small ones, so that a weight ratio significantly lower than this value would be indicative of a low level of RNase activity. It was seen (Figure 6.1a) that the ratio of 28S to 18S rRNA in the total nucleic acid extract was quite high - in the example shown the ratio, determined by integrating the areas under the peaks, was 2.33:1. The slowly migrating peak described as DNA in the figure was found to be DNA by both its different staining with stains-all (Dahlberg et al., 1969) and by its

FIGURE 6.1.

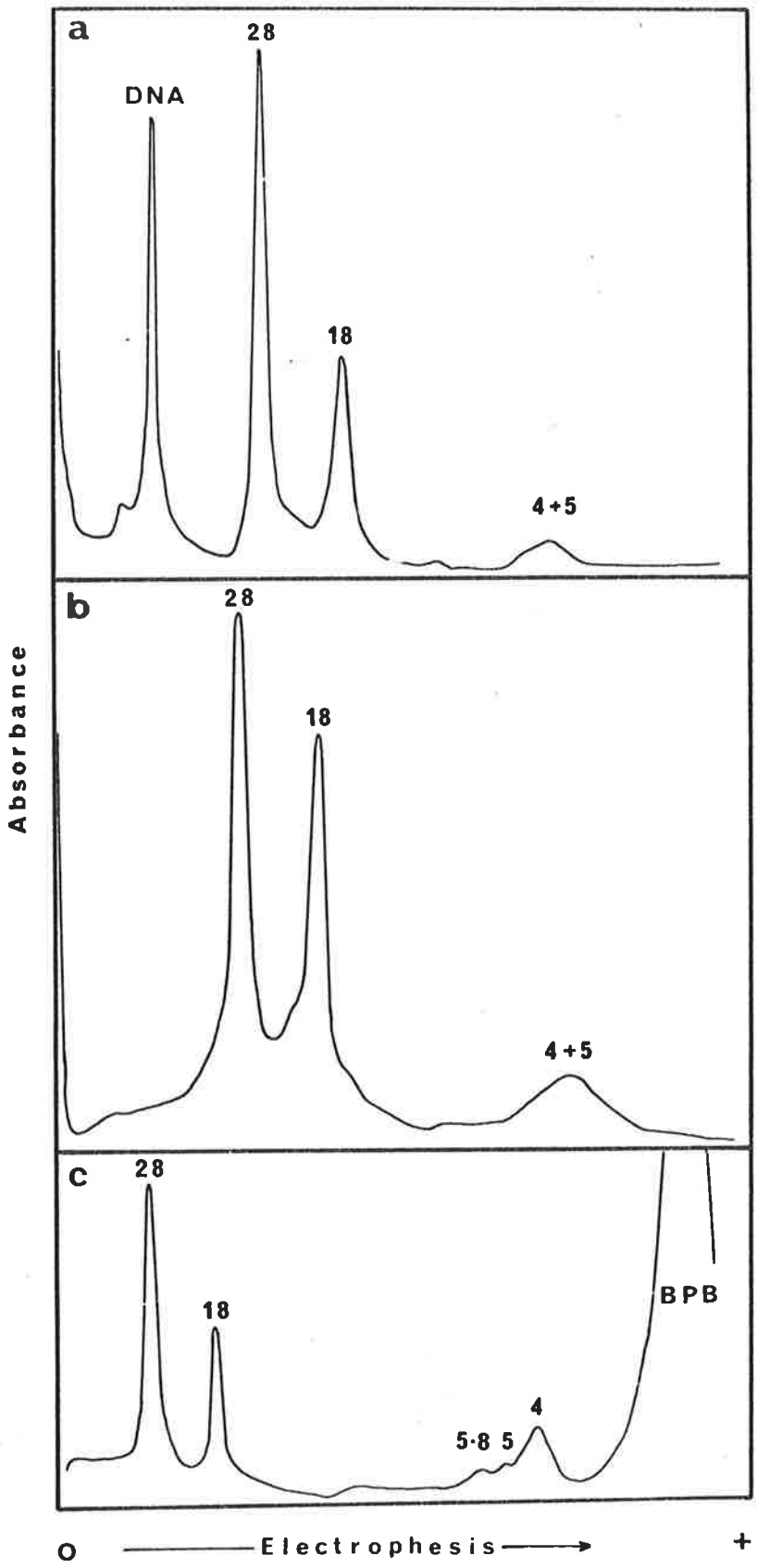
Isolation of Total 12-day feather RNA.

(a) Total nucleic acid was isolated by phenol-$CHCl_3$ extraction as described in the text, and an aliquot (~10 μg) subjected to polyacrylamide gel electrophoresis in the presence of formamide (Pinder et al., 1974) The gel was stained with 0.005% stains-all (Dahlberg et al., 1969), and scanned at 600nm using a Gilford spectrophotometer coupled to a W + W recorder.

(b) Gel electrophoresis of a sample (10 μg) of the RNA obtained after digestion of the material in (a) with DNase I. Staining and scanning are as in (a).

(c) Gel electrophoresis of RNA prepared in a separate experiment, but in which the DNase I had been pretreated with iodoacetic acid (Zimmerman and Sandeen, 1966). The gel was scanned at 280nm immediately after electrophoresis.

The approximate S-values of the major RNA species are given by the numbers. BPB represents the bromophenol blue marker dye.


a
28
DNA
18
4+5
b
28
18
4+5
c
28
18
BPB
4
5·8 5
Absorbance
o
Electrophesis
+

susceptibility to digestion with DNase (of Figure 6,1 b and c).

When total nucleic acid was digested with commercial "RNase-free" DNase, which had not been treated with iodoacetic acid to inactivate any residual RNase (Zimmerman and Sandeen, 1966), a significant reduction in the 28S:18S ratio was observed; the value obtained after digestion of the material in Figure 6.1a was 1.60:1 (Figure 6.1b). In other experiments where iodoacetate-treated material was used the ratio was considerably higher, ranging from 2.0 to 2.2:1 (e.g. Figure 6.1c). Provided that stringent precautions were taken to eliminate the possibility of RNase contamination, it was found that the procedures employed gave RNA which was undegraded. RNA isolated in this manner was used for all the experiments described below.

(ii) Enrichment of mRNA sequences by oligo(dT)-cellulose chromatography.

Oligo(dT)-cellulose chromotography was performed by the method of Aviv and Leder (1972), modified as described in Methods (6.B(iv)), and the bound and non-bound fractions collected. In a large number of experiments using this procedure between 3% and 7% of the RNA was recovered in the bound fraction.

The distribution of mRNA sequences in the bound and unbound fractions was examined by hybridisation

with keratin cDNA. Both fractions contained mRNA sequences (Figure 6.2), although it was necessary to carry the reaction to extremely high $R_o t$ values to achieve significant hybridisation with the unbound RNA*, which hybridises at a rate some 200 fold lower than the bound RNA.

Calculation of the level of mRNA in each fraction (Table 6.1) revealed that ~ 93% of the mRNA sequences were located in the bound fraction, which contained only 5 - 6% of the total RNA. This represented a 16fold enrichment of mRNA sequences in the bound fraction. The results presented here are the averages of two experiments, in which 90% and 95% of the keratin mRNA bound to the column.

It was apparent that the proportion of mRNA sequence in the bound RNA was quite low (0.072%, Table 6.1). This could represent, in part, the low level of keratin mRNA (Chapter 4) and keratin synthesis in 12 day tissue (Kemp et al., 1974a). It was also a function of the high level of non-specific binding observed with the batch of oligo(dT)-cellulose used under the conditions employed. When 14-day polysomal RNA was applied to the column, as in a routine mRNA preparation, and the bound fraction examined by sucrose gradient centrifugation, substantial levels of 18S and particularly 28S rRNA were observed (Figure 6.3). Despite the high level of nonspecific binding, the

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*Although hybridization did not reach more than 80%, direct comparison of $R_o t_{\frac{1}{2}}$ values is valid since they were within the one experiment.

FIGURE 6.2.

Binding of mRNA to Oligo(dT)-Cellulose.

12-day RNA (~650 μg) was fractionated on oligo(dT)-cellulose as described in the text. The bound and non-bound fractions were collected, concentrated by ethanol precipitation, and hybridised with keratin cDNA as described (Chapter 2). The extent of hybridisation was assayed with nuclease S_1, and the data were fitted to the mRNA : cDNA $R_o t$ curve obtained by Kemp (1975). Arrows indicate the $R_o t_{\frac{1}{2}}$ values.

a. Bound RNA

b. Non-bound RNA

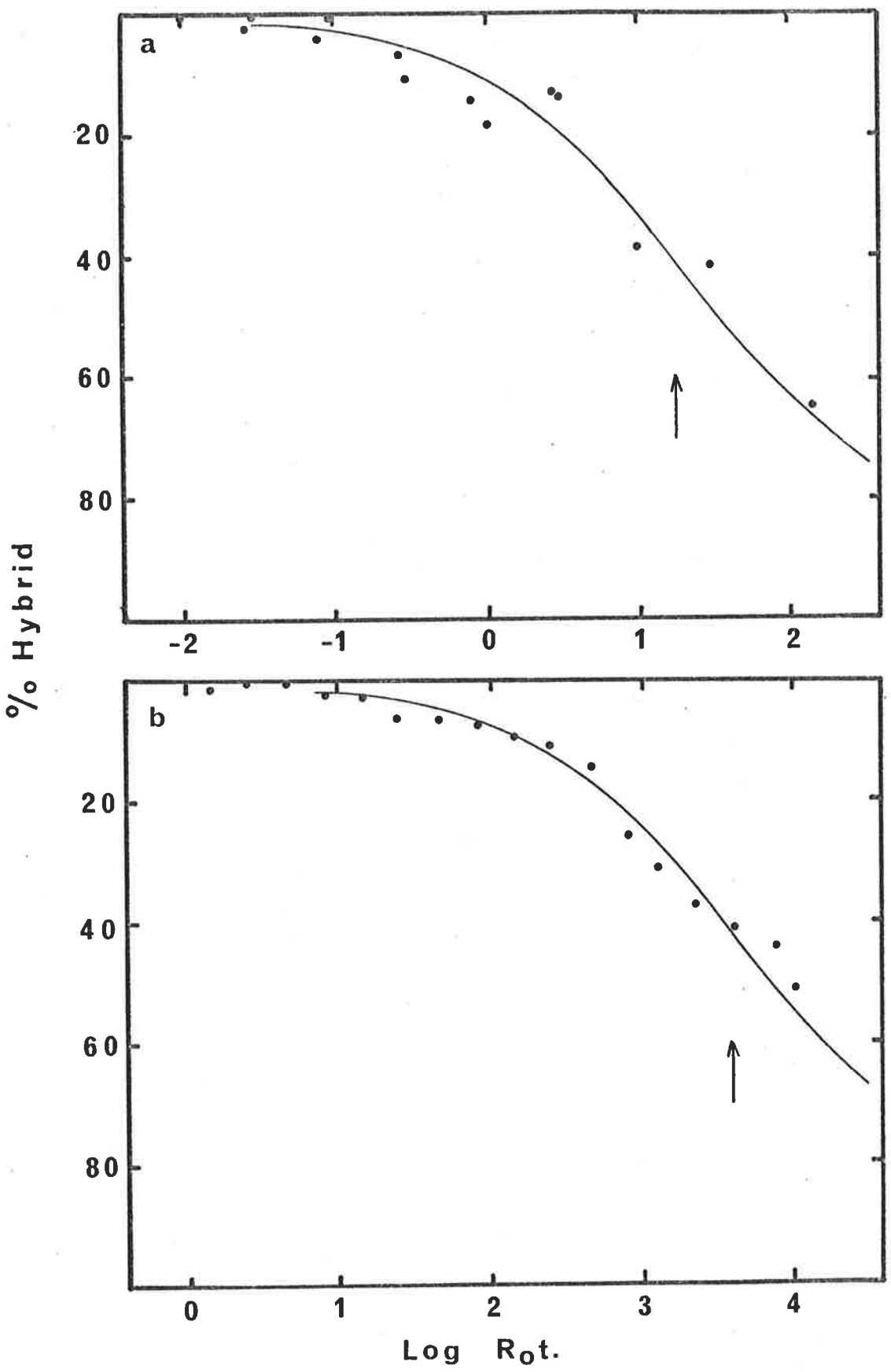

a
b
20
40
60
80
% Hybrid
-2
-1
0
1
2
0
1
2
3
4
Log Rot.

TABLE 6.1.

Proportion of mRNA sequences binding to oligo(dT)-cellulose.

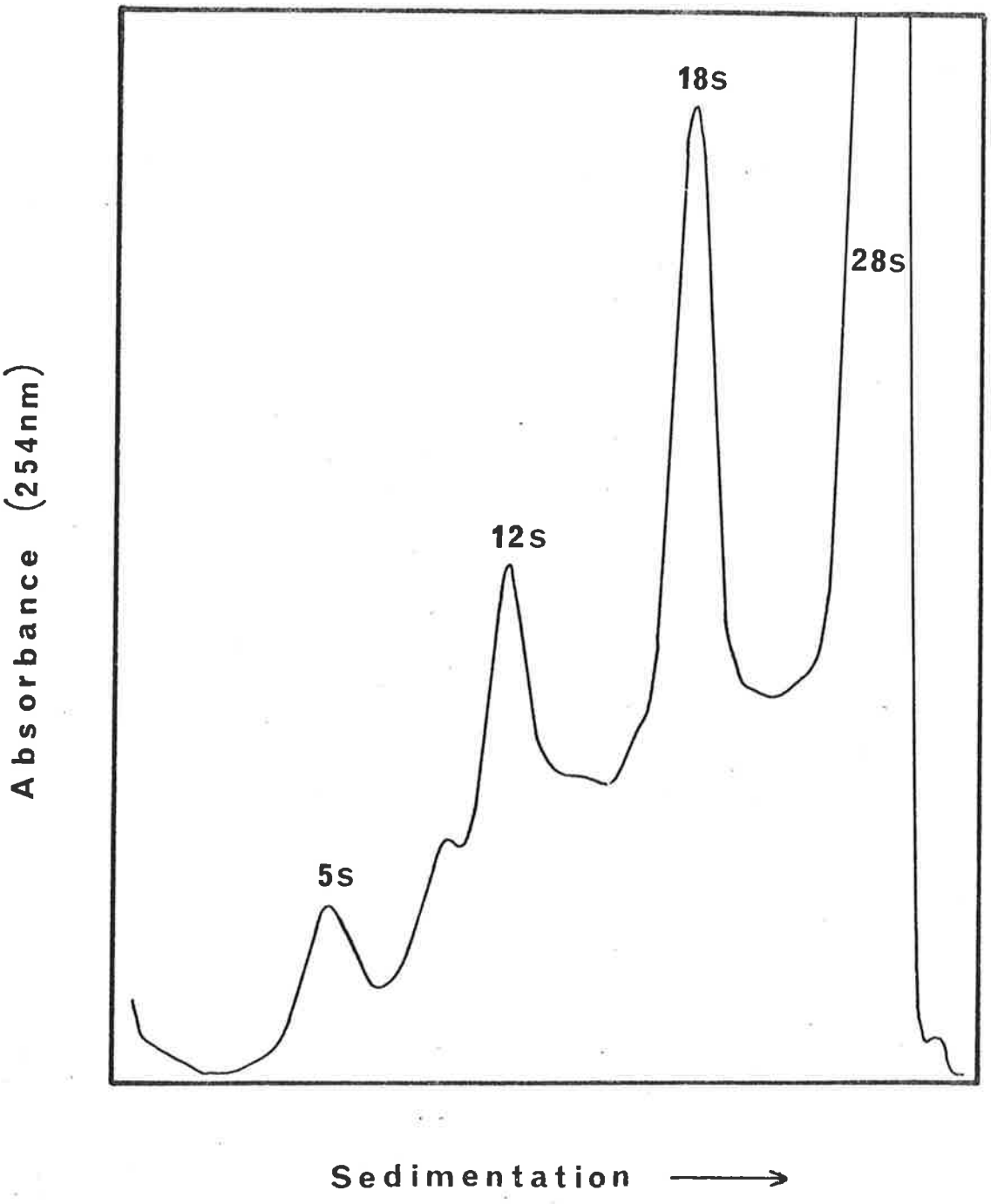
	Non-bound	Bound	Total
wt. RNA, μg	611	35	646
$R_o t_{\frac{1}{2}}$ (cDNA)	3.9×10^{3}	1.8×10^{1}	-
% mRNA	3.3×10^{-4}	7.2×10^{-2}	(4.2×10^{-3})
wt. mRNA, ng	2.0	25.3	27.3
% of total RNA in fraction.	94.6	5.4	100
% of total mRNA in fraction.	7.0	93.0	100

Total 12-day RNA was fractionated on a column of oligo(dT)-cellulose, as described in methods (B(iv)), and the bound and non-bound fractions collected. After concentrating by ethanol precipitation, samples of bound and non-bound RNA were hybridised with keratin cDNA (Figure 6.2) and the $R_o t_{\frac{1}{2}}$ for each fraction determined. The mRNA content was determined from the $R_o t_{\frac{1}{2}}$ as described in the legend to Table 4.4. Figures for unfractionated RNA are the sum of those for the bound and non-bound fraction, except for the figure for % mRNA which was calculated from the weights of RNA and of mRNA.

FIGURE 6.3.

Sedimentation Analysis of 14-day Polysomal RNA Bound by Oligo(dT)-Cellulose.

Polysomes were isolated from the feathers of ~ 20 14-day embryos, and the RNA isolated from them by extraction with phenol and chloroform, as described for total 12-day RNA in the text, except that 0.1 M NaCl was present in the aqueous phase. The RNA was fractionated on oligo(dT)-cellulose as described, the bound fraction collected, precipitated with ethanol and loaded onto 4 linear 10 - 40% sucrose gradients in 0.1 M NaCl, 1 mM EDTA, 10 mM tris-HCl(pH 7.4). The gradients were centrifuged at 38,000 rpm at 4° for 15 hours in an SW41 rotor, and were fractionated on an ISCO gradient fractionator. The absorbance at 254 nm was monitored by the optical system of the ISCO, which was coupled to a W + W recorder.


18s
28s
12s
5s
Absorbance (254nm)
Sedimentation

enrichment of mRNA sequence obtained was found to be adequate for the experiments performed using oligo(dT)-cellulose-bound RNA, and no further purification was attempted.

(iii) Recovery of RNA from formamide-acrylamide gels.

After several abortive attempts to recover mRNA sequences from polyacrylamide gels, the method finally adopted employed elution at low temperature in a neutral, low salt buffer. Although precautions were taken against ribonuclease contamination of gel slices during cutting and elution, there was a strong possibility that they were inadequate. To minimise degradation of the eluted RNA, yeast RNA was added during the elution.

The recovery of RNA from gels was estimated using hybridisation of mRNA to cDNA. A sample of messenger RNA was subjected to electrophoresis, the gel sliced and slices corresponding to the position of 12S RNA were eluted with buffer. In one experiment (Table 6.2), the recovery of mRNA was 34%. In this experiment, however, the RNA eluate had been extracted with phenol, a procedure which in itself leads to non-specific losses of mRNA sequence, as noted in other experiments (Figure 4.6). In experiments where the mRNA sequence in unfractionated RNA was estimated by hybridisation prior to electrophoresis, the yields were found to be somewhat higher, ranging from 45% to 100%.

TABLE 6.2.

Recovery of mRNA from formamide gel.

0.84 μg of keratin mRNA was loaded onto a formamide-acrylamide gel, and, after electrophoresis, the gel was sliced and the slices containing mRNA (determined from the migration of marker RNAs in parallel gels) eluted with 0.25 ml of 10 mM tris-HCl (pH 7.4), 1 mM EDTA, 0.1% SDS containing 15 μg of yeast RNA. 0.2 ml of eluate was collected from each slice, pooled, and extracted with phenol-chloroform (1:1) at pH 9.0. The RNA was concentrated by ethanol precipitation, dissolved in 0.3 ml hybridisation buffer (Chapter 2,C.(v)), and 20 μl aliquots taken for hybridisation. To each was added 5 μl of [^{3}H]-cDNA (1000 cpm), after which the samples were sealed in capillary tubes, denatured by boiling, and incubated at 60°. At appropriate times samples were removed and assayed with nuclease S_1. The per cent hybridised was plotted as a function of time (see Figure 6,5, for example), and the $t_{\frac{1}{2}}$ determined after fitting the data to a standard mRNA-cDNA $R_o t$ curve. A sample of mRNA was also hybridised in this manner. The yield of mRNA recovered from the gel has been calculated for the total mRNA eluted after correcting for dilution and sampling volumes.

TABLE 6.2.

Recovery of mRNA from formamide gel.

Wt loaded	0.84 g.
Volume recovered	0.30 ml.
$R_o t_{\frac{1}{2}}$ (mRNA used)	1.2×10^{-2}
$t_{\frac{1}{2}}$ (mRNA recovered)	1.53 h.
R_o	0.63 µg/ml.
R_o (prior to dilution for hybridisation)	0.78 µg/ml.
Wt mRNA recovered	.234 µg.
Wt mRNA eluted	.294 µg.
i.e. % eluted	35 %

Because only a limited number of points were used for these analyses, the accuracy of estimation is questionable. Despite this reservation, a 60% recovery of mRNA seems likely, and the real value may be higher.

Several attempts were made to estimate the recovery of mRNA by translation in a cell free system from wheat embryos (Shih and Kaesberg, 1973, Kemp et al., 1974c). These attempts proved futile, partly because of polyacrylamide which eluted from the gels and also precipitated with the RNA by ethanol. This material was found to completely inhibit the wheat embryo cell-free translation system. Although it was found that this material could be removed by sucrose gradient centrifugation, the recovery of mRNA was too low to be effectively quantitated.

(iv) Hybridisation of keratin cDNA to gel eluates.

The hybridisation experiments described in this section were performed under conditions of mRNA excess. Although in general it was assumed prior to starting the experiment that this condition was fulfilled, in many situations this appeared to be justified by the results obtained.

In initial experiments, eluates were pooled from groups of 5 slices (each slice was 1 mm), the RNA concentrated by ethanol precipitation, and hybridised with keratin cDNA. The result of such an experiment

is shown in Figure 6.4. Both 12-day total RNA and 13-day cytoplasmic RNA showed a small, apparently real level of hybridisation in the high molecular weight regions of the gel. There appeared to be a small amount of mRNA sequence in the 12-day material which migrated slightly slower than 12S RNA (Figure 6.4b), but this could be explained by the 12S peak being slightly more diffuse in that gel, as mRNA was also observed in the more rapidly migrating fraction.

Messenger RNA was quantitated in each pool by comparison with a standard mRNA:cDNA $R_o t$ curve, using the extent of hybridisation observed to estimate $R_o t$, and hence the concentration of mRNA. With low levels of hybridisation, the method of quantitation became subject to error, both because of the shape of the curve, which made $R_o t$ estimation most inaccurate, and also because of a variability in the background resistance of cDNA alone to S1 nuclease under the conditions used. The reason for the variability was unclear, but could have been due to other material eluting from the gels, or to contamination of precipitated RNA with SDS from the buffer used for elution.

These difficulties in quantitation were not overcome directly, but an alternative approach, based on the kinetics of the hybridisation of gel eluates with keratin cDNA was attempted. Material eluted from slices was pooled, concentrated by ethanol precipitation,

FIGURE 6.4.

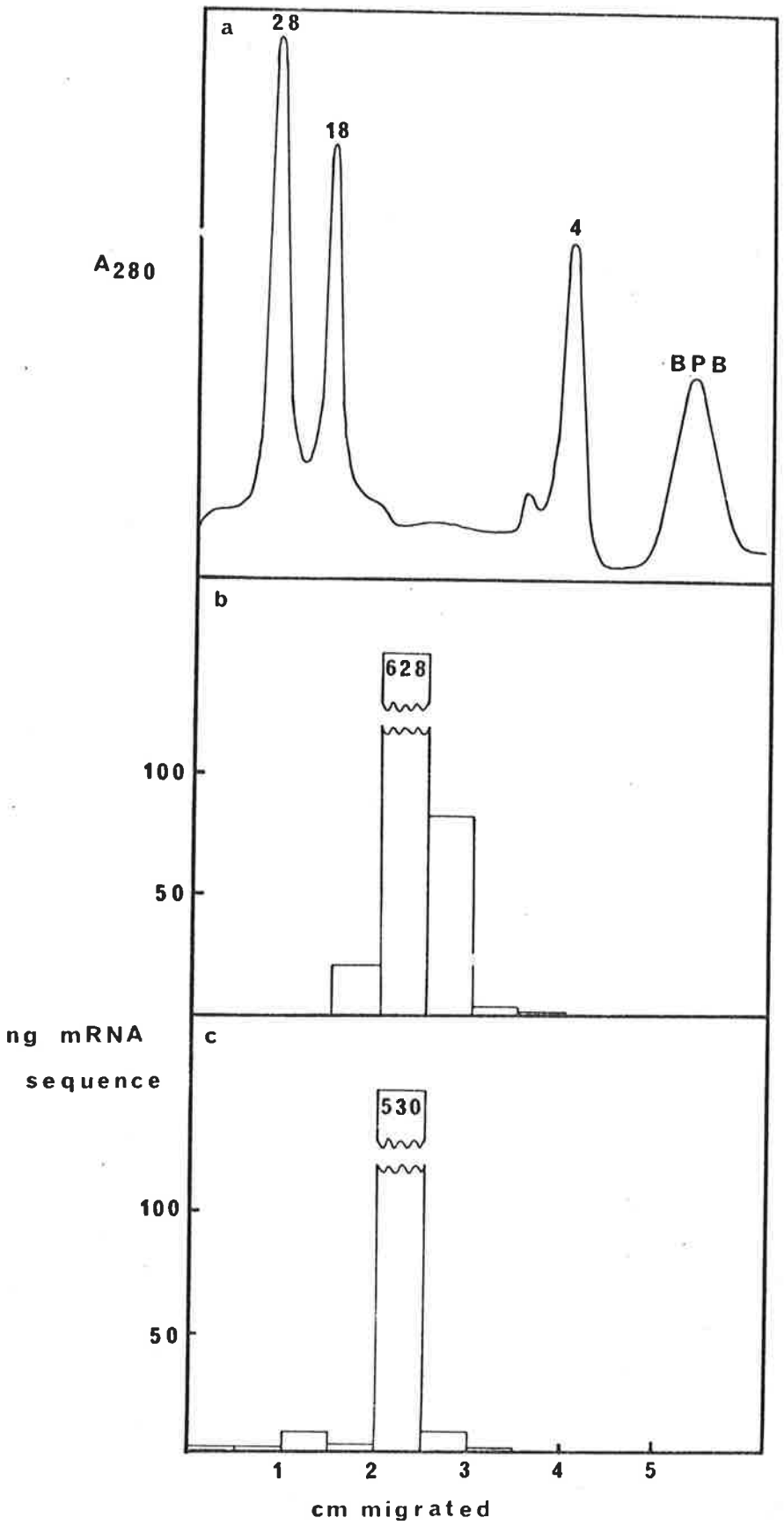
Formamide Gel Electrophoresis of Keratin mRNA Sequences.

12-day total RNA (27.3 μg) and 13-day cytoplasmic RNA (27.8 μg) were subjected to gel electrophoresis in the presence of formamide, after which the gels were sliced, and the RNA eluted as described in the text. Eluates from groups of five 1 mm slices were pooled, concentrated by ethanol precipitation, and duplicate aliquots of each hybridised with cDNA. The extent of hybridisation was assayed with S_1 nuclease, and the concentration of mRNA in each pool estimated from this value, using a standard curve for the hybridisation of keratin mRNA with its cDNA to estimate $R_o t$, and hence R_o.

(a) Scan at 280 nm of a parallel gel of marker rRNA and tRNA (28S, 18S and 4S). BPB = bromophenol blue dye marker.

(b) 12-day total RNA

(c) 13-day cytoplasmic RNA


a
28
18
4
BPB
A_{280}
b
628
100
50
ng mRNA
sequence
c
530
100
50
1
2
3
4
5
cm migrated

and aliquots hybridised for varying periods of time (e.g. ranging from 0.1 to 100 hours). The extent of hybridisation was then assayed using nuclease S1, and the data fitted to a standard mRNA:cDNA $R_o t$ curve (taken from Kemp, 1975). From this curve an estimate could be made of the $t_{\frac{1}{2}}$ (i.e. the time required for half-maximal hybridisation), which enabled the concentration of mRNA to be determined, using the $R_o t_{\frac{1}{2}}$ for keratin mRNA:cDNA hybridisation (1.3×10^{-2}, see Table 4.4). Examples of such fitted curves are shown in Figure 6.5.

When appropriate fractions were pooled after electrophoresis of 12-day total or 13-day cytoplasmic RNA, and analysed by hybridisation kinetics, the distributions of mRNA sequence shown in Figure 6.6 were obtained. In each case the bulk of the material is in the pool corresponding to 12S RNA (molecular weight 2.5×10^5, i.e. the pool from 1.6 to 4.0×10^5). Some material was seen in the higher molecular weight pools, but the distributions of messenger sequence were seen to be virtually identical, suggesting that these sequences could arise as a consequence of residual aggregation of messenger sequences.

Essentially identical results were obtained when 12-day total RNA which had been enriched for keratin mRNA sequence by oligo(dT)-cellulose chromatography was compared to 14-day cytoplasmic RNA (Figure 6.7). The

FIGURE 6.5.

Kinetic analysis of RNA pools to Determine mRNA Concentrations.

12-day total RNA or 14-day cytoplasmic RNA was subjected to formamide-acrylamide gel electrophoresis, and the gel sliced as described in the text. RNA was eluted from the slices, pooled from appropriate regions of the gel, and precipitated by the addition of 0.1 volume of 1 M Na acetate, pH 5 and 2.5 volumes of ethanol. The precipitates were collected by centrifugation, dried, and dissolved in hybridisation buffer. Aliquots were mixed with a small quantity of cDNA, denatured at 100°, and hybridised at 60° for periods of from 0.1 to 100 hours. The extent of hybridisation for each aliquot was estimated by resistance to S_1 nuclease. The data were fitted (by eye) to the curve for keratin mRNA : cDNA hybridisation obtained by Kemp (1975). The examples shown here are for the 12S mRNA pool of 14-day RNA (●), a higher molecular weight pool from 14-day RNA (□) and a high molecular weight pool of 12-day RNA (○). Midpoints are indicated by arrows.

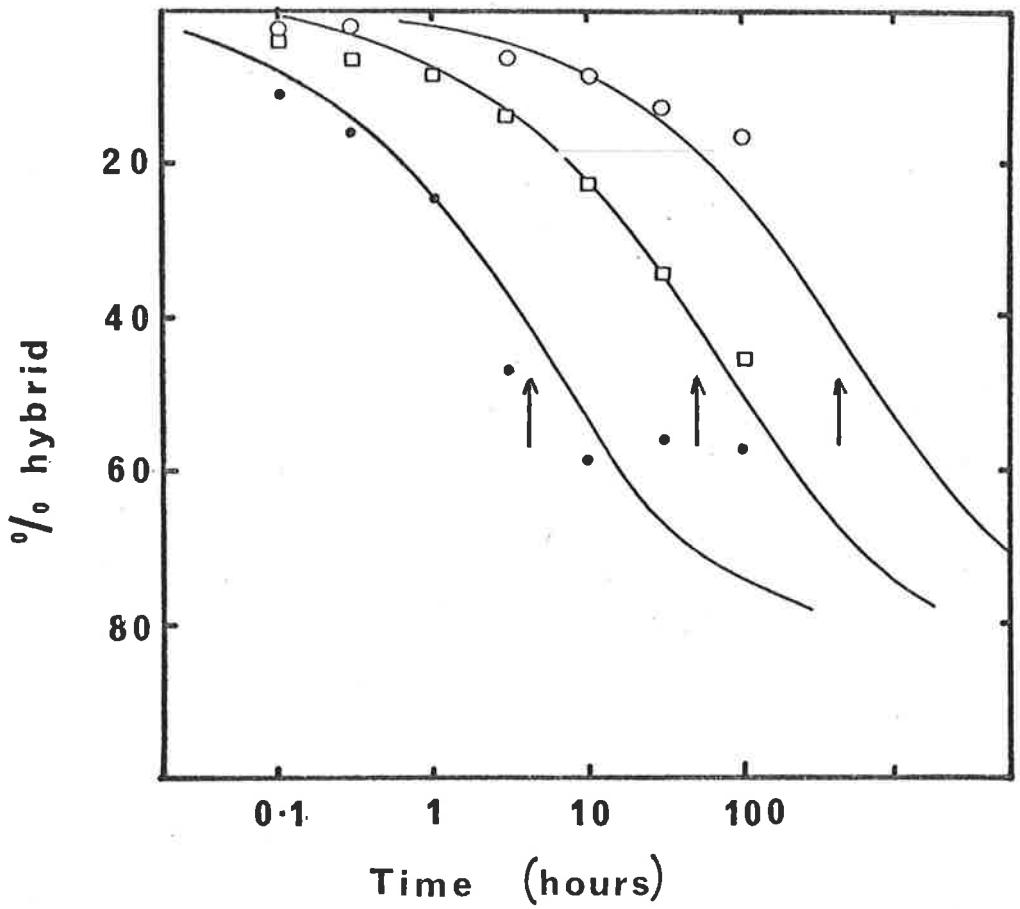

20
40
60
80
% hybrid
0·1
1
10
100
Time (hours)

FIGURE 6.6.

Distribution of Keratin mRNA Sequences in 12-day total RNA.

Aliquots (28 μg) of 12-day total RNA and 13-day cytoplasmic RNA were subjected to polyacrylamide gel electrophoresis in the presence of formamide, the gels sliced, and the RNA eluted as described in the text. Eluates of slices in the molecular weight ranges indicated in the figure were pooled, concentrated by ethanol precipitation, and hybridised with keratin cDNA as shown in Figure 6.5. The concentration of mRNA was determined from the kinetics of hybridisation.

(a) Densitometer trace at 280 nm of a parallel gel with marker 28S and 18S rRNA, and tRNA. BPB represents the bromophenol blue dye marker.

(b) 12-day total RNA

(c) 13-day cytoplasmic RNA

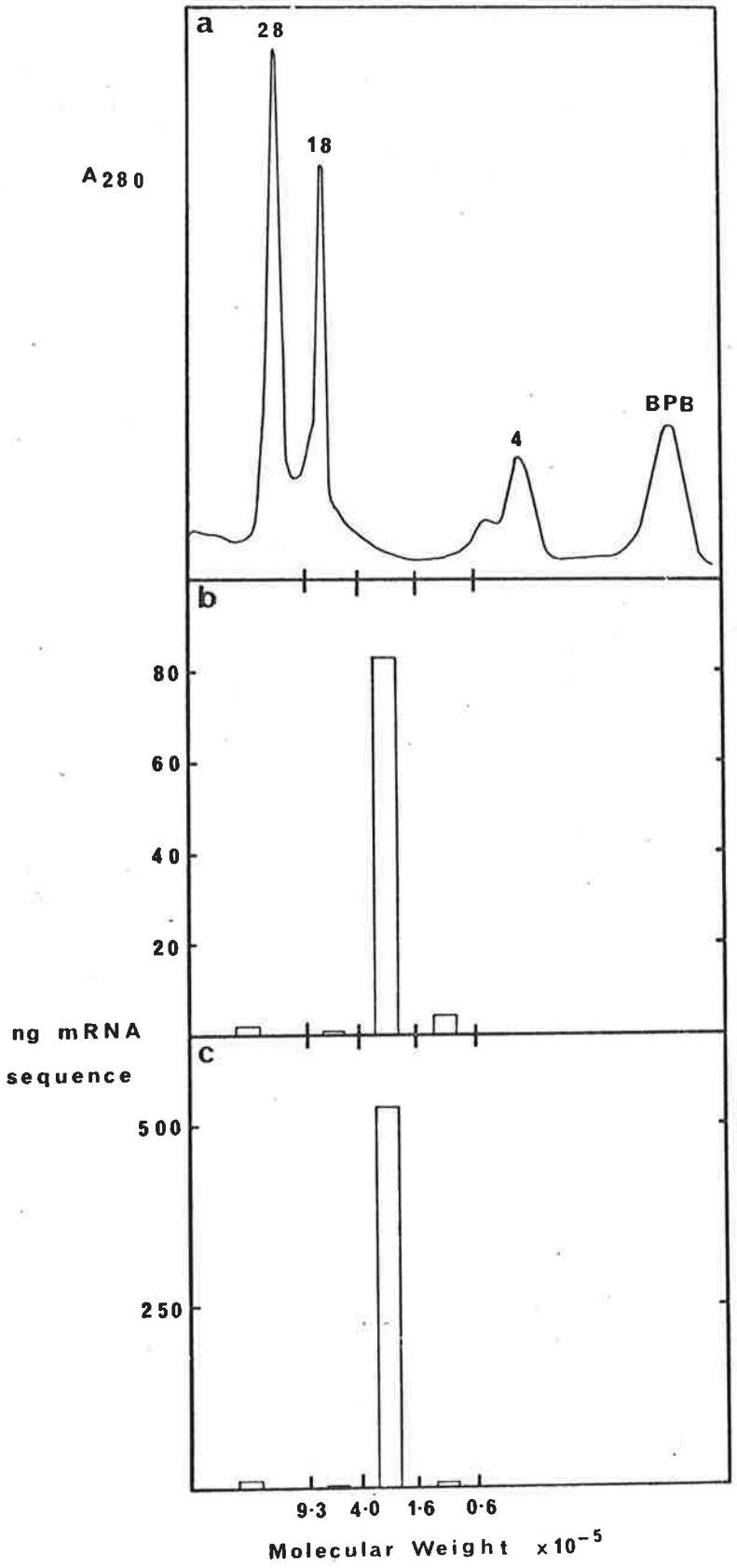

a
28
18
4
BPB
A280
b
80
60
40
20
ng mRNA
sequence
c
500
250
9·3
4·0
1·6
0·6
Molecular Weight x10⁻⁵

FIGURE 6.7.

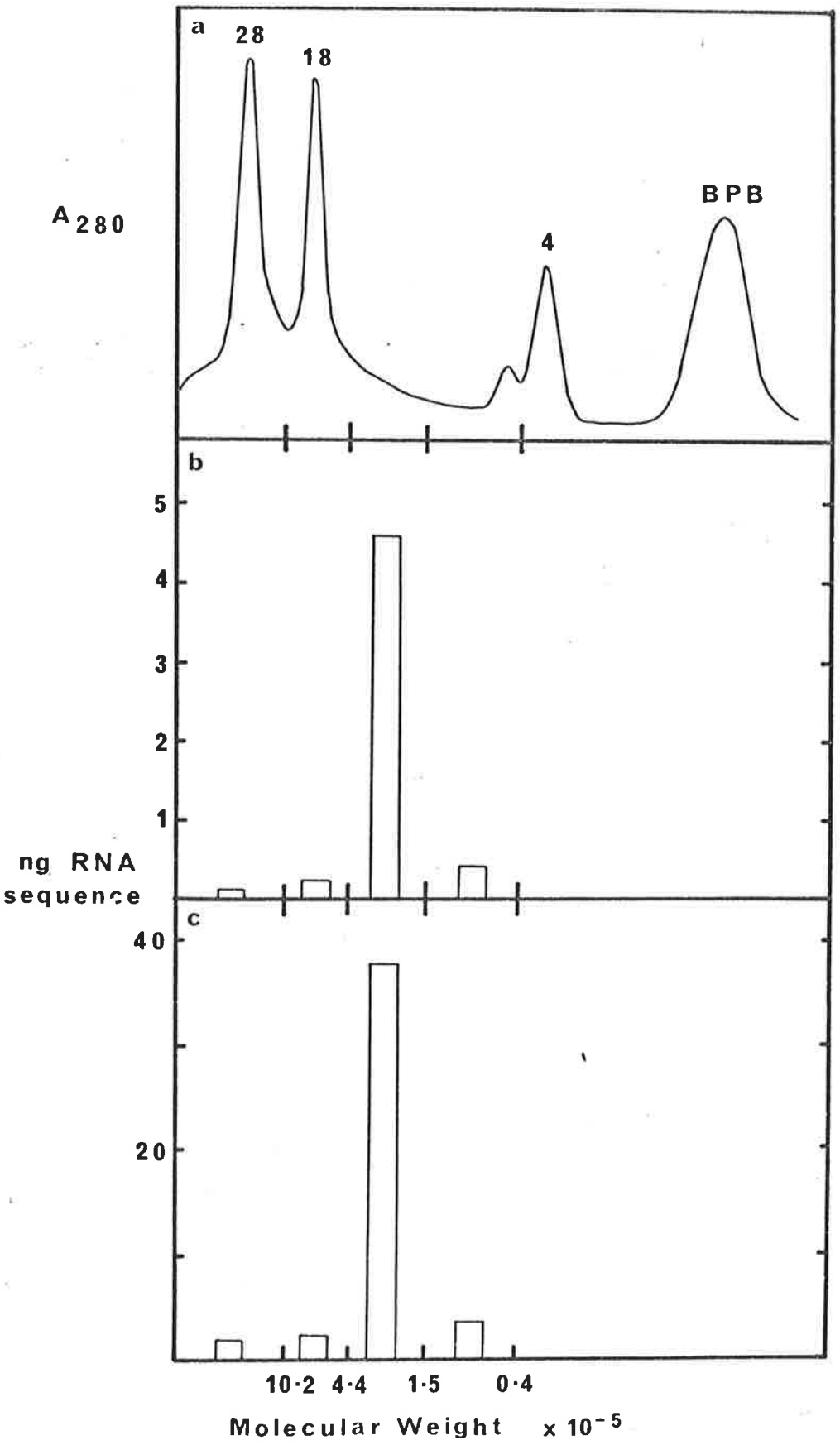
Distribution of Keratin mRNA Sequences in 12-day RNA Bound by Oligo(dT)-Cellulose.

10.9 μg of 12-day RNA retained by oligo(dT)-cellulose, and 12.1 μg 14-day cytoplasmic RNA were subjected to polyacrylamide gel electrophoresis, and the distribution of mRNA sequences analysed, as described in the legend to Figure 6.6

(a) Densitometer trace at 280 nm of rRNA and tRNA markers; figures represent S-values, BPB marks position of dye marker.

(b) 12-day oligo(dT)-cellulose-bound RNA

(c) 14-day cytoplasmic RNA


a
28
18
4
BPB
A_{280}
b
5
4
3
2
1
ng RNA
sequence
c
40
20
10·2
4·4
1·5
0·4
Molecular Weight x 10^{-5}

recovery of mRNA from the gels was somewhat lower in this experiment, but it is apparent that no significant difference in the relative levels of mRNA in each pool could be detected between the two samples.

These results argue against a precursor of substantially higher molecular weight than keratin mRNA being detectable in the samples examined. There are, however, limitations which must be placed on this interpretation. The first of these is purely technical, and can best be described diagramatically (Figure 6.8). It is apparent that a number of curves can be fitted to certain sets of experimental points. To distinguish between the alternatives shown would require hybridisations to be carried out for excessively long periods of time. This problem does not particularly interfere with the interpretation of the results, since the curve fitted to the data was that which gave the highest concentration of mRNA in the pool. If one of the alternative curves was more appropriate, in general this would strengthen the conclusion that a high molecular weight precursor was undetectable.

While the kinetic analyses revealed apparently similar distributions of mRNA sequence in the gel, it is nonetheless arguable that, within each pool, the mRNA sequences could be localised in different apparent molecular species for 12-day RNA compared to RNA from older embryos. Thus, this material in the cytoplasmic

FIGURE 6.8.

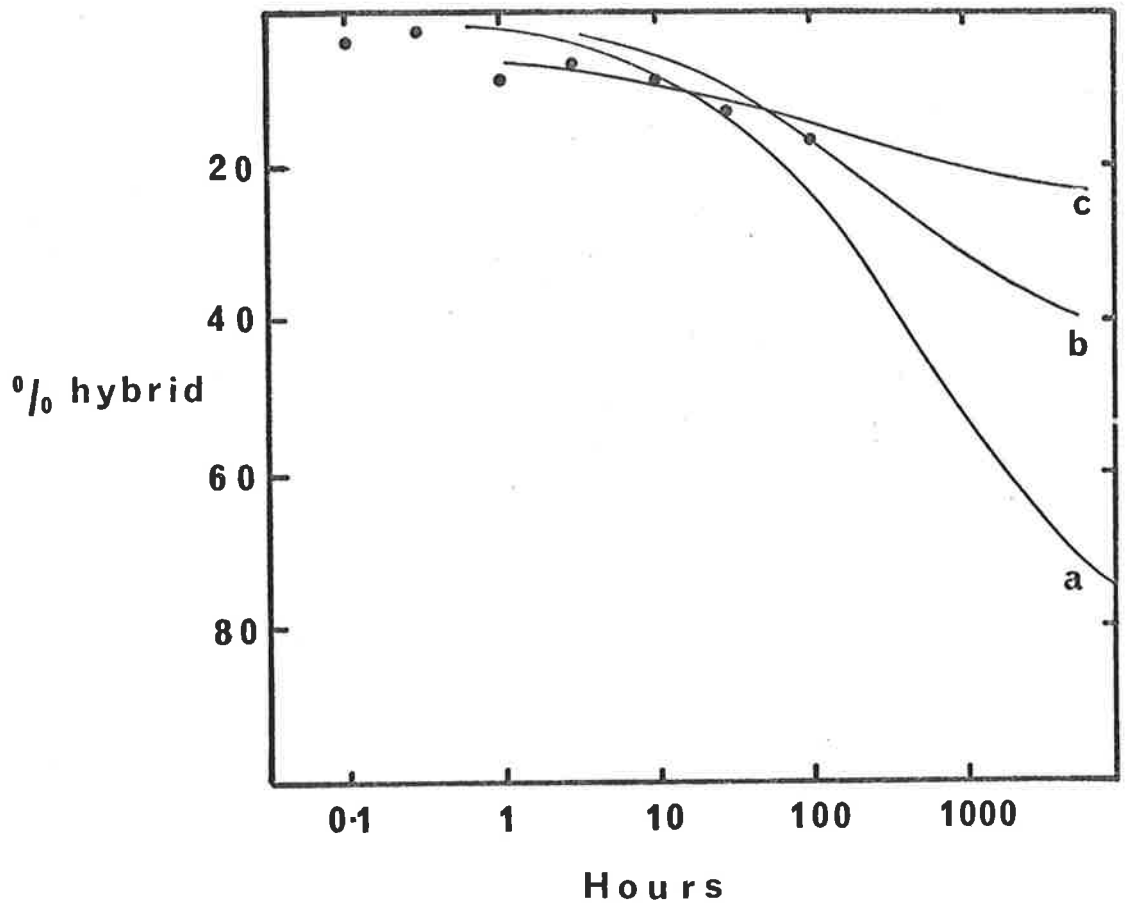
Problem with Analysis of Low Levels of mRNA Sequence.

Pooled RNA eluted from a formamide-acrylamide gel was concentrated and hybridised with cDNA as described in the legend to Figure 6.5, and assayed for resistance to S_1 nuclease. The data obtained were plotted (•) and have been fitted to the different curves shown.

(a) The standard curve for mRNA : cDNA hybridisation, as used in Figure 6.5. The calculated mRNA concentration using this curve approximately equals that of the cDNA used in the reaction.

(b) Theoretical curve, assuming the mRNA concentration was 0.5x that of the cDNA.

(c) Theoretical curve, assuming the mRNA concentration was 0.2x that of the cDNA, and allowing a 5% background resistance of the cDNA to S_1 nuclease.


20
40
60
80
% hybrid
0·1
1
10
100
1000
Hours
a
b
c

extracts could be a consequence of aggregation, while that in 12-day RNA could be due to precursor(s) of the mRNA. This would require that the 13 and 14-day cytoplasmic RNA contains a species with which mRNA aggregates, but which is absent from 12-day total RNA. This alternative, which seems rather unlikely, can only be dismissed if the profiles of mRNA sequence on the gels are examined by hybridisation to the eluates of single slices.

A third possibility is that the mRNA and its precursor are similar in molecular weight. None of the experiments above would distinguish the presence of a precursor of molecular weight in the range 3 - 4 x 10^5, compared to a molecular weight of 2.5 x 10^5 for the final, cytoplasmic, mRNA (Kemp et al., 1974b).

Each of these reservations requires that an alternative approach to the detection of mRNA precursors be undertaken, to enable the steady state level of any precursors to be estimated.

(v) Hybridisation of keratin cDNA to gel eluates in cDNA excess.

The principal problem encountered in interpreting the kinetic experiments described above was the quantitation of low levels of mRNA, in experiments where it was not technically feasible to take reactions to completion. Under conditions of cDNA excess, however,

it is possible to take the reaction to completion (the kinetics of hybridisation of cDNA to mRNA are discussed in detail in an appendix to this chapter (part E, below), at a $D_o t$ approximately 40 x $D_o t_{\frac{1}{2}}$ for a reaction of equal concentrations of mRNA and cDNA - for keratin mRNA-cDNA, this can be achieved at a $D_o t$ of 7.5 - 8 x 10^{-1}.

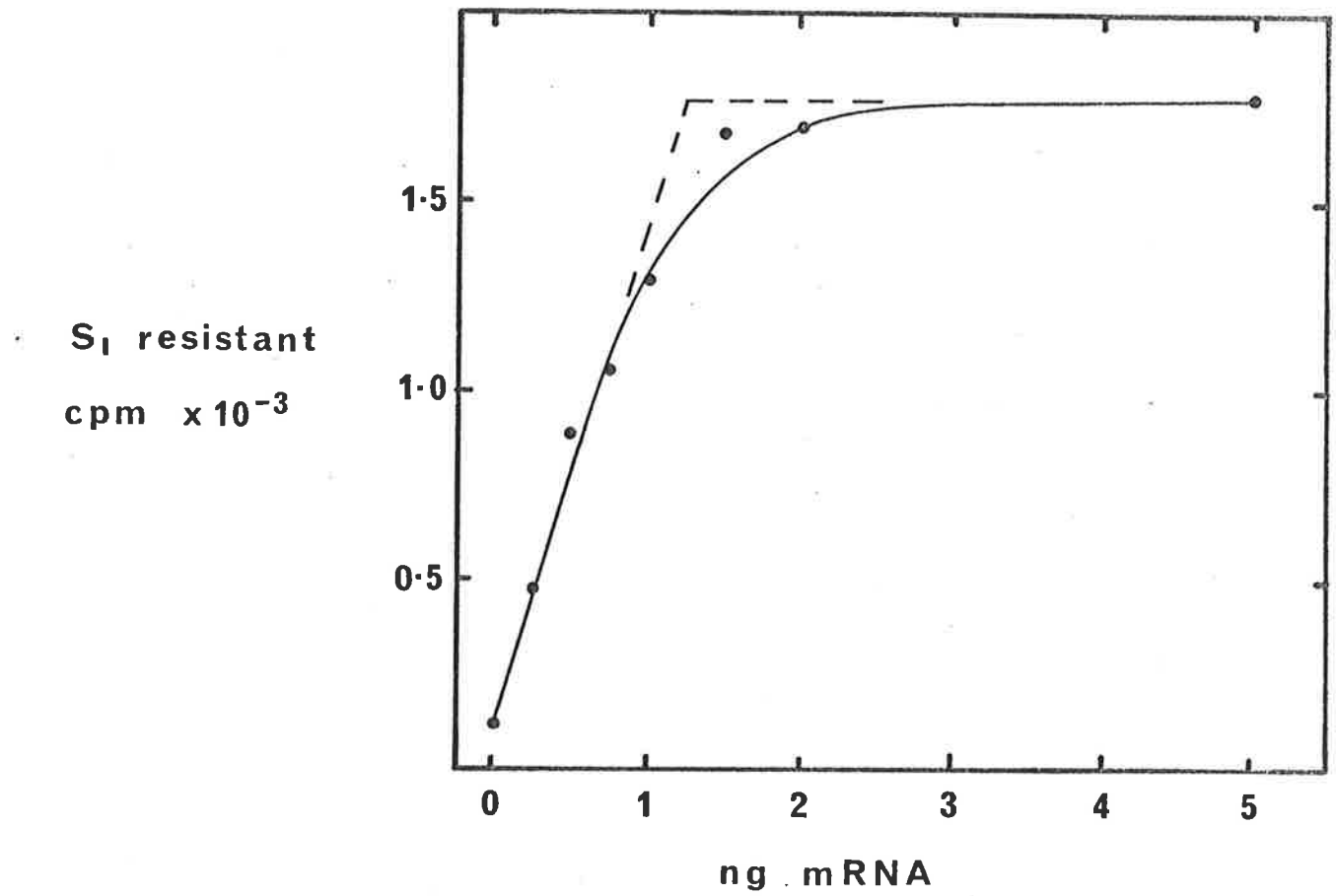
The heterogeneous population of messenger RNAs (Kemp 1975) may complicate this analysis. From the data of Walker and Rogers (1976a) it is unlikely that the different keratin species are present in equal concentrations in the tissue, suggesting that these differences in concentration may also be reflected in the concentrations of the appropriate messenger RNAs. Secondly, each mRNA contains two sequences of vastly different hybridization characteristics (Kemp 1975). It may be difficult to achieve true completion under these conditions, as it is difficult to assess the kinetics of the complex reaction.

Despite these reservations, it is possible to titrate mRNA sequences by this method. Increasing quantities of mRNA were hybridised with a constant amount of cDNA (1.25 ng) in 2 µl of buffer for 96 hours (i.e. to a $D_o t$ of 7.6 x 10^{-1}). The result is shown in Figure 6.9. It can be seen that the curve obtained approximates that for an idealised titration of equal length complementary sequences. Furthermore, the

FIGURE 6.9.

Saturation Hybridisation of Keratin mRNA with cDNA.

Samples of mRNA (0 - 5 ng) were dried *in vacuo*, and redissolved in 2 μl of buffer containing 1.25 ng (2260 cpm) of $[^{32}P]$-cDNA. The samples were sealed in capillary tubes, denatured at 100°, and incubated at 60° for 80 hours, after which they were assayed for resistance to S_1 nuclease. The solid curve represents a best-fit of the data obtained, while the dashed line is the hypothetical curve for the reaction, assuming that 79% resistance to S_1 represents completion of the reaction.


S1 resistant
cpm x 10-3
1·5
1·0
0·5
0
1
2
3
4
5
ng mRNA

plateau level of counts is equivalent to an extent of hybridisation of 79%, similar to that achieved in kinetic experiments. These data suggest that the reaction is in fact being carried to a state closely approximating completion. It is possible to quantitate small quantities (up to 0.5 ng) of mRNA sequence by this procedure more accurately than by the approaches used earlier.

Hybridisation in cDNA excess was used to estimate the mRNA content of eluates from individual slices from formamide gels. Aliquots of eluted material were dried *in vacuo* (larger aliquots were freeze-dried), dissolved in a small volume of buffer containing cDNA (2 - 3 µl), and hybridised to completion. The size of the aliquot taken was varied to allow detection of mRNA sequence with maximum efficiency - in high molecular weight regions of the gel large aliquots were taken (up to 80% of the gel eluate in some 12-day samples), while in the "peak" regions of some gels the size of the aliquot was kept fairly small, so that the final hybridisation was in the "linear" region seen in Figure 6.9. Because the aliquot size was varied across individual gels, the extent of hybridisation in each case was corrected to give the total mRNA content of each slice, using a standard curve such as that shown in Figure 6.9.

The profiles for mRNA content of 12-day

oligo(dT)-cellulose-bound RNA and 14-day cytoplasmic RNA are compared in Figure 6.10. The two curves are virtually superimposable, and it is evident that in the higher molecular weight region of the gel there is very little mRNA sequence; the levels observed were only marginally above background in each case. The broad peak observed could conceivably be due to the presence of impurities in the mRNA from which the cDNA had been prepared. The distribution of mRNA sequence indicates that no poly(A)-containing precursor is detectable, under the conditions used, which has a molecular weight of greater than 5×10^5. Because of the somewhat scattered points over the peak, detection of precursors of slightly greater molecular weight than mature mRNA (2.5×10^5) may be complicated.

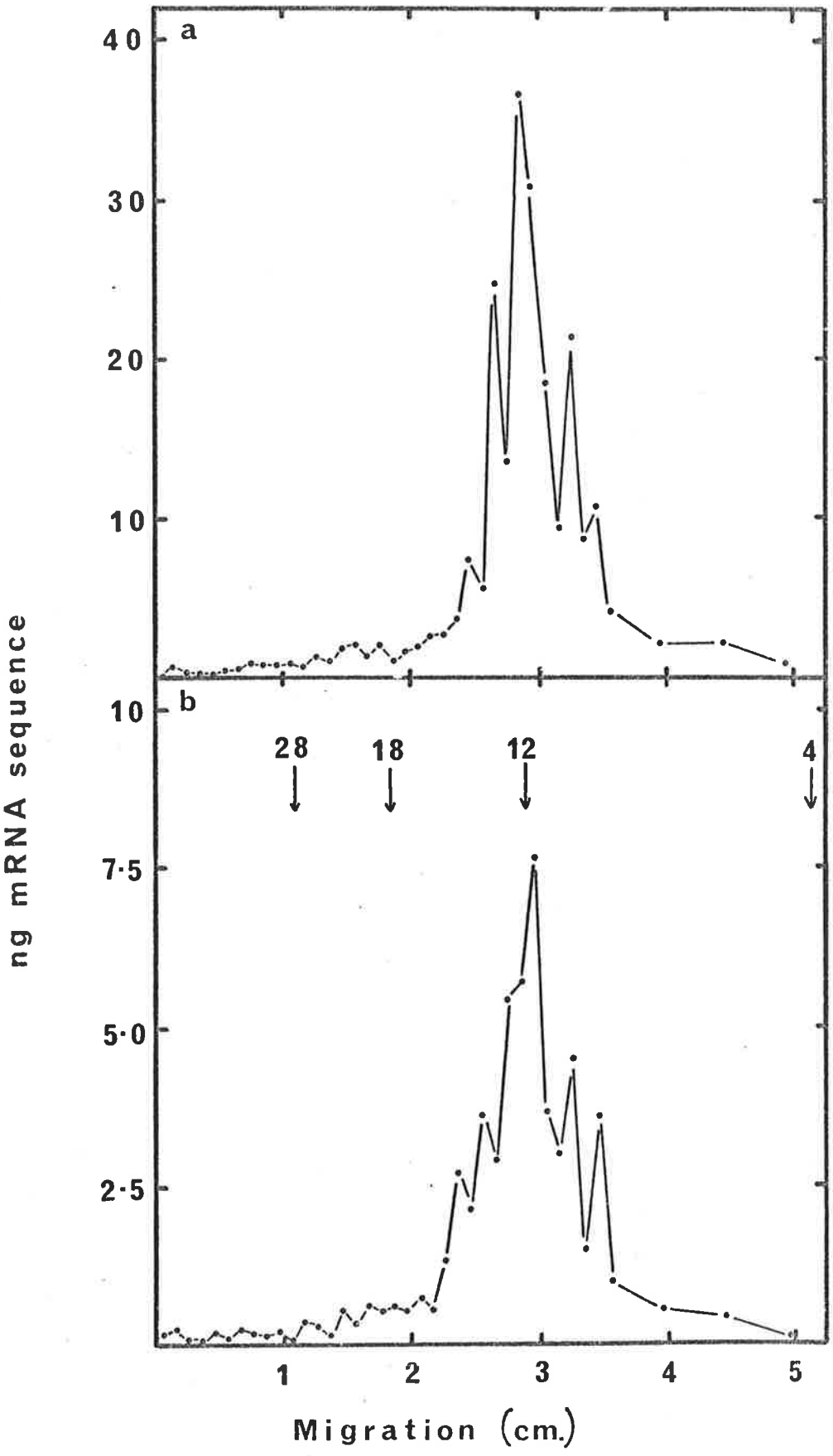
In the same experiment, unfractionated 12-day RNA was also examined, but it is found that, even using large aliquots of the eluted RNA, the total mRNA sequence content was too low to allow a meaningful assignment of the mRNA sequence content of each slice. The extent of hybridisation, even in the peak regions, was only slightly above background.

In a second experiment, unfractionated RNA was examined using an overloaded gel - 150 μg of RNA was loaded onto a single gel. Again, it is evident that there are no significant differences between the

FIGURE 6.10.

Analysis of 12-day Poly(A)-containing mRNA Sequences.

24 μg of 14-day cytoplasmic RNA (a), and 50 μg of 12-day RNA which had been bound to oligo(dT)-cellulose (b) were subjected to electrophoresis on 98% formamide polyacrylamide gels, which were then sliced, and RNA eluted from the slices as described in the text. Aliquots of the total eluate (200 μl) were taken and dried *in vacuo* (2 - 20 μl in (a), 10 - 20 μl in (b)). The dried samples were then dissolved in 2 μl of buffer containing 2,000 cpm of keratin cDNA (specific activity 10^6 cpm/μg) and were sealed in capillary tubes. The samples were denatured for 5 minutes at 100°, incubated at 60° for 80 hours, and assayed with nuclease S_1. The concentration of mRNA in each sample was determined from a curve similar to that shown in Figure 6.9, and from this, the total mRNA content of each eluate was determined. The arrows on (b) indicate the positions of 28A, 18S and 4S RNA markers on a parallel gel, and the predicted position of 12S mRNA.


a
b
40
30
20
10
10
7·5
5·0
2·5
28
18
12
4
1
2
3
4
5
ng mRNA sequence
Migration (cm.)

profiles of 14-day cytoplasmic and 12-day total RNA (Figure 6.11). In each gel, the material which apparently hybridises to cDNA is barely above the S1 nuclease resistance of cDNA alone. The peaks in this experiment are neither as broad nor are the points as scattered as those in Figure 6.10. This suggests that if a precursor of molecular weight significantly different to that of mature mRNA exists in the extracts, it should have been detected by this technique.

The conclusion which may be drawn from these results, both from the hybridisations in RNA excess and those in cDNA excess, is that no precursor to keratin mRNA can be detected in these nucleic acid samples. While this suggests strongly that keratin mRNA is not synthesised as a higher molecular weight precursor species, such a conclusion is subject to limitations, which are discussed below.

D. DISCUSSION.

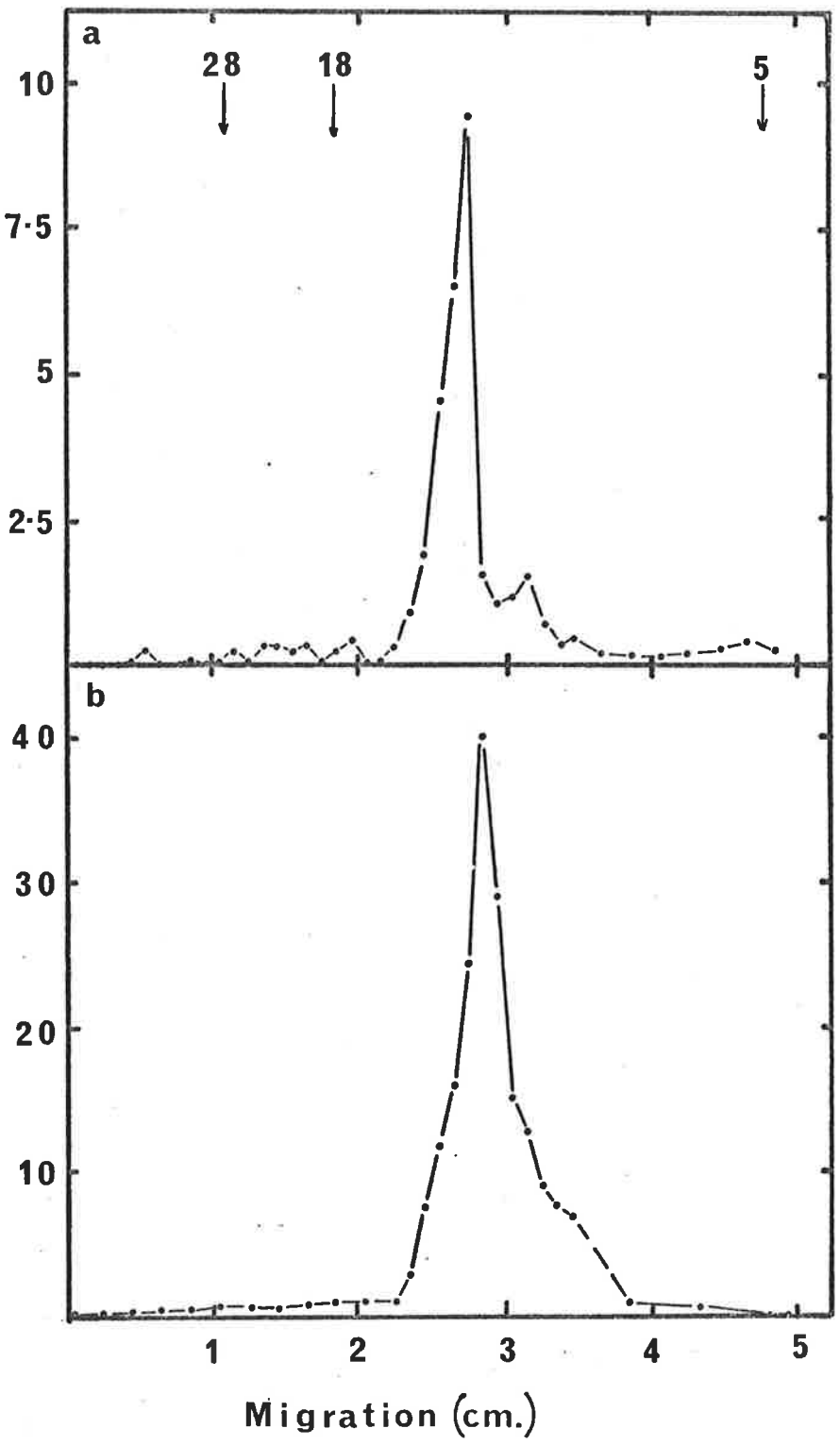
(i) Extraction of total RNA.

The method chosen for the isolation of total RNA from 12-day feathers was designed to halt intracellular processes as quickly as possible after plucking the feathers from the embryo. Lysis of feathers was judged to be rapid, as aggregates of feather cells were quickly destroyed in the emulsified mixture of aqueous detergent and phenol: the nature of the aggregates is

FIGURE 6.11.

Analysis of mRNA Sequences in Unfractionated 12-day RNA.

130 μg of unfractionated 12-day RNA (a) and 24 μg of 14-day cytoplasmic RNA (b) were submitted to polyacrylamide gel electrophoresis, as described in Figure 6.10. Other details are as in Figure 6.10, except that 150 μl aliquots from 12-day eluates were used, and the input of cDNA was slightly less (1400 cpm in 3 μl). The latter was compensated for by increasing the incubation time to 170 hours.



ng mRNA

sequence

uncertain, and it could be that these cells were lysed quickly, leaving a temporarily insoluble aggregate of denatured protein. Aggregates were removed quickly by vigorous vortex mixing, and it is unlikely that any intact tissue survived for longer than about 1 - 2 minutes. The combination of two protein denaturants (sarkosyl and phenol) and the low temperature at which tissue was collected would be expected to almost completely retard nucleolytic degradation of the RNA.

This method appeared to give RNA which was undegraded as judged by the high ratio of 28S to 18S rRNA obtained when the RNA was subjected to electrophoresis under strongly denaturing conditions. There was also little sign of partial degradation products on the leading edges of the two rRNA peaks.

There is a possibility that specific classes of RNA, such as the keratin mRNA precursors, might not be extracted by this technique, although a priori it would seem unlikely that all of the RNA in the nucleus could be lost. The presence of high levels of DNA in the total nucleic acid extracts (Figure 6.1a) suggests that considerable extraction of nuclear contents was achieved. Poly(A)-containing RNA in general appears to be refractory to phenol extraction, unless the extraction is performed at high pH (Brawerman et al., 1972) or in the presence of chloroform (Perry et al., 1972). In earlier work (Figure 4.6) it was observed

that significant losses of keratin mRNA occurred during extraction at pH 9.0, so it is conceivable that, despite the presence of chloroform, losses of keratin mRNA and its precursors could occur in this extraction. Loss of precursor sequences would be virtually impossible to quantitate - ideally, however, if it is the mRNA sequence which determines the partitioning of mRNA sequences to the phenol phase, there should be no change in the relative abundance of mRNA and precursor in the extracted RNA.

(ii) Detection of precursors to keratin mRNA.

The distribution of keratin mRNA sequences in high molecular weight fractions of total RNA was examined using hybridisation with keratin cDNA. Reactions were assayed with nuclease S1 (Ando, 1966; Vogt, 1973) rather than hydroxyapatite (cf. Chapter 5). Although the apparent rate of reaction is slower by this technique (Kemp, 1975), the conditions of assay are more stringent, in that only well-matched hybrids are detected.

Two essentially dissimilar approaches to hybridisation with cDNA were used. Hybridisation was performed in RNA excess using either a single hybridisation to quantitate mRNA (estimating the $R_o t$, and hence mRNA concentration, using a cDNA-mRNA hybridisation curve) in a given pool, or a kinetic

approach, similar to that used by Imaizumi et al., (1973) to quantitate globin mRNA precursors. The kinetic data were fitted to a mRNA:cDNA $R_o t$ curve (taken from Kemp, 1975), and although the fit was made by eye, a reasonable estimate of the half-time for hybridisation, and hence of mRNA concentration, could be made (e.g. Figure 6.5). The principal problem encountered with this approach was in quantitation of low levels of RNA; with a single point, it was frequently difficult to distinguish the extent of hybridisation from the non-specific background, while with kinetic estimates the reactions could not be taken to completion. In the latter case, some data could be fitted to several different curves (Figure 6.8), although this problem is less serious than it may seem. In general, the alternative curves favour lower levels of mRNA, and as the difficulty was more frequently encountered with 12-day RNA pools, it is possible that the levels of "precursors" may be lower than those given in the figures.

Hybridisation in cDNA excess was performed in order to overcome these difficulties with taking reactions to completion. The value of $C_o t$ at which the reaction should have attained completion was calculated (Appendix 1, below). The validity of the calculation may be open to question, as it is based on the average $R_o t_{\frac{1}{2}}$ of keratin mRNA:cDNA hybridisation,

which is a complex reaction (Kemp, 1975) compared to the simple reaction on which the calculations have been based. There is, however, some evidence that the reaction may be essentially complete under the conditions used. The plateau of S_1 resistance is at about 80%, very similar to that observed for "completion" in kinetic experiments in RNA excess (Kemp, 1975; see also Figure 4.5). Furthermore, the shape of the curve approximates that for an idealised titration, assuming that the end-point is at 80%, and that all cDNA and mRNA molecules are of equal length (Figure 6.9).

Both hybridisation techniques yielded the same result; i.e. that no significant differences could be detected between cytoplasmic RNA from 13- or 14-day tissue, and 12-day total mRNA. This then leaves the question of what level of precursor could be detected by each of the techniques.

Values for limits of detection can be calculated for the precursor, assuming a constant rate of synthesis for mRNA between 12 and 13 days of development (appendix 2, below). This assumption is probably not entirely valid, as the rate of mRNA synthesis probably increases over the 24 hour period, so that the rate at 12-days may be somewhat lower. The effect of this modification in the calculations will be to increase the difficulty of detection.

A lower limit for detection was calculated assuming that transcription was halted immediately on placing the tissue into the emulsion, while processing continued unabated until complete tissue destruction occurred. The half-time for processing used in the calculation is that at 0^{o}. Clearly, if processing did continue in the absence of transcription, this would represent the worst possible situation which could arise during isolation. It is probable, however, that the conditions for lysis of the tissue would prevent either transcription or processing of the RNA.

The kinetic experiments to obtain mRNA sequence concentrations indicated little difference in the relative sequence content of various RNA pools. The problem with these estimations (and, indeed, with those performed in cDNA excess) was the presence in cytoplasmic RNA of mRNA sequences which migrated on the gels as residual aggregates, despite the use of denaturing conditions. Thus a given high molecular weight pool in cytoplasmic RNA might contain 1.4% of the total mRNA on the gel, while the same pool for total RNA might contain 1.9% of the total mRNA detected. In several experiments, the greatest differences detected in this way were of the order of 0.4 - 0.6%, and frequently it was found that the relative level in the cytoplasmic pools was greater.

Similarly, no differences could be detected between the profiles for hybridisations performed in cDNA excess. Because some small variations were observed, particularly where low levels of cDNA were used (e.g. Figure 6.11), it is not clear whether these represent true precursors or variations in background. Electrophoresis of the oligo(dT)-bound fraction for this RNA also showed minor peaks in the high molecular weight region (result not shown), none of which can be correlated with those in the unfractionated material, suggesting that the "peaks" observed were due to variations in background. Despite these variations, a peak amounting to 1% of the mRNA in the 12S region for unfractionated RNA, and to 0.5% in poly(A)-containing RNA, would have been detected.

Thus in either type of experiment, a precursor amounting to 0.5 -1% of the steady-state level of mRNA would have been detectable, if it was reproducibly present. This corresponds to a precursor with a half-life of approximately one to two minutes (see Appendix 2 and Figure 6.13). It can be calculated from the data of Greenberg and Penman (1966) for the synthesis of 45S rRNA precursor, that the elongation rate for RNA synthesis is 5-6,000 nucleotides per minute. The synthesis of the mRNA sequence would thus require approximately 0.15 minutes,

while a precursor of molecular weight 10 times that of the mRNA would require a synthesis time close to the predicted half-life for mRNA precursors. Thus high molecular weight precursors to keratin mRNA may not exist in embryonic feather.

The estimate for half-life made here is considerably less than estimates for hnRNA in other systems. In mouse L-cells, it has been shown that total hnRNA has a half-life of 23 minutes (Brandhorst and McConkey, 1974), while estimates of 30 minutes were made in HeLa cells (Penman et al., 1968) and duck reticulocytes (Attardi et al., 1966). In sea urchins (Brandhorst and Humphreys, 1971, 1972) the half-life was estimated to be 7 minutes. These studies in effect estimate total processing time, and it is not clear to what extent polyadenylation and capping of the mRNA contribute to this time - suggestions have been made that polyadenylation may not take place in the nucleus until several minutes after transcription is complete (Jelinek et al., 1973 a,b). Thus it is probable that these estimates do not clearly differentiate between the distinct stages in processing, and measure only the overall rate.

It is possible, too, that in some cell types a fraction of specific gene transcripts are never released from the nucleus. Spohr et al., (1974) claimed that globin mRNA sequences could be detected

in higher molecular weight species, some of which had extremely long half-lives. Egyhazi (1976) found that only a very low proportion of molecules transcribed from Balbiani rings in Chironimus tentans could be transported to the cytoplasm and translated. The presence of sequences of this type in feather nuclei would increase the chances of finding "precursors" to keratin mRNA.

While it seems likely from the data presented in this chapter that keratin mRNA is either not synthesised as a high molecular weight precursor, or that such a precursor has only a transient existence and is not detectable in steady-state conditions, there is one further limitation which must be placed on this conclusion. The peaks obtained on hybridisation to individual gel slices are broad (Figures 6.10 and 6.11) making it extremely difficult to estimate differences between the profiles in the molecular weight range 1.5 -5 x 10^5. Thus it is conceivable that a low level of precursors of molecular weight slightly greater (but less than 2-fold greater) than mRNA could be present, but not be detectable by difference.

Comparison of the levels of high molecular weight mRNA sequence in total 12-day RNA and in the poly(a)-containing fraction suggests that there is unlikely to be a significant level of precursor which

lacks poly(A). That this could have been the case was suggested by a recent report which claimed that the majority of nuclear globin mRNA sequences in avian erythroblasts are devoid of poly(A) (Spohr et al.,1976).

The data obtained in these experiments suggest that keratin messenger RNA is not synthesised as a high molecular weight precursor species, or alternatively if such species are intermediates in the synthesis of the mRNA, then they must have only a transient existence in the cell, and are not detectable by the methods adopted here. Thus the biosynthesis of keratin mRNA resembles that of hen ovalbumin (McKnight and Schimke, 1974) and silk fibroin (Lizardi, 1976) mRNAs, neither of which appears to be synthesised as a high molecular weight species.

E. APPENDIX 1. Kinetics of mRNA - cDNA hybridisation

(i) Rate equations

The equations given are based on those of Young et al., (1974). The hybridisation reaction can be described by the equation

$$D + R \longrightarrow H$$

where D, R and H represent the molar concentrations of DNA, RNA and hybrid, respectively. The rate equation of the reaction is then

$$\frac{dH}{dt} = k.D.R.$$

$$= k(D_o - H)(R_o - H) \qquad (1)$$

where D_o and R_o are the initial concentrations of DNA and RNA respectively, and k is the rate constant for the reaction.

When R does not equal D, equation 1 may be integrated to give

$$H = \frac{R_o D_o (1 - e^{(R_o - D_o)kt})}{D_o - R_o e^{(R_o - D_o)kt}}$$

or, since the degree of hybrid formation is usually expressed as the proportion of the cDNA hybridised,

$$\frac{H}{D_o} = \frac{R_o (1 - e^{(R_o - D_o)kt})}{D_o - R_o e^{(R_o - D_o)kt}} \qquad (2)$$

Where R_o and D_o are equal, however, equation 1 simplifies to

$$\frac{dH}{dt} = k(D_o - H)^2 \qquad (3)$$

which integrates to give

$$H = \frac{D_o^2 kt}{1 + D_o kt}$$

$$\frac{H}{D_o} = \frac{D_o kt}{1 + D_o kt} \qquad (4)$$

These equations allow the calculation of the

course of the hybridisation reaction as a function of either $D_o t$ (the product of the initial DNA concentration and time) or of $R_o t$ (where the initial RNA concentration is used). Examples of calculated curves to describe such reactions are shown in Figure 6.12, and are compared with experimental data obtained in this laboratory for a simple hybridisation reaction, using rabbit globin mRNA and its cDNA. The experimental points are taken from Kemp (1975). Details of the values used for the calculations are given in the legend to the figure. The rate constant k was calculated using procedures described below. Comparison of the curves in Figures 6.12a and b suggests that the rate of reaction is least when R_o and D_o are equal. This is verified in the following section.

(ii) Midpoints of hybridisation reactions

The midpoint of the reaction can be simply defined as the stage at which 50% of the limiting component has reacted to form hybrid. The equations below give the midpoints for the reactions described by equations 2 and 4 above. The midpoint is expressed as the $D_o t_{\frac{1}{2}}$ or $R_o t_{\frac{1}{2}}$.

When R_o exceeds D_o, the midpoint is reached when $H = \frac{1}{2}D_o$. Substituting this into equation 2 gives

$$D_o t_{\frac{1}{2}} = \frac{\ln(2 - \frac{D_o}{R_o})}{k\,(\frac{R_o}{D_o} - 1)} \qquad (5)$$

FIGURE 6.12.

Kinetics of mRNA : cDNA Hybridisation.

Theoretical curves for the reaction of mRNA with its cDNA, generated from the rate equations given in the text.

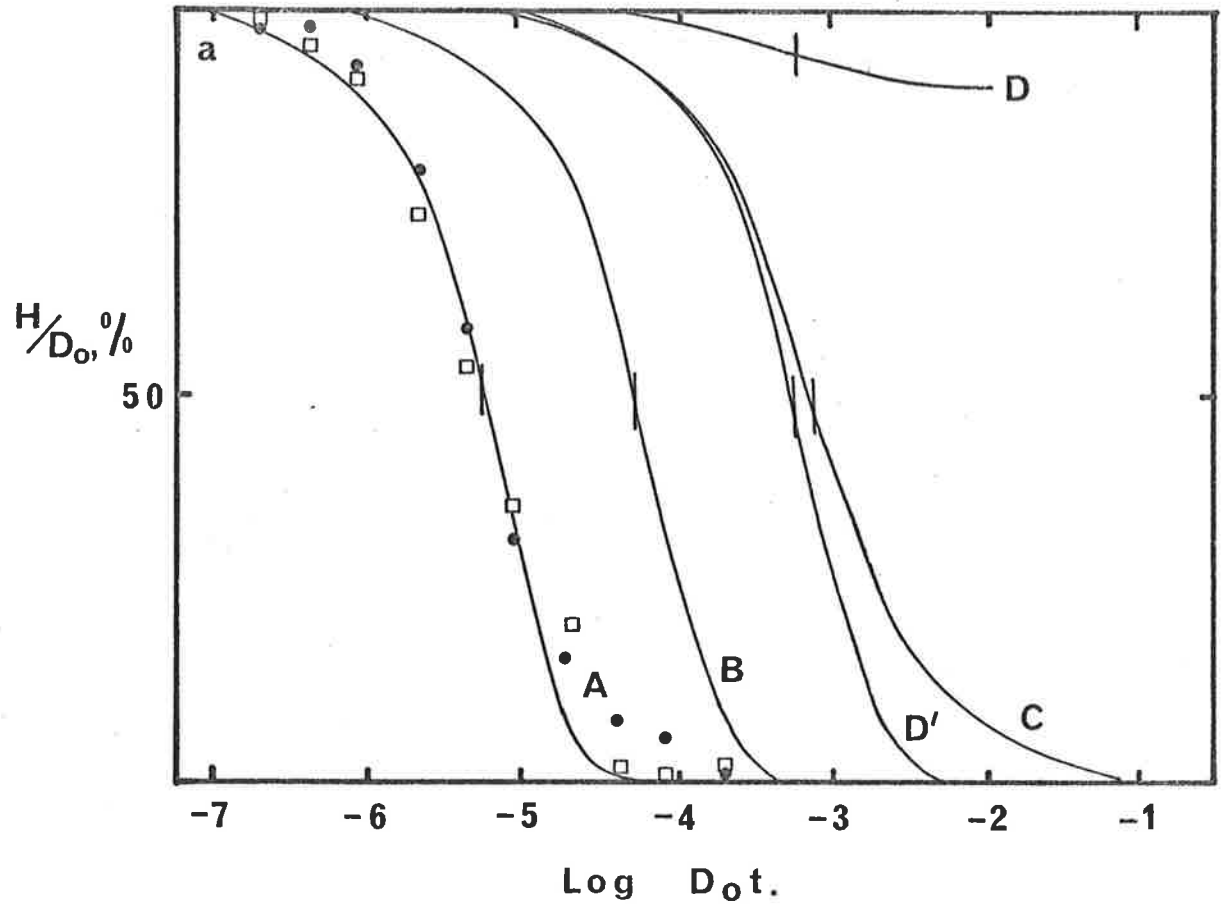
(a) The reaction is plotted as a function of $D_o t$, the product of the initial cDNA concentration and time. The rate constant, k, was taken as 1520 $l.mol.^{-1}sec.^{-1}$ The experimental points are for the hybridisation of rabbit globin mRNA to its cDNA, and are taken from Kemp (1975); the data has been corrected to take the reaction to a value of 100% for completion. The conditions of reaction were those normally employed in this laboratory; reactions were assayed with S_1-nuclease (•) or hydroxyapatite (□).

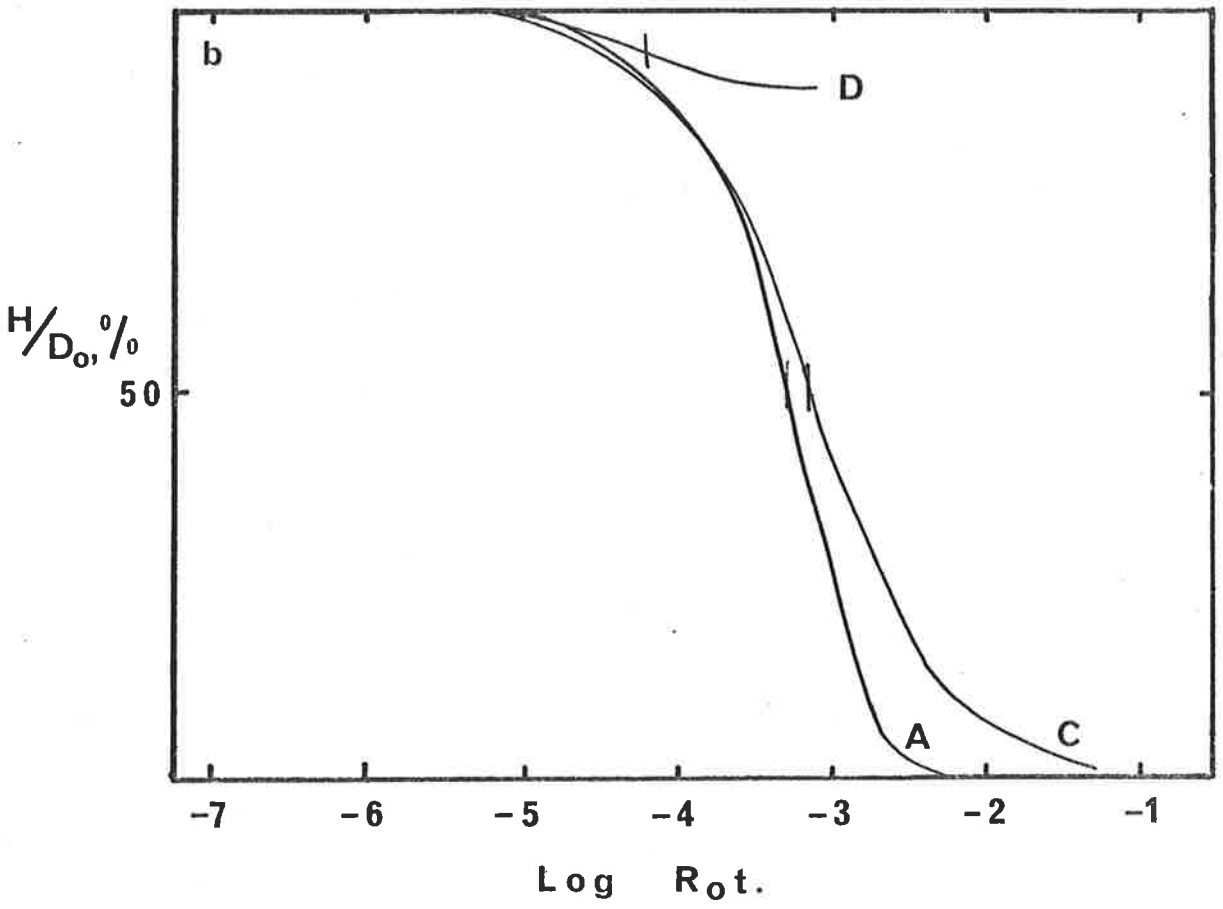
A. RNA 2 μg/ml, DNA 0.02 μg/ml
B. RNA 0.2 μg/ml, DNA 0.02 μg/ml
C. RNA 0.02 μg/ml, DNA 0.02 μg/ml
D. RNA 0.002 μg/ml, DNA 0.02 μg/ml

D' is equivalent to D, but is plotted as percentage of the maximum achievable hybridisation.

Mid-points are marked with vertical bars.

(b) The reaction plotted as a function of $R_o t$. A, C and D are exactly equivalent to the same curves in (a). Curve B, which is not shown, is virtually superimposable on A.


a
D
B
A
D′
C
H/Do, %
50
-7
-6
-5
-4
-3
-2
-1
Log Dot.


b
D
A
C
H/Do, %
50
-7
-6
-5
-4
-3
-2
-1
Log Rot.

$$\text{or} \qquad R_o t_{\frac{1}{2}} = \frac{\ln(2 - \frac{D_o}{R_o})}{k\ (1 - \frac{D_o}{R_o})} \qquad (6)$$

For $R_o = D_o$ equation 4 gives

$$R_o t_{\frac{1}{2}} = D_o t_{\frac{1}{2}} = \frac{1}{k} \qquad (7)$$

Where R_o is less than D_o, the midpoint is reached where $H = \frac{1}{2}R_o$, and hence

$$D_o t_{\frac{1}{2}} = \frac{\ln(2 - \frac{R_o}{D_o})}{k\ (1 - \frac{R_o}{D_o})} \qquad (8)$$

$$\text{and} \qquad R_o t_{\frac{1}{2}} = \frac{\ln(2 - \frac{R_o}{D_o})}{k(\frac{D_o}{R_o} - 1)} \qquad (9)$$

Thus the midpoint of reaction is a function of the rate constant, k, and the ratio of RNA:DNA in the reaction.

For RNA in excess over DNA, the midpoint occurs at a higher value of $R_o t_{\frac{1}{2}}$ than for $D_o t_{\frac{1}{2}}$, since in the one reaction the $t_{\frac{1}{2}}$, or time required to reach the midpoint will be the same irrespective of which mode of description is chosen, and the value for R_o exceeds that for D_o. In a similar fashion, it is obvious that when

DNA is in excess, the value for $D_o t_{\frac{1}{2}}$ is greater than that for $R_o t_{\frac{1}{2}}$.

To calculate the conditions for which the rate of reaction is slowest, requires the maximum value for the $R_o t_{\frac{1}{2}}$, or $D_o t_{\frac{1}{2}}$, as appropriate. For RNA excess, it can be shown that

$$R_o t_{\frac{1}{2}} < \frac{1}{k}$$

since, where for any number, x, such that $0 < x < 1$,

$$\ln(1 + x) = \sum_{n=0}^{\infty} \frac{(-1)^{n-1} . x^n}{n}$$

from which it can be shown that

$$\ln(1 + x) < x$$

Thus, since $D_o < R_o$,

$$\frac{\ln(2 - \frac{D_o}{R_o})}{(1 - \frac{D_o}{R_o})} < 1$$

and hence, from equation 6,

$$R_o t_{\frac{1}{2}} < \frac{1}{k}$$

Similarly, where DNA is in excess,

$$D_o t_{\frac{1}{2}} < \frac{1}{k}$$

The slowest rate of reaction therefore occurs when $R_o = D_o$, since it was shown in equation 7 that, under these

conditions,

$$R_o t_{\frac{1}{2}} = D_o t_{\frac{1}{2}} = \frac{1}{k}$$

The rate constant k can be determined simply from the midpoint of a reaction employing equal concentrations of RNA and DNA. However, when one component is in vast excess over the other; e.g. with a vast RNA excess, then

$$\frac{D_o}{R_o} \longrightarrow 0,$$

and hence,

$$R_o t_{\frac{1}{2}} = \frac{\ln 2}{k}$$

Thus k can be determined simply in reactions where one reactant is in vast excess. This was used to calculate the average value for k in the reaction between keratin mRNA and keratin cDNA. With RNA in excess, the $R_o t_{\frac{1}{2}}$ is 1.3×10^{-2}, from which

$$k = \frac{\ln 2}{1.3 \times 10^{-2}}$$

$$= 53.3 \text{ l.mol}^{-1}.\text{sec}^{-1}$$

(iii) Completion of reaction

As shown above the rate of reaction is slowest when $R_o = D_o$, and if any reaction is allowed

to proceed to a $D_o t$ at which the slowest possible reaction has achieved completion, that reaction will also be complete. Thus, to predict the point at which completion will have occurred, it is necessary only to calculate an appropriate value for $D_o t$ in the situation where the two reactants are present in equal concentrations.

As suggested by Young et al., (1974), the endpoint of the reaction has essentially been reached at a $D_o t$ which is 40x the $D_o t_{\frac{1}{2}}$ of the reaction with equal concentrations of the two reactants. Since under these conditions,

$$k = \frac{1}{D_o t_{\frac{1}{2}}} ,$$

substitution of this value into equation 4 enables the value for the extent of reaction to be calculated as

$$\frac{H}{D_o} = \frac{40}{41}$$

so that by this $D_o t$ value the reaction is more than 97% completed.

Using the calculated value for k determined above for the hybridisation of keratin mRNA and its cDNA, the reaction will be essentially complete at

$$D_o t = \frac{40}{k}$$
$$= 0.75 \text{ mol.sec. } 1^{-1}.$$

F. APPENDIX 2. Limits of detection of precursors.

The rate of accumulation of a molecule which is also being degraded is defined by the equation (McKnight and Schimke, 1974)

$$\frac{dX}{dt} = k_s - k_d X,$$

where X is the molecule (in this case the precursor), k_s is the zero-order rate constant for its synthesis, and k_d is the first order rate constant for degradation. At steady-state

$$\frac{dX}{dt} = 0$$

i.e.

$$X = \frac{k_s}{k_d}$$

The rate of synthesis of the precursor will be equal to the rate of accumulation of mRNA, provided that all molecules of precursor finally appear as mRNA, and that the mRNA is itself stable. Hence the value for k_s for keratin mRNA precursor may be calculated from data in Table 4.7 and Figure 4.7. The value so calculated for 12-day feather is 34 molecules per minute per cell.

On the other hand, the value of k_d is not calculated directly. However, if the half-life of the precursor is taken as $t_{\frac{1}{2}}$, then

$$k_d = \frac{\ln 2}{t_{\frac{1}{2}}}$$

Thus it should be possible to calculate the value of X as a function of the *in vivo* half-life of the precursor. A graph depicting the values of X is shown in figure 6.13.

The above calculation assumes that either all the processes which normally occur in the cell are halted instantaneously, or that no imbalance occurs between the normally observed synthesis and degradation. Should an imbalance occur, such that synthesis is halted but that degradation proceeds for a limited period of time after the cessation of synthesis, the observed value for X, X', will be given by the equation

$$X' = Xe^{-(k'_d t)}$$

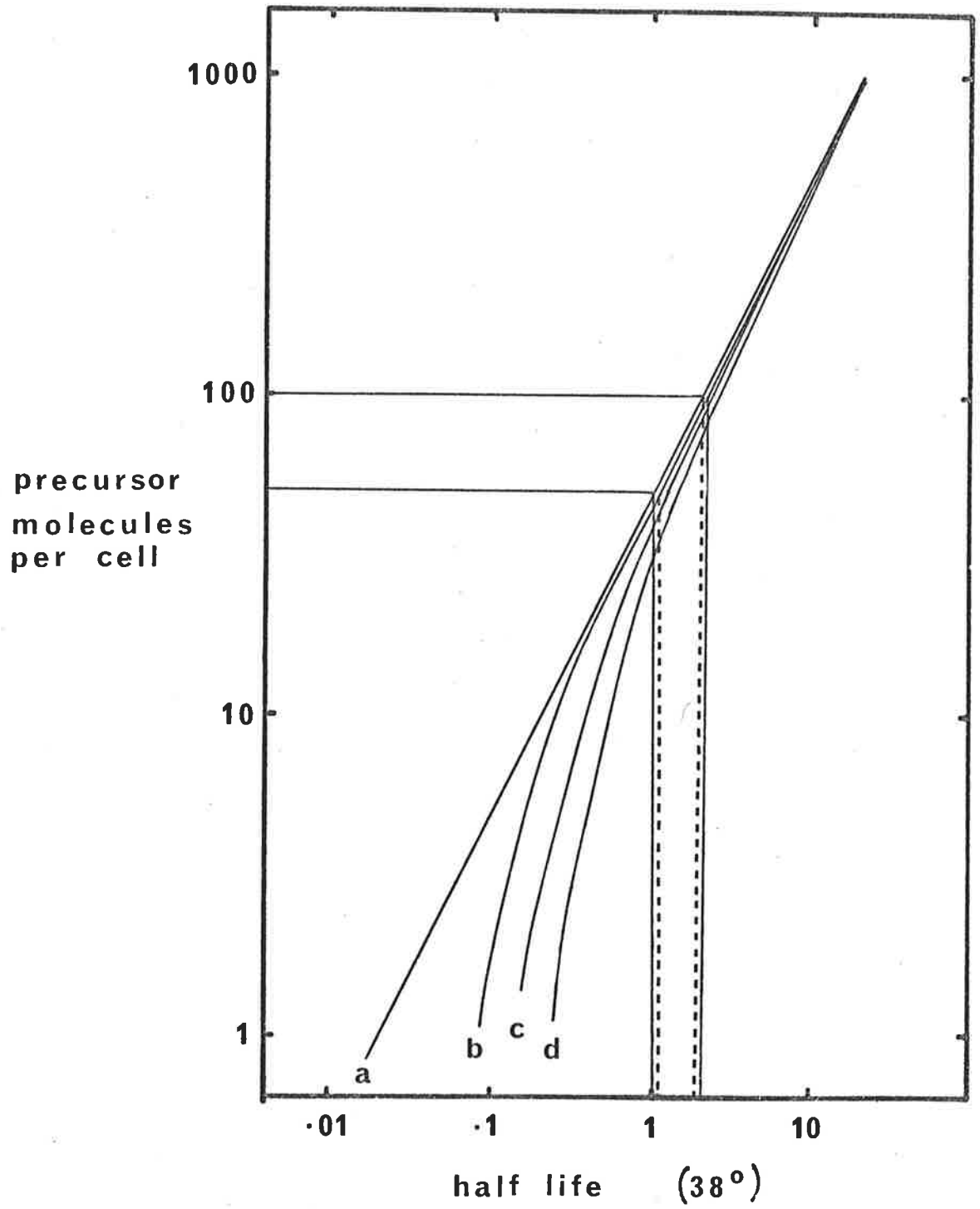
where k'_d is the rate constant for degradation at the reduced temperature, in this case 0°. An approximate value for k'_d can be calculated if it is assumed that a reduction in temperature of 10° produces a 50% reduction in reaction rate. Since the *in vivo* temperature is 37°, at which temperature the values for k_d were calculated, the value of k'_d at 0° is calculated to be

$$k'_d = \frac{k_d}{14}$$

FIGURE 6.13.

Detection Limits for Precursor mRNA.

Theoretical curves describing the number of molecules of precursor to keratin mRNA per cell as a function of the *in vivo* half-life. Curve a. is calculated from the equation of McKnight and Schimke (1974), as described in the text, and assuming that no processing, or degradation, occurs during isolation. Curves b, c and d were calculated using the modifications described in the text, assuming that processing continues (at a reduced rate) for 2, 5 and 10 minutes, respectively. The horizontal lines indicate the levels of precursor which would have been detected under the experimental conditions employed, and yield estimates of the *in vivo* half-life of the mRNA precursor.


1000
100
10
1
precursor
molecules
per cell
a
b
c
d
·01
·1
1
10
half life (38°)

Using this relationship, values of X' were calculated allowing for different periods of degradation after the cessation of precursor synthesis. The calculated curves are also shown in Figure 6.13.

The curves shown were used to estimate the maximum value for the half-life of the messenger precursor *in vivo*. The minimum levels of detection were taken to be 50-100 molecules per cell, assuming that there were 10,000 molecules of mRNA per cell (Table 4.7) at 12 days, and that it was possible to detect a precursor at a level of 0.5-1.0% of the total mRNA sequences present in the cell. The half-life calculated was one to two minutes (Figure 6.13).

CHAPTER 7

CONCLUDING DISCUSSION

The work described in this study may be divided into two clearly distinct parts. The overall significance of each part is discussed separately below, with a view to relating each to the control of keratin synthesis, and to the conclusions which may be drawn from other systems. Potential future directions of study are also discussed.

(i) Nucleic acids in feather development.

It was observed that feather DNA synthesis had essentially ceased by the onset of keratinisation of the tissue, and, as the cells were found to be diploid, this suggests that they are in the G1 stage of the cell cycle. This is the stage of the cell cycle which Holtzer et al., (1972, 1973) have suggested as being essential for expression of a terminally differentiated state, marked by the synthesis of tissue-specific proteins. The findings agreed with an earlier limited study using autoradiography at the electron microscope level (Kemp et al., 1974a).

The increase in the total RNA in the cells has been suggested as being a method whereby the cell may optimise its capacity for protein synthesis, by ensuring an excess of ribosomes and transfer RNA over mRNA. Indeed, it seems that very little of the mRNA is not actively translated, since the levels are barely adequate to support the level of protein

synthesis observed; the calculations required a slightly elevated value for the rate of elongation of the protein chains above that normally observed in eukaryote cells. Further, most of the cytoplasmic mRNA is associated with polysomes. It seems, therefore, that keratin synthesis is a most efficient process in terms of utilisation of translational machinery.

Both RNA and DNA are lost from the tissue late in the development of the feather, and enzyme activities which may be responsible for these processes were identified. It is also probable, from the curves in Figure 4.9, that some proteolysis is also involved in the later stages, to remove the non-keratin proteins from the cell. These observed rises in the enzyme activities may occur by any of three mechanisms: the activation of nuclear genes which code for the enzymes, the activation of cryptic forms of the enzymes, such as zymogens, or by transport of the enzymes to the cell from another tissue, for example <u>via</u> the blood circulation. The last seems the least likely, as it is difficult to conceive of a mechanism whereby an active protein molecule could be selectively transported to the most highly developed cells, which lie farthest from the blood supply to the tissue, or a means whereby they could be transported through the cell membrane.

If the cell employs either of the first two mechanisms, some interesting problems in timing of development arise. If a pre-programmed quantal mitosis, and entry into the subsequent G1 phase (Holtzer et al., 1972, 1973) is a suitable stimulus for the expression of luxury protein synthesis by the cell, these results argue that at least some genes must be activated later in development. Thus, even if zymogen forms of the degradative enzymes are synthesised soon after entry into the final G1 phase and in parallel with the onset of keratin synthesis, a mechanism must exist whereby the zymogens are activated. This activation may also be a sequential process, since it was found that the DNase activities reached maximum values prior to the RNases. Thus, in attempting to characterise the stimuli which result in feather development and keratinisation, it may also be necessary to characterise the stimulus which may result in the cessation of protein synthesis in the cells.

Similar late functions have been proposed in the terminal differentiation of the lens (Modak, 1972), which suggests that a time-dependent mechanism may be a common feature of certain types of differentiated cells. However, it is certain that such a sequence is not characteristic of all terminally differentiating cells, even in the avian embryo. The avian erythrocyte, for example, retains all of its

nuclear DNA.

(ii) Keratin mRNA precursors.

The search for precursors to keratin mRNA proved fruitless. It was concluded from this that either no precursor exists, or that it is short-lived in the cell; a half-life of at most 1 - 2 minutes was calculated, as a precursor of this half-life would have been seen. This half-life for the keratin mRNA "precursor", if any, is considerably lower than figures obtained for the half-life of bulk hnRNA (23 minutes; Brandhorst and McConkey, 1974) or for globin mRNA precursor (45 minutes; Ross, 1976). Rapid processing, however, could have advantages in that the temporal separation between gene and cytoplasm would be minimal.

The calculated half-life for any presumptive precursor to keratin mRNA was shown to be 5 - 10 times greater than the time required for synthesis of the messenger sequence. This suggests that a precursor may exist, but that it would require the use of more specific, and sensitive, methods of isolation and detection of the molecules containing mRNA sequence. Various highly selective procedures, based on affinity chromatography, have been developed for the purification of specific sequences. One such method, that used by Lizardi (1976) in the search for fibroin mRNA precursors,

employed a sequence of nucleotides, bound to a Sephadex matrix, complementary to an internally repeated sequence in the mRNA. This approach may not be applicable to feather keratin mRNA, at least until detailed sequence data is available for the mRNA, but might be applicable to scale keratin mRNA, as the scale proteins contain a repeating tripeptide sequence in part of the molecule (Walker and Bridgen, 1976).

Other workers have extended the concept of oligo(dT)-cellulose by using this material as the primer for cDNA synthesis, thereby obtaining covalently coupled cDNA-cellulose (Levy and Aviv, 1976; Anderson and Schimke, 1976). Such a column would be highly specific for mRNA-containing sequences, and could conceivably be employed for studies of the type proposed in searching for precursors. An alternative, equally selective, procedure uses cDNA to which a poly(dC) tail has been attached. This is then used to hybridise the mRNA sequences, and the hybrids selectively purified by chromatography on poly(I)-sephadex (Coffin et al., 1974). Each of these methods, however, requires the use of much larger quantities of cDNA, and therefore larger quantities of purified mRNA, than can be obtained currently in this laboratory.

It is possible that the presence of a precursor to keratin mRNA in the cells may be inferred

from analysis of the 5'-termini of the cytoplasmic mRNA. Initiation of mRNA synthesis in some eukaryote systems appears to utilise only purine nucleotides (Schibler and Perry, 1976; Schminke et al., 1976). Hence if the penultimate nucleotide, X, in the 5'-terminal sequence m^7G ppp X^mpYp.... was a pyrimidine, the processing of at least a short 5'-sequence must have occurred. Studies on the 5'-sequence of keratin mRNA have been severely restricted by the difficulty in labelling the mRNA.

This problem with labelling also interferes with the approaches which would conclusively prove that a high molecular weight, messenger-containing species could serve as a precursor to the cytoplasmic mRNA. This requires that, in a "pulse-chase" type experiment, label initially incorporated into the higher molecular weight species eventually appears in the lower. It is difficult to obtain high specific activity in feather RNA labelled in tissue culture. This is probably due to the permeability of the tissue being low; the pool sizes for nucleotide triphosphates have not been investigated, but these may be high and thus further compound the difficulties in labelling.

It was suggested, in the discussion of feather differentiation, above, that at the level of translation keratinisation is an efficient process. It is conceivable that at the transcriptional level it will also be

efficient. There is a large battery of keratin genes active in the feather cell (Kemp, 1975), and it has been suggested earlier that the presence of these genes may be effective in promoting the rapid synthesis of the mRNA. It seems, for reasons of efficiency alone, to be a wasteful procedure to synthesis the mRNA as a high molecular weight precursor, only to discard a substantial portion of the mRNA within a short time of transcription. In the mRNA is required in the cytoplasm in the shortest possible time, it would be advantageous to have only the minimum of processing. It is apparent that a significant portion of the mRNA is not translated, and these sequences may represent the type of sequence discarded in, say, the processing of globin mRNA precursor.

The primary aim of these studies was to characterise the non-messenger portions of the precursor, which should provide information as to the nature of the sequences adjacent to the structural genes in the genome. It is apparent that, even should a precursor form exist, it will not be usable for this type of study, as workable quantities would be difficult to isolate. Other approaches to studying neighbouring sequences are becoming more feasible - it is possible, for example, to extensively purify specific genes from cellular DNA by a variety of methods (Birnsteil et al., 1974; Kedes et al., 1975; Anderson and Schimke, 1976; Woo et al, 1976).

Clearly, the large number of keratin genes is a potential advantage in the purification either of the total keratin gene population by physical methods, or of a single gene by cloning techniques (c.f. Kedes et al., 1975). Some preliminary experiments suggest that keratin genes, and neighbouring sequences, may be amenable to purification by physical and cloning methods (Lockett and Kemp, 1975; R. B. Saint, personal communication). Results obtained by this more direct approach may be more meaningful than these based on precursors, particularly if the latter could not be shown to be direct precursor species.

(iii) Final comments.

It seems, from the above discussion and from the work described elsewhere in this thesis, that keratinisation of the tissue, once initiated, is an efficient process, which does not appear to be regulated at post-transcriptional levels. Thus, in investigating the molecular events which control keratin synthesis, it will be necessary to examine the earliest stages, to determine the stimuli which prompt the expression of the specific gene set. It may be that it is a series of environmental effects, such as the influence of the dermis, and of surrounding epidermal cells, and the hormonal balance of the embryo which will be the crucial factors in feather development and keratinisation.

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APPENDIX - PUBLICATIONS.

Papers Published:

Control of feather keratin synthesis by the availability of keratin mRNA: B. C. Powell, D. J. Kemp, G. A. Partington, P. E. M. Gibbs and G. E. Rogers (1976).

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CONTROL OF FEATHER KERATIN SYNTHESIS BY THE AVAILABILITY OF KERATIN mRNA

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SUMMARY

The relative timing of the synthesis of keratin and its mRNA in the developing chick embryo feather has been examined. Study of both active mRNA in polysomes, and of the total number of mRNA sequences in the tissue, leads to the conclusion that the rate-limiting step in the synthesis of keratin is the accumulation in the cytoplasm of its mRNA.

INTRODUCTION

In the embryonic chick feather, keratin synthesis is first detectable at 12 days of development, and accounts for most of the protein synthetic activity at 14 days [1]. Feather keratins are a large family of homologous polypeptide chains, all of MW about 10,000 [2, and I.D.Walker and G.E.Rogers, unpublished observations]. The 12S mRNA coding for most or all of the feather keratins [3] has been isolated in highly purified form [4,5] and used to direct the synthesis of complementary DNA (cDNA) by the DNA polymerase from avian myeloblastosis virus [6,7]. We now report studies which demonstrate that during feather development both the amount of polysomes synthesizing keratin and the rate of keratin synthesis correlate with the total amount of keratin mRNA present in feather tissue, as determined by hybridization of both cytoplasmic RNA and total feather nucleic acid with keratin cDNA.

MATERIALS AND METHODS

Lysates, post-mitochondrial supernatants, polysomes and poly-

somal RNA were prepared from 11-14 day embryonic chick feather as described [3]. Total cytoplasmic RNA was prepared by phenol extraction [3] of the post-mitochondrial supernatant. Keratin mRNA was prepared by dissociation of mRNP particles with Na dodecyl sulphate [4]. Keratin cDNA labelled with [^{3}H]-dCTP (26.2 Ci/mmole) was transcribed from keratin mRNA using the DNA polymerase from avian myeloblastosis virus [7]. The total feather nucleic acids were prepared by phenol extraction of a high-speed homogenate of feathers using a microhomogeniser (Sorvall Omnimix) in a buffer containing 10 mM tris-HCl (pH 9.0), 5 mM EDTA and 1% Na dodecyl sulphate. The total nucleic acid concentration was determined by absorbance measurement at 260 nm, and the RNA contents by the orcinol reaction [8]. All other methods are given in the Figure legends.

RESULTS AND DISCUSSION

The ratio of polysomes to monosomes increases in embryonic feathers over the period of 12 - 14 days [9,10], with a major peak of polysomes containing 4 - 5 ribosomes. Polysomes containing about 4 ribosomes are of the size expected for the synthesis of feather keratin (MW 10,000) on monocistronic mRNA when compared with other systems [e.g. 11]. Studies on cell-free protein-synthesizing systems demonstrated that most of the radioactive nascent chains were initially associated with polysomes containing 3 - 5 ribosomes [G.A.Partington, D.J.Kemp and G.E.Rogers, unpublished observations].

Labelled proteins from post-ribosomal supernatants of 12 and 14 day cell-free systems were investigated by gel electrophoresis at pH 7.5 (Figure 1). The products of the 12 day system (Figure 1a) were predominantly non-keratin proteins, migrating more slowly than the added keratin markers (bands β2 - β5), while the major products of the 14 day system (Figure 1b) co-electrophoresed with keratin bands β2 - β5. This change in synthetic products was strikingly similar to that observed *in vivo* [1]. Identification of the major products of the 14 day system as keratins was confirmed by co-electrophoresis of the products with keratin markers at pH 2.7, by Sephadex G-100 and DEAE-cellulose chromatography and

by isolation of N-terminal peptides [G.A.Partington, D.J.Kemp and G.E.Rogers, unpublished observations]. The keratin mRNA content of 12 and 14 day feather polysomes was determined in order to establish the basis for this change in synthesizing capacity. When 12 day feather polysomal RNA was fractionated on cellulose [4] very little 12S mRNA was present (Figure 2a) in comparison with a large peak of 12S mRNA from 14 day polysomal RNA (Figure 2b).

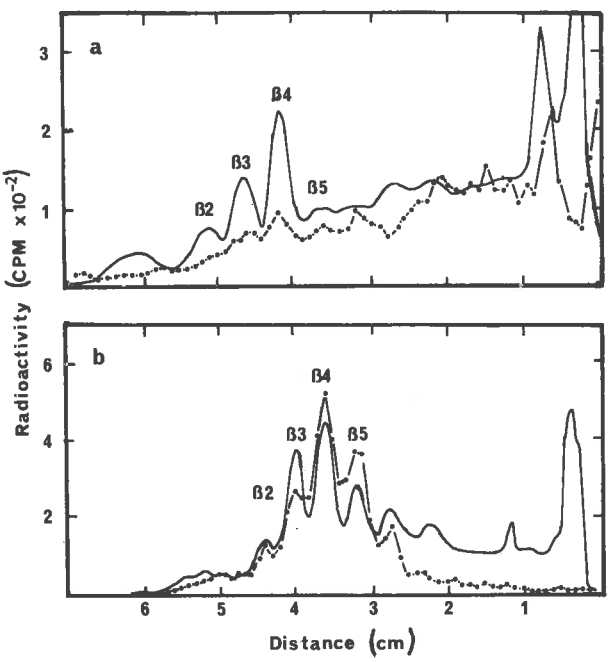

FIGURE 1. Gel electrophoresis at pH 7.5 of the protein products of 12 and 14 day feather cell-free systems. The cell-free systems (0.5 ml) contained 0.75 volumes of the 16,000 g supernatant and final concentrations of 1.0 mM ATP, 0.25 mM GTP, 4 mM $MgCl_2$, 10 mM tris-HCl, pH 7.4, 150 mM KCl, 15 mM phosphocreatine, 100 µg creatine kinase and either 10 µCi ^{14}C-algal-hydrolysate (a) or 25 µCi ^{3}H-serine (b). After incubation for 1 hour at 37°, ribosomes were removed by centrifugation. Proteins in the post-ribosomal supernatants were reduced, carboxymethylated, mixed with 100 µg of S-carboxymethylated 21 day embryonic feather keratins electrophoresed on pH 7.5 polyacrylamide gels, stained and analysed by densitometry and for radioactivity exactly as described [1]. ——— densitometer traces. •—•—• radioactivity. The densitometer peaks corresponding to the added marker keratin bands β2-β5 [2] are indicated, and were clearly distinguishable from the background due to proteins in the lysate. (a) 12 day feather (b) 14 day feather.

When 12 and 14 day polysomal RNA was centrifuged on sucrose gradients and keratin cDNA was used to perform hybridizations across the gradients (Figure 3), marginal keratin mRNA content was observed in the 12 day polysomal RNA whereas at 14 days the keratin mRNA content of the polysomal RNA increased by at least 25-fold. It should be noted that some variation was observed in the keratin mRNA content between embryos within the same age group. The increase in polysomal keratin mRNA during feather development could arise by an increase in the total amount of keratin mRNA in feather cytoplasm or by activation for translation of keratin mRNA already present in 12 day feather cytoplasm. To discriminate between these alternatives the total amount of keratin mRNA present in 11 - 14 day total cytoplasm was measured by hybridization with keratin cDNA.

As detailed previously [6,7], pure keratin mRNA hybridized to keratin cDNA in a broad transition (Figure 4) with a $R_o t_{\frac{1}{2}}$ [12] of 1.3×10^{-2} mol.s.l^{-1} when assayed by the nuclease S_1 procedure.

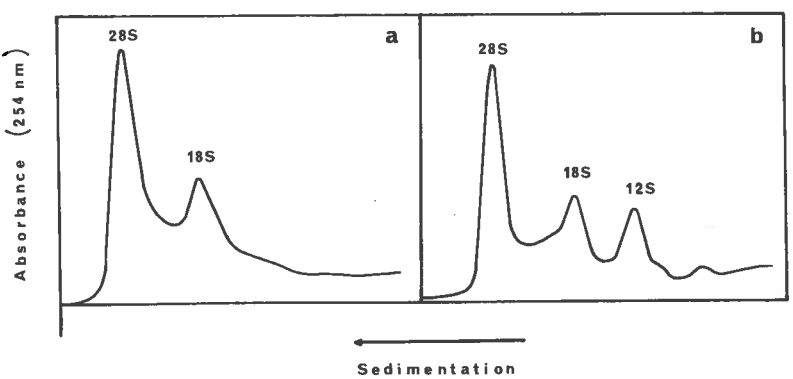

FIGURE 2. Sucrose gradient centrifugation of the cellulose-bound fraction of 12 and 14 day feather polysomal RNA. Polysomal RNA (65 A_{260} units) from 12 and 14 day feathers was fractionated on 1 x 15 cm columns of Whatman cellulose and the cellulose-bound fractions were dissociated with formamide and fractionated on sucrose gradients as described [4]. (a) 12 day cellulose-bound RNA (b) 14 day cellulose-bound RNA.

This broad curve has been shown to result from sequence heterogeneity of the multiple homologous keratin mRNA species [7]. Total cytoplasmic RNA from 14-, 13-, 12- and 11- day feathers hybridized in transitions of similar shape to that of pure keratin mRNA (Figure 4a) but with $R_0t_{\frac{1}{2}}$ values of 7×10^0, 1.7×10^1, 4.0×10^1 and 4.8×10^2 mol.s.l^{-1} respectively. Similarly, total nucleic acid showed $R_0t_{\frac{1}{2}}$ values for the same ages (Figure 4b) of 4.5×10^0, 1.7×10^1, 5.0×10^1 and 4.0×10^2 mol.s.l^{-1}. The differences between the figures for each age are within the range found for individual embryos of the same age. The $R_0t_{\frac{1}{2}}$ values (Figure 4) were used to calculate the fraction of the RNA that is keratin mRNA sequence [cf.13]. However, it was necessary to include a

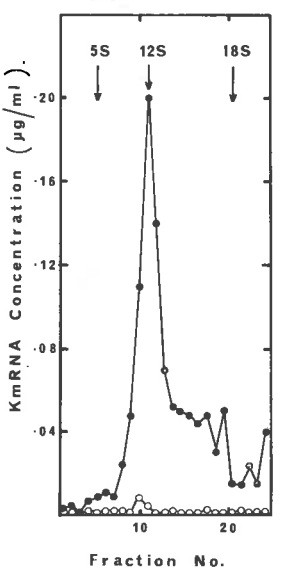

FIGURE 3. Polysomal RNAs from 12 and 14 day feathers were isolated [3] and run on sucrose gradients. Fractions were collected and aliquots hybridized with cDNA at 60° in 0.18 M Na^+ for 4 hr. and assayed for resistance to the single-strand specific nuclease S_1 of Aspergillus oryzae under low salt conditions [6,7]. The keratin mRNA concentration of each fraction was calculated using a standard R_0t curve for keratin mRNA with its cDNA (see Figure 4) and the observed hybridization value. 12 day O—O, 14 day ●—●.

correction factor of 5 in the calculation since it was found that if a known amount of mRNA was added to a 12-day lysate only 20% of the expected mRNA was recovered as judged by hybridization kinetics. These values were combined with the figures for the RNA content of embryonic feathers at 11 - 14 days, expressed as either mg/ml of tissue or the content per cell [P.E.M.Gibbs, B.C.Powell and G.E. Rogers, unpublished observations] to yield the mRNA content of the

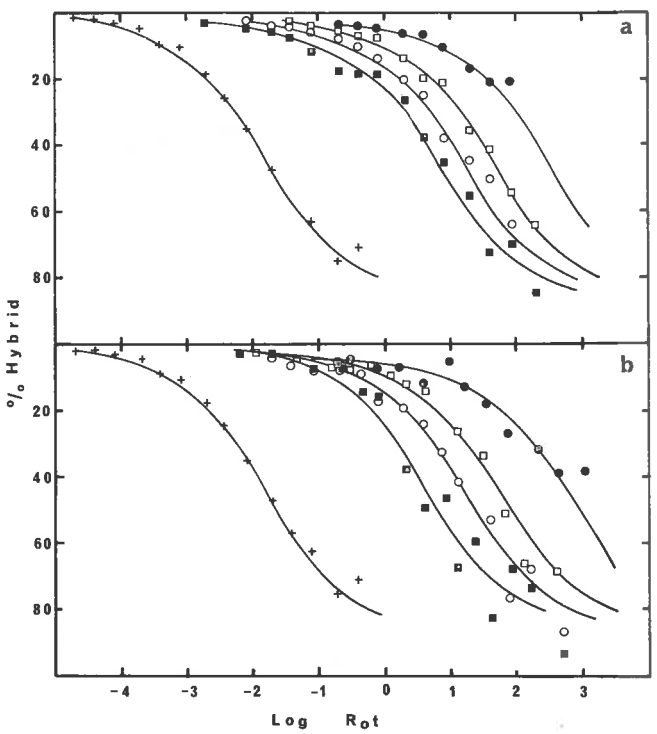

FIGURE 4. Kinetics of hybridization of feather RNA with keratin cDNA. After denaturation by boiling, hybridization mixtures were incubated at 60° in 0.18 M Na^+ for up to 110 hr. to achieve the R_ot values indicated and assayed for resistance to nuclease S_1 as in Figure 3. Results (% hybrid) are expressed as the % of input cpm in cDNA resistant to nuclease S_1 after hybridization.

(a) Cytoplasmic RNA - the 11-, 12-, 13-day curves are the average of 4 experiments; the 14-day curve is the average of 2 experiments. (b) Total nucleic acid (sonicated before use). All curves are the average of 2 experiments. The values are uncorrected for any sequences contributed by DNA. Pure keratin mRNA +——+, 11 day ●——●, 12 day □——□, 13 day O——O, 14 day ■——■.

tissue (Figure 5). It was found that the mRNA content increased 60 - 90 fold on a packed tissue basis (Figure 5a), or some 600 - 750 fold in terms of molecules per cell (Figure 5b) over the interval of 11 - 14 days. The values shown are average figures for the entire tissue, since at any age the feather cells are at different

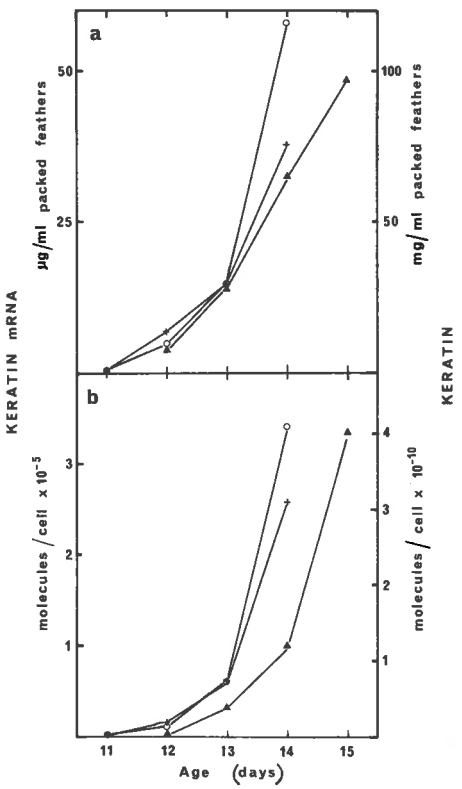

FIGURE 5. Keratin mRNA and keratin accumulation during embryonic feather development.

(a) The amounts of keratin mRNA given as µg/ml of packed tissue were calculated from the $R_o t_{\frac{1}{2}}$ values relative to that of pure keratin mRNA (1.3×10^{-2} mol.s.l^{-1}) in Figure 4, and the quantity of RNA per ml of tissue [P.E.M.Gibbs, B.C.Powell and G.E.Rogers, unpublished observations]. The amounts of keratin (mg/ml of packed feathers) are from [1]. Cytoplasmic RNA +——+, total nucleic acid O——O , keratin ▲—▲

(b) The number of keratin mRNA molecules per cell. This was calculated from the $R_o t_{\frac{1}{2}}$ values of Figure 4, the RNA content per cell [P.E.M.Gibbs, B.C.Powell and G.E.Rogers, unpublished observations], and the molecular weight of keratin mRNA [4]. The number of molecules of protein was calculated in a similar manner. Cytoplasmic RNA +——+ , total nucleic acid O——O , keratin ▲—▲

stages of development [14,15] and, in addition, there is a contribution to the cell number from erythrocytes in the central capillaries. It is possible that the keratin mRNA molecules are concentrated in a portion of the feather cells, so that the total number in the cells most active in keratin synthesis might be greater than the figure of ∿300,000 observed for 14 day tissue. In order to discount the possibility that the differences in amounts of keratin mRNA were artifacts caused by RNAse contamination of the younger (11 and 12 day) feather lysates, equal amounts of 11 and 14 day lysate were mixed, RNA was extracted and the amount of keratin mRNA determined in both the mixture and the batch of 14 day lysate used. Essentially all (97%) of the keratin mRNA contributed by 14 day lysate was recovered in the presence of 11 day lysate.

We conclude that the dramatic increase in the level of keratin mRNA in the embryonic chick feather between 11 and 14 days of development is the direct result of an accumulation of keratin mRNA in the feather cytoplasm. The existence of any significant amount of inactive cytoplasmic keratin mRNA present prior to the onset of keratin synthesis, or release of presynthesized mRNA from the nucleus, can be discounted. As the ratio of polysomes to monosomes increases greatly while keratin mRNA accumulates, it is most likely that availability of keratin mRNA in the cytoplasm is the rate-limiting step in keratin synthesis. It is of relevance to note that it has has recently been shown that in developing erythroid cells [16,17] and in oviduct magnum cells stimulated with estrogen [13,18] the mRNA is synthesized immediately prior to its use, and is not stored.

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